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# Microbe participation in aroma production during soy sauce fermentation

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**Soy sauce is a traditional Japanese fermented seasoning that contains various constituents such as amino acids, organic acids, and volatiles that are produced during the long fermentation process. Although studies regarding the correlation between microbes and aroma constituents have been performed, there are no reports about the influences of the microbial products, such as lactic acid, acetic acid, and ethanol, during fermentation. Because it is known that these compounds contribute to microbial growth and to changes in the constituent profile by altering the moromi environment, understanding the influence of these compounds is important. Metabolomics, the comprehensive study of low molecular weight metabolites, is a promising strategy for the deep understanding of constituent contributions to food characteristics. Therefore, the influences of microbes and their products such as lactic acid, acetic acid, and ethanol on aroma profiles were investigated using gas chromatography/mass spectrometry (GC/MS)-based metabolic profiling. The presence of aroma constituents influenced by microbes and chemically influenced by lactic acid, acetic acid, and ethanol were proposed. Most of the aroma constituents were not produced by adding ethanol alone, confirming the participation of yeast in aroma production. It was suggested that lactic acid bacterium relates to a key aromatic compound, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone. However, most of the measured aroma constituents changed similarly in both samples with lactic acid bacterium and acids. Thus, it was clear that the effect of lactic acid and acetic acid on the aroma profile was significant.**



[**Keywords:** Soy sauce; Metabolic profiling; Fermentation; Constituent profile; Fermentation microbe; Lactic acid; Acetic acid; Ethanol; *Tetragenococcus halophilus*; *Zygosaccharomyces rouxii*]

Soy sauce is a traditional Japanese fermented seasoning, and its demand is increasing worldwide. It is fermented by *koji* mold (*Aspergillus oryzae* or *A. sojae*), lactic acid bacterium (*Tetragenococcus halophilus*), and yeast (*Zygosaccharomyces rouxii*), using steamed soybeans and roasted wheat as ingredients. Each microbe is intricately involved in the change in the constituents during soy sauce fermentation (1,2). The *koji* mold produces various enzymes that assist in the degradation of soybeans and wheat (3). The lactic acid bacterium produces lactic and acetic acids, which lower the pH of moromi (soy sauce *koji* mixed with saturated saline solution) and contribute to its acidic odor (4). Yeast produces ethanol and several hundred aroma compounds through alcoholic fermentation. Therefore, yeast is considered to be essential for the aroma of soy sauce (5), which contains various constituents such as amino acids, sugars, organic acids, volatiles, and other compounds that are produced through fermentation and aging to produce the unique flavor of soy sauce (6–8).

Studies regarding the relationship between constituents and the flavor of soy sauce have been performed on umami taste and its contributing constituents (7,9). Correlation analysis of the sensory evaluation and constituent profile was also previously reported (10). Among the studies of constituents, aroma constituents have often been reported, including a comparison of aroma profiles in soy sauce (11) and the flavor dilution (FD) factor of volatile constituents by aroma extract dilution analysis (AEDA) (6,11). Studies on the aroma compounds of soy sauce and microbes involved in their generation include investigations of the production pathway of specific compounds such as 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2*H*)-furanone (HEMF) by yeast (12) and involved random mutagenesis and selection of strains suitable for specific indices of aroma compounds (13). Despite the existing analyses on the relationship between microbes and aroma constituents, the factors involved in the production of each constituent during fermentation have not yet been determined.

Recently, due to the development of metabolomics technology, the relationships between quality and constituents, as well as taste and constituents, of foods such as cheese (14), coffee (15), and sake (16) have been examined using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). In soy sauce research, the contribution of constituents to soy sauce flavor has been explored by analyzing the correlation between constituent profiles and sensory attributes (8,17,18). Meanwhile, we have applied metabolomics strategies to evaluate soy sauce fermentation in previous studies and found a novel correlation between aroma constituents and microbes (19). In a previous report, we focused only on the influence of microbial fermentation. In addition, the lactic acid, acetic acid, and ethanol produced by the microbes are also important factors in soy sauce

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fermentation that make effects on not only microbial growth (20), but also chemical reactions, such as the Maillard reaction (21), by changing the pH of the moromi environment. Furthermore, these compounds also contribute to the formation of various other compounds, acting as reaction substrates. In miso, it has been suggested that the concentration of ethanol produced by yeast contributes to the formation of ethyl esters, which are key aroma constituents (22). In sake and wine, it is clear that ethyl esters are produced not only through the enzymatic reactions of yeast, but through chemical reactions as well (23,24). These reports indicate that simple comparisons of the foods produced in the presence and absence of microbes is not sufficient to explain the unpredictable constituent changes. Furthermore, changes due to the presence of lactic acid, acetic acid, and ethanol could influence the quality of the resulting soy sauce. Thus, clarifying the aroma constituents related to microbes and the presence of lactic acid, acetic acid, and ethanol is important in the development of desirable quality soy sauce.

Therefore, we investigated the effects of the fermentation of soy sauce by lactic acid bacterium and yeast and the presence of lactic acid, acetic acid, and ethanol on the aroma constituent profile. To clarify the influence of lactic acid bacterium and yeast on aroma constituents, a classification of the constituents that are influenced by microbes and chemicals is needed. It was necessary to distinguish between the influence of microbes and that of pH changes due to the change in lactic and acetic acids and ethanol concentrations. Therefore, we performed soy sauce fermentation with lactic acid bacterium and yeast, and we also directly added chemicals in the absence of microbes. We also compared the resulting constituent profiles of the moromi filtrates at various sampling times using GC/MS and discuss their differences herein.

## MATERIALS AND METHODS

**Reagents** Ribitol, pyridine, ethyl acetate, ultrapure water, phosphoric acid, sodium dihydrogen phosphate dehydrate, 2-ethyl-1-hexanol, *n*-hexane, and *n*-heptane were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methoxyamine hydrochloride and 1-propanol-1,1-$d_2$ were purchased from Sigma–Aldrich (Milwaukee, WI, USA). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), *n*-alkanes (C9–C40), *n*-pentane, and *n*-octane were purchased from GL Science, Inc. (Tokyo, Japan). Other chemicals for compound annotation were purchased from Kanto Chemical, Co. (Tokyo, Japan).

**Soy sauce samples** Soy sauce fermentation was performed as previously described (19). Briefly, equal quantities of steamed soybeans and roasted wheat were mixed with a pre-culture of *Koji* mold (*A. sojae* NBRC 4239). In this experiment, a low concentration of penicillin (2 U/g-*koji*) was added to inhibit contamination of the natural microbes and to obtain reliable results. Soy sauce *koji* was prepared using the cultured mixture. When soy sauce *koji* is mixed with saturated saline solution, it is called moromi; a pure culture of lactic acid bacterium (*T. halophilus* NBRC 12172) was subsequently added to this moromi. Six weeks later, a pure culture of yeast (*Z. rouxii* NBRC 1876) was added at a pH of 5.0. The moromi was stored at 15–30 °C for 10 weeks with occasional brief aeration and then stored anaerobically for alcohol fermentation. The moromi was filtered using filter paper (Advantec No. 2, Toyo Roshi, Inc., Tokyo, Japan), and the filtrates were isolated at 10 time points (0, 1, 3, 4, 5, 6, 7, 15, 17, and 18 weeks) and stored at −20 °C prior to analysis. To compare the effect of the chemicals, lactic acid and acetic acid were added to the moromi at 6 weeks, and ethanol was added at 7 and 10 weeks to adjust the concentrations in each sample to be the same as those in the control samples. The control samples were inoculated with lactic acid bacterium and yeast. Samples labeled LAB + EtOH and Acids + Yeast were inoculated with only lactic acid bacterium or yeast, and ethanol or lactic and acetic acids were added, respectively. The sample labeled Acids + EtOH was not inoculated with any microbe, but both acids and ethanol were added (Fig. 1). Each sample was prepared in triplicate.

**Derivatization of hydrophilic compounds for GC/MS analysis** Pretreatment of hydrophilic compounds was performed as previously reported (19). Briefly, soy sauce samples were diluted 10-fold with ultrapure water. Each diluted sample (20 μL) was dispensed into a 1.5-mL microfuge tube, and 60 μL of ribitol (0.2 mg/mL in ultrapure water) was added as an internal standard. The mixtures were lyophilized at 22 °C for 15 h. For derivatization, 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to the lyophilized samples. The mixtures were subsequently incubated in a thermomixer (Eppendorf, Ltd., Hamburg, Germany) at 30 °C for 90 min for the methoxylation reaction to proceed to completion. Then, 50 μL of MSTFA was added as a second derivatization agent and the mixtures were incubated at 37 °C for 30 min to induce the trimethylsilylation reaction. The derivatized solutions were then transferred to vials for GC/MS analysis.

**Extraction of volatile compounds using ethyl acetate for GC/MS analysis** Pretreatment of volatile compounds using an ethyl acetate extraction was performed as previously reported (19). Soy sauce samples (1 mL) were dispensed into 2 mL microfuge tubes and saturated with sodium chloride. Ethyl acetate, containing 0.01 mg/mL of 2-ethyl-1-hexanol, was used for the extraction. The samples were added to 400 μL of ethyl acetate and homogenized with a MM 301 mixer mill at 25 Hz for 30 min (Retsch, Haan, Germany). The samples were centrifuged at 16,100 ×*g* for 15 min at 22 °C to separate the organic layer, which was then transferred to vials for GC/MS analysis.

