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Metabolite profile of *koji* amazake and its lactic acid fermentation product by *Lactobacillus sakei* UONUMA

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The *koji* amazake is a traditional sweet Japanese beverage. It has been consumed for over a thousand years in Japan; nonetheless, little is yet known of the ingredients in *koji* amazake. Therefore, this study aimed to analyze the metabolites of *koji* amazake using a metabolomics approach. Additionally, we reformed the flavor of *koji* amazake by lactic acid fermentation (LAF-amazake) using *Lactobacillus sakei* UONUMA, which was isolated from snow caverns. The purpose of this article is to identify the ingredients in these beverages. In LAF-amazake and *koji* amazake, sugars, amino acids, organic acids, and vitamin B complex were determined in the two beverages, and over 300 compounds were detected in total. Thirteen saccharides were identified including two unknown trisaccharides, and there were no differences in these between the two beverages. In LAF-amazake, lactic acid, vitamin B2 (riboflavin), B3 (nicotinic acid and nicotinamide), and B6 (pyridoxine) were significantly increased as compared to *koji* amazake, whereas malate and glutamine decreased. These results suggