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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.

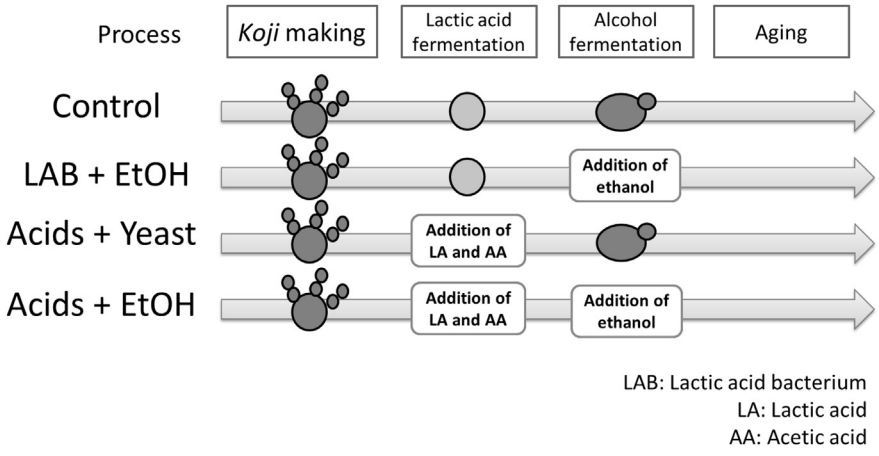

FIG. 1. Moromi conditions during fermentation. *Koji* mold, *Aspergillus sojae* NBRC 4239; lactic acid bacterium, *Tetragenococcus halophilus* NBRC 12172; yeast, *Zygosaccharomyces rouxii* NBRC 1876.