**Sampling of volatile compounds using static headspace sampler for GC/MS analysis** Soy sauce samples (2.5 mL) were dispensed into 10 mL vials and saturated with sodium chloride. Sodium phosphate solution (phosphoric acid was added to sodium dihydrogen phosphate solution to adjust pH to 2,15, 1 mol/L, 500 μL) was added to control the pH of the vial solution. 1-Propanol-1,1-$d_2$ (10 μL, 1 μL/mL) was added to the samples as an internal standard. HS-20 headspace samplers (Shimadzu, Co., Kyoto, Japan) were used for extraction of volatiles according to the following conditions. Loop mode was used with 1 mL of sample loop. The temperature of the sample line and transfer line were both 150 °C. The vials were kept at 50 °C for 30 min, and then the gas phase was transferred to the sample loop. The vial pressure time, pressure equilibration time, load time, load equilibration time, injection time, and needle flash time were 0.5, 0.1, 0.5, 0.1, 0.5, and 5 min, respectively. The extracted gas phase was then transferred to the GC–MS.

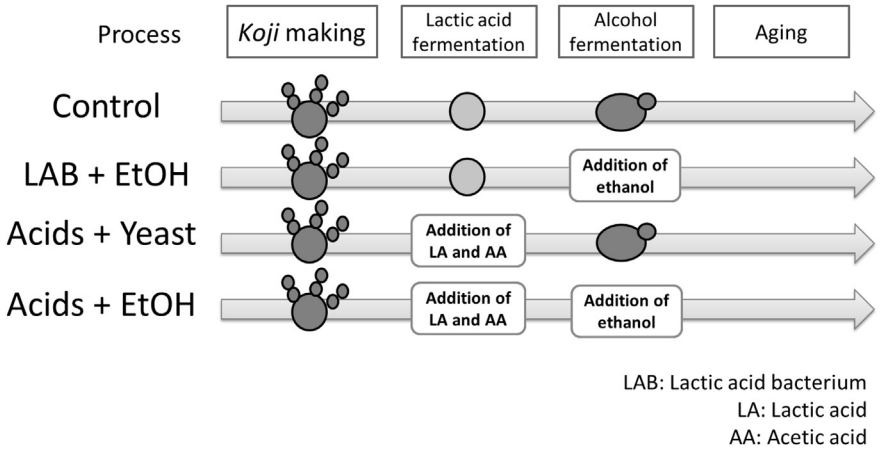


FIG. 1. Moromi conditions during fermentation. *Koji* mold, *Aspergillus sojae* NBRC 4239; lactic acid bacterium, *Tetragenococcus halophilus* NBRC 12172; yeast, *Zygosaccharomyces rouxii* NBRC 1876.

**GC/MS analysis of hydrophilic compounds and volatile compounds (ethyl acetate extraction and headspace extraction)** In this study, a GCMS-QP2010 Ultra (Shimadzu) was used for the analysis of hydrophilic and volatile compounds from the ethyl acetate extraction. The hydrophilic compound analysis used a CP-SIL 8 CB low bleed/MS column (30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 μm, Agilent Technologies, CA, USA). For the volatile compound analysis from the ethyl acetate extraction, an InertCap Pure WAX column (30 m × 0.25 mm i.d. with 0.25 μm film thickness, GL Sciences, Inc.) was used. Mass spectra were recorded over the mass range of *m*/*z* 85–500 for hydrophilic compound detection and *m*/*z* 35–350 for volatile compound detection with ethyl acetate extraction. The analytical conditions were the same as previously reported (19).

A GCMS-TQ8040 (Shimadzu) was used for volatile compound analysis from headspace (HS) sampling A DB-1 column (60 m × 0.25 mm i.d. fused silica capillary column coated with 1.00 μm, Agilent Technologies) was used. The injection temperature was 25 °C. The carrier gas was helium at a linear velocity of 32.2 cm/s. The column temperature was held at 35 °C for 5 min isothermally, raised at 8 °C/min to 230 °C, and then held for 10 min. Subsequently, the column temperature was decreased at 5 °C/min to 200 °C. The transfer line and the ion source temperatures were 280 °C and 230 °C, respectively.

The acceleration energy of electrons for ionization was 70 eV. Mass spectra were recorded at 10 scans per second over the mass range of *m*/*z* 34–500 using Q3 scan mode. A standard C5–C40 alkane mixture was injected to calculate the retention indices of the volatile compounds from HS sampling.

**Data processing** The GC/MS data of hydrophilic and volatile compounds from the ethyl acetate extraction were converted to the netCDF format. Peak detection and alignment were performed using the freely available software MetAlign (Wageningen UR, Netherlands; http://www.pri.wur.nl/UK/products/MetAlign/). The processed data were then exported to a CSV-format file, and the data matrices were constructed by AIoutput2 (25). Data obtained for the volatile compounds from HS sampling were analyzed by GCMS Browser (Shimadzu, Co.). Data matrices were exported in Microsoft Excel format, and tentative peak identification was performed according to the retention indices and mass spectra of the peaks. Retention indices were calculated based on the retention time of the standard *n*-alkane mixture.

For hydrophilic compound data, the retention indices and their mass spectra were compared with an in-house library prepared from authentic standards. The NIST11 MS spectral library was used to confirm the annotations. For volatile compound data from both ethyl acetate and HS sampling, the retention times and their mass spectra were compared with authentic standards. The mass spectra of other volatile peaks that were not compared with these standards due to a lack of availability were instead compared with the NIST11 MS spectral library. Mass spectrum and retention index of some peaks were compared with those of the literature (26–28). The assigned peak intensities were normalized against the peak intensities of the spiked internal standards, which were ribitol for hydrophilic compounds, 2-ethyl-1-hexanol for volatile compounds from the ethyl acetate extraction, and 1-propanol-1,1-$d_2$ for volatile compounds from HS sampling.

**Multivariate analysis** The annotated compound data from the hydrophilic and volatile compound analyses were integrated into one Excel sheet. Principal component analysis (PCA) was performed using commercial software, SIMCA-P+ version 13 (Umetrics, Umeå, Sweden). Auto scaling was performed for preprocessing. AIoutput2 was used to perform a *t*-test and the volcano plots were made using Microsoft Excel 2013.

## RESULTS AND DISCUSSION

**Compounds detected by GC/MS** Constituent profiling of soy sauce was performed using three analytical methods. A total of 36 compounds from the hydrophilic compound analysis (Table S1), 52 compounds from the ethyl acetate extraction (Table S2), and 45 compounds from HS sampling were detected (Table S3). These constituent profiles were used for further analysis.

PCA was performed using the analysis results of the samples measured at 18 weeks (Table S4) to confirm the data structure (Tables S5 and S6). The samples were plotted separately based on the fermentation conditions for each sample (the addition of yeast or ethanol, and the addition of lactic acid bacterium or acids) on a score plot (Fig. 2). Thus, it was confirmed that the obtained constituent profile displayed less error in repetition and could distinguish unique characteristics of each sample.

**Comparison of microbe and chemical influence at 18 weeks** To compare the influence of the addition of microbes, lactic acid, acetic acid, and ethanol, a *t*-test was conducted using the GC/MS peak intensities of the samples at 18 weeks (Table S4). To compare the effects of yeast and ethanol addition, the control (LAB + Yeast) and LAB + EtOH samples were compared. To evaluate the effects of lactic acid bacterium and addition of lactic and acetic acids, the control and Acids + Yeast were compared. In each comparison, a volcano plot, which is a scatter-plot of the negative $\log_{10}$-transformed *p*-values from the *t*-test vs. the $\log_2$ fold change in the ratio of the relative peak intensities in each sample, was generated (Tables 1 and S7, Fig. 3). In the control vs. LAB + EtOH plot (Fig. 3A), which shows the differences in constituents resulting from the addition of yeast and ethanol, it is found that some aroma constituents increased with and are characteristic of the presence of yeast because many appeared on the right side of the plot (the addition of yeast). On the other hand, the control vs. Acids + Yeast plot (Fig. 3B), which shows the differences in constituents from the addition of lactic acid bacterium vs. lactic and acetic acids, some constituents appeared on the far ends of either the positive or negative sides. Compared with the plot of the yeast and ethanol additions, there were few constituents that differ between the lactic acid bacterium, and lactic and acetic acids additions. In other words, each volcano plot revealed the constituents that were influenced by microbial fermentation as well as constituents that were chemically influenced by lactic acid, acetic acid, and ethanol. To investigate

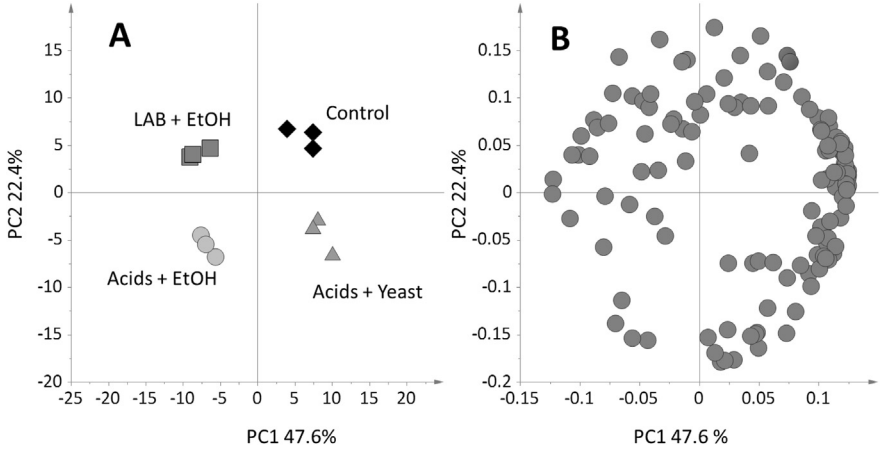


FIG. 2. Principal component analysis of the constituent profile data. (A) Score plot; diamonds represent control samples inoculated with *Zygosaccharomyces rouxii* and *Tetragenococcus halophilus*; squares represent LAB + EtOH samples inoculated with *T. halophilus* and with added ethanol; triangles represent Acids + Yeast samples with added lactic and acetic acids and inoculated with *Z. rouxii*; circles represent Acids + EtOH samples with added lactic and acetic acids and ethanol. (B) Loading plot.

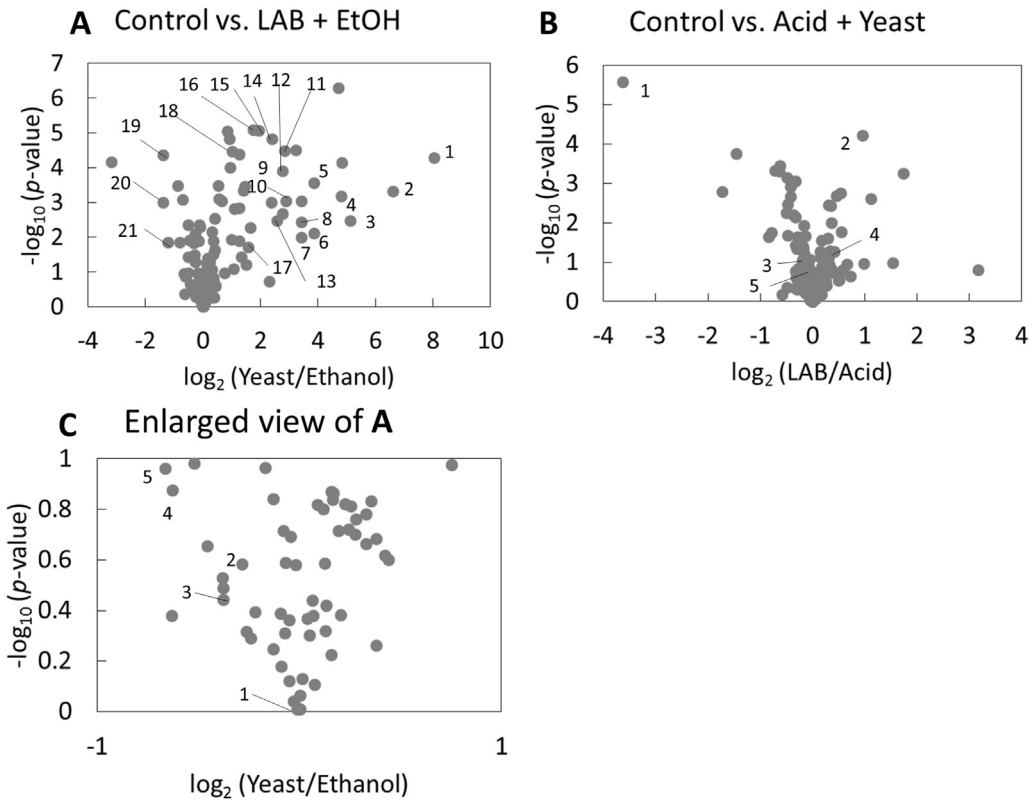


FIG. 3. Volcano plots using the constituent profile data at 18 weeks. (A) Volcano plot of control vs. LAB + EtOH. (B) Volcano plot of control vs. Acid + Yeast. (C) Enlarged view of panel A. Each circle indicates a constituent; the vertical axis indicates the log of the change between the two samples; the horizontal axis indicates the negative $\log_{10}$ of the p-value. The discussed constituents were numbered as follows: (A) 1: 1-butanol; 2: 3-hydroxy-2-butanone (acetoin); 3: 2,3-butanedione (diacetyl); 4: 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2*H*)-furanone (HEMF); 5: 2,3-butanediol; 6: 2-phenylethyl alcohol; 7: 2-methylpropyl acetate (isobutyl acetate); 8: 3-methyl-1-butanol (isoamyl alcohol); 9: 2-(4-hydroxyphenyl) ethanol; 10: 2-methyl-1-propanol (isobutyl alcohol); 11: dihydro-5-methyl-2(3*H*)-furanone (*gamma*-valerolactone); 12: butyl acetate; 13: 3-methylbutyl acetate (isoamyl acetate); 14: 2-methyl-1-butanol (active amyl alcohol); 15: 3-(methylthio)-1-propanol (methionol); 16: glycerol; 17: 2-methylbutyl acetate; 18: 5-ethyldihydro-2(3*H*)-furanone; 19: 4-hydroxy-5-methyl-furanone; 20: ethyl formate; 21: ethyl 2-methylbutanoate. (B) 1: 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (HDMF), 2: 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), 3: 2-furancarboxaldehyde (furfural), 4: 2-furanmethanol (furfuryl alcohol), 5: 3-(methylthio)-1-propanal (methional). (C) 1: ethyl butanoate, 2: ethyl benzoate, 3: ethyl hexanoate (ethyl caproate), 4: ethyl 3-methylbutanoate (ethyl isovalerate), 5: ethyl 2-methylpropanoate (ethyl isobutyrate).

further, we chose $\log_2 = 1$ or $-1$ on the x-axis and $\log_{10} = 0.1301$ on the y-axis (corresponding to $p = 0.05$) as the thresholds that defined constituents with large variations between samples (Tables 1 and S7).

**Influence of replacing yeast with ethanol** After fermentation by the lactic acid bacterium, a yeast-added moromi and an ethanol-added moromi were prepared. The constituents that increase due to the effect of yeast should only increase in the yeast-added moromi, while the constituents that increase with the addition of ethanol should increase in both moromi, since yeast also produces ethanol during fermentation. Therefore, it is predicted that the constituents that are more pronounced in yeast added-moromi are influenced only by yeast, and the constituents that appear in both moromi are solely influenced by ethanol. In this comparison, the number of constituents considered to be significant from the volcano plot (Table S7) was 35. The selected constituents are plotted in the same way on the end of the loading plot of the PCA, which shows that they are characteristic constituents for each condition (Tables S5 and S6).

The largest difference between the yeast and ethanol additions was the production of 1-butanol (Fig. 4). It has been reported that *Saccharomyces cerevisiae* produces 1-butanol (29), and the detection of this constituent has also been reported in studies regarding the aroma of soy sauce (30–32). Even in moromi, it is possible that 1-butanol is produced by yeast from amino acids that are derived from soybeans. Moreover, the alcohols 2-methyl-1-propanol (isobutyl alcohol, Fig. 4), 2-methyl-1-butanol (active amyl alcohol, Fig. S1), 3-methyl-1-butanol (isoamyl alcohol, Fig. S1), 2-phenylethyl alcohol (Fig. S1), and 2-(4-hydroxyphenyl)ethanol (Fig. S1), and 3-(methylthio)-1-propanol (methionol, Fig. S1) were also selected. These constituents are generated from the corresponding amino acids valine (Fig. 4), isoleucine (Fig. S2), leucine (Fig. S2), phenylalanine (Fig. S2), tyrosine (Fig. S2), and methionine (Fig. S2), respectively, by the Ehrlich pathway (33,34). According to these studies, it is reasonable to assume that these alcohols are produced by the addition of yeast herein.

Glycerol (Fig. 4), which was also selected as a significant constituent, is produced by yeast as a compatible solute in soy sauce moromi (35). It imparts sweetness and can possibly change the flavor of soy sauce through its production by yeast.

Most furanones that are important for the soy sauce aroma also increased in the presence of yeast. Production of 5(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2*H*)-furanone (HEMF, Fig. 4) by yeast has previously been reported (12). Furthermore, increases of dihydro-5-methyl-2(3*H*)-furanone (*gamma*-valerolactone, Fig. S3), which is present in coffee and has a sweet/vanilla aroma (36), and 5-ethyldihydro-2(3*H*)-furanone (Fig. S3), which has a herb, green, incense (37), suggested that yeast is involved in furanone production. Additionally, 4-hydroxy-5-methyl-furanone (Fig. S3), which has a caramel-like, sweet aroma (38), decreased significantly when yeast was present. Although an increase in 4-hydroxy-5-methyl-furanone has been reported due to the browning reaction, there are no such reports related to yeast. The correlation between furanones

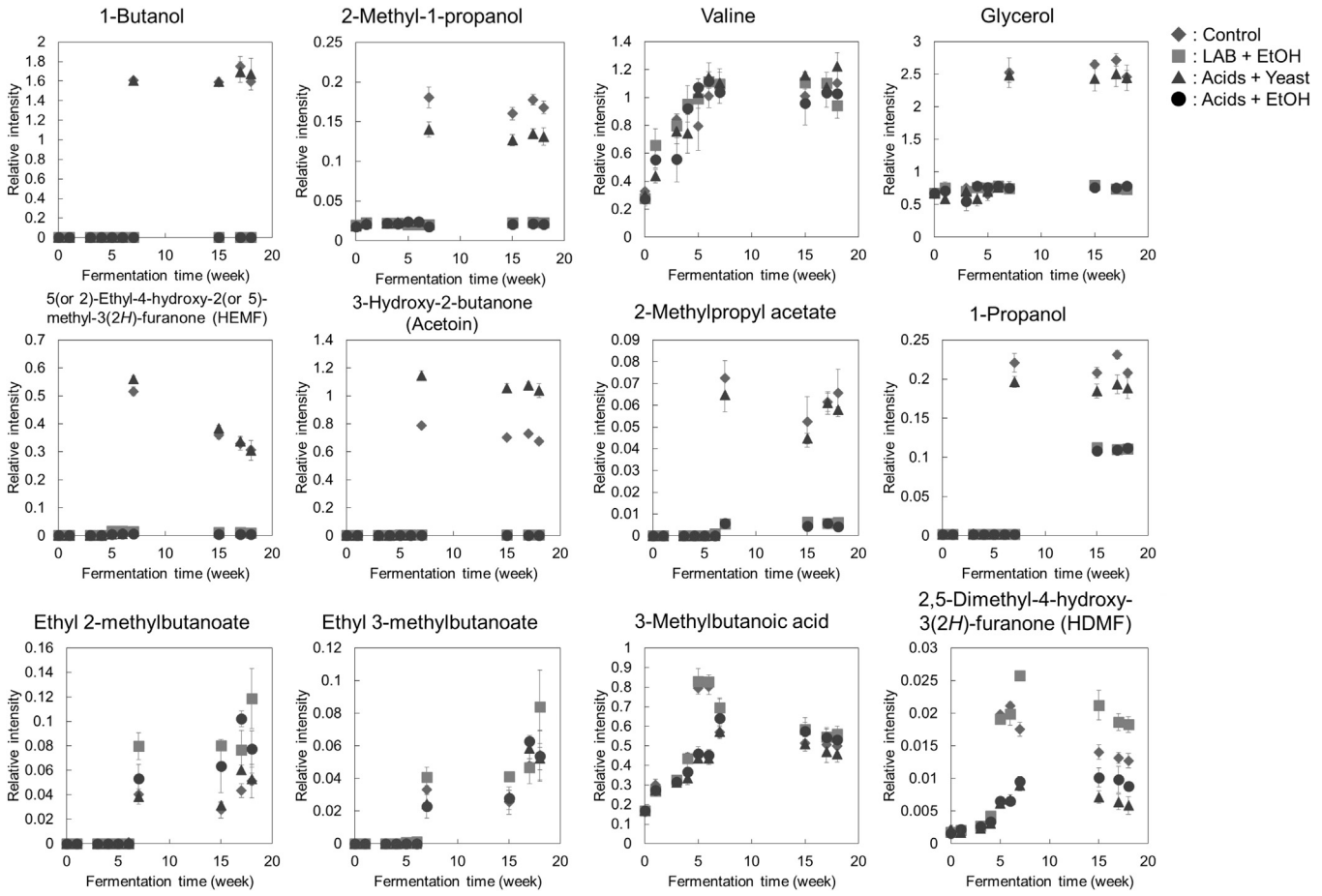


FIG. 4. Constituent changes during fermentation. The vertical axis shows the relative intensity (normalized using an internal standard) of each constituent, and the horizontal line indicates fermentation time (week). The error bars show the standard deviations for three independent experiments. Diamonds represent control samples inoculated with *Z. rouxii* and *T. halophilus*; squares represent LAB + EtOH samples inoculated with *T. halophilus* and with added ethanol; triangles represent Acids + Yeast samples with added acetic and lactic acids and inoculated with *Z. rouxii*. Circles represent the Acids + EtOH samples with added lactic acid, acetic acid, and ethanol. Samples were retrieved at 10 time points (0, 1, 3, 4, 5, 6, 7, 15, 17, and 18 weeks) and analyzed by GC/MS.

such as *gamma*-valerolactone, 5-ethyldihydro-2(3*H*)-furanone, and 4-hydroxy-5-methyl-furanone and yeast is newly suggested from these results.

3-Hydroxy-2-butanone (acetoin, Fig. 4) and 2,3-butanediol (Fig. S4) showed significant differences upon addition of yeast which was 10 times greater than with the addition of ethanol. *S. cerevisiae* produces acetoin and 2,3-butanediol instead of ethanol in a high salt environment (39). In addition, 2,3-butanedione (diacetyl, Fig. S4), which is converted to acetoin when reduced by butanediol reductase, was present in the yeast-added samples. Increases in these constituents have also been reported in soy sauce yeast (2), thus the participation of yeast in their production is strongly supported.

Some acetates were increased only in the presence of yeast. 2-Methylpropyl acetate (isobutyl acetate, Fig. 4), butyl acetate (Fig. S5), 3-methylbutyl acetate (isoamyl acetate, Fig. S5), 2-methylbutyl acetate (Fig. S5), and *n*-propyl acetate (Fig. S5) could be produced by reacting 2-methyl-1-propanol (Fig. 4), 1-butanol (Fig. 4), 3-methyl-1-butanol (Fig. S1), 2-methyl-1-butanol (Fig. S1), and 1-propanol (Fig. 4), respectively, with acetic acid. Since these precursors are produced by yeast, it is reasonable that acetates only increased in the presence of yeast. Acetate esters have various fruity aromas, for example butyl acetate (apple) (40), isoamyl acetate (banana-like aroma) (23), 2-methylbutyl acetate (fruity, apple) (41), isobutyl acetate (fruity aroma) (23), *n*-propyl acetate (fruity, floral) (41). These are important aroma constituents not only in fruits, but also in wine (42), beer (23), and sake (43). In *S. cerevisiae*, these acetates are formed from alcohol and acetyl-CoA precursors (23). In sake and beer, some acetate esters are produced by the action of alcohol acetyltransferase (AATFase). There are studies that have reported AATFase activity in soy sauce as well (44). Therefore, the generation of acetate esters by a similar mechanism is likely in soy sauce (2).

Conversely, ethyl 2-methylbutanoate (Fig. 4) and ethyl formate (Fig. S6) decreased in the presence of yeast. It has been previously reported that yeast produce esterase which degrade esters (45), and thus it is not surprising that these ethyl esters decreased in the presence of yeast. However, other detected ethyl esters did not differ between the yeast-added and ethanol-added samples, suggesting that those ethyl esters increased in the presence of ethanol. Volcano plots also showed that the constituents located in the center of the plot exhibit little difference with the addition of yeast and ethanol (Fig. 3C).

Ethyl 3-methylbutanoate (ethyl isovalerate, Fig. 4), ethyl benzoate (Fig. S7), ethyl 2-methylpropanoate (ethyl isobutyrate, Fig. S7), ethyl 2-methylbutanoate (Fig. 4), ethyl hexanoate (ethyl caproate, Fig. S7), and ethyl butanoate (Fig. S7) could be increased nonenzymatically through dehydration condensation with the corresponding acids, 3-methylbutanoic acid (isovaleric acid, Fig. 4), benzoic acid (Fig. S8), 2-methylpropanoic acid (isobutyric acid, Fig. S8), 2-methylbutanoic acid (Fig. S8), hexanoic acid (caproic acid, Fig. S8), and butanoic acid (butyric acid, Fig. S8), respectively,

and ethanol during fermentation. Additionally, in our previous report (19), among the soy sauce samples produced without microbes or chemical addition, none of these ethyl esters were present (data not shown), suggesting that they are not originally present in the moromi.

From the results of this study, it was found that more than half of the alcohols, furanones, and acetates excluding ethyl esters found in the microbial samples were absent with the addition of ethanol alone. Among these constituents, some furanones and acetates not previously discussed in the context of soy sauce yeast were also included. It was confirmed that fermentation with yeast plays an important role in soy sauce fermentation producing constituents that give soy sauce its characteristic aromas. Conversely, it was revealed that ethyl esters were formed without yeast, and these constituents are present in the moromi as long as ethanol is also present, even if the microbial strain is changed. Therefore, these ethyl esters could be controlled by varying the concentrations of the ethanol and organic acid precursors.

**Influence of replacing lactic acid bacterium with lactic and acetic acids** Lactic acid bacterium-added moromi and lactic and acetic acids-added moromi were prepared, and yeast was added to each moromi. The constituents that increase due to the influence of the lactic acid bacterium should increase only with the addition of lactic acid bacterium. Conversely, the constituents that increase with the addition of lactic and acetic acids should increase in both kinds of moromi, since lactic acid bacterium produces both lactic and acetic acids. Five constituents were selected by the volcano plot of the lactic acid bacterium vs. lactic and acetic acids (Table 1). For yeast, the increases in most constituents occurred only in the presence of yeast, and the effect of adding ethanol was remarkable only among the ethyl esters. However, in the case of the lactic acid bacterium, most of the constituents, including those noted in our previous paper (i.e., 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), 2-furancarboxaldehyde (furfural), 2-furanmethanol (furfuryl alcohol), and 3-(methylthio)-1-propanal (methional) (19)), showed little change between the treatments. Thus, the production of lactic and acetic acids had a significant influence on the constituent profile. As with the comparison between yeast and ethanol, the selected constituents were plotted at the end of the loading plot of the PCA in the samples containing lactic acid bacterium or lactic and acetic acids. This result confirmed these constituents were characteristic of each sample (Tables S5 and S6).

2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (HDMF), an important aroma constituent was not selected as it depended on the presence or absence of lactic acid bacterium (19). However, in the results of this study, the intensities of the constituents were higher in the samples with lactic acid bacterium (control and LAB + EtOH) than in the other samples, and was more pronounced in the LAB + EtOH samples. Therefore, the addition of lactic acid bacterium, and changes in the moromi environment due to the increase in ethanol concentration could influence the production of HDMF.

According to these results, it can be proposed that the lactic acid bacterium may be correlated with HDMF production. On the other hand, most constituents did not show a significant change between samples containing lactic acid bacterium and those with only acids. This revealed that many constituents are influenced by the lactic and acetic acids produced by the lactic acid bacterium.

**TABLE 1.** Selected constituents by volcano plot (influence of lactic acid bacterium addition and acids addition).

| Constituent name | Log2 (LAB/acids) | −Log10 (*p*-value) |
|---|---|---|
| Ornithine | 1.735 | 3.255 |
| 2,5-Dimethyl-4-hydroxy-3 (2*H*)-furanone (HDMF) | 1.120 | 2.617 |
| Citric acid + isocitric acid | −3.628 | 5.585 |
| Fructose | −1.731 | 2.784 |
| Arabinose | −1.460 | 3.750 |

**Conclusion** The results of this study based on GC/MS metabolomics allowed the clarification of constituents influenced by fermentation by lactic acid bacterium and yeast, and those more chemically influenced by the presence of lactic acid, acetic acid, and ethanol. Specifically, ethyl esters, which were historically thought to be related to yeast (46), increased even in the presence of ethanol alone, confirming that ethyl esters could be produced nonenzymatically during fermentation. However, the importance of yeast in soy sauce fermentation was confirmed, because many volatiles, such as acetates, are not produced by ethanol alone. HDMF, a key aroma constituent in soy sauce, was newly revealed to be related to the presence of lactic acid bacteria. Moreover, these results suggested that lactic and acetic acids produced by lactic acid bacteria influenced the moromi constituent profile.

To our knowledge, this study is the first to compare the influence of microbes on the constituent profile with that of lactic acid, acetic acid, and ethanol alone. The findings obtained in this study will be useful for further analysis of the involvement of microbes in the production of each aroma constituent.

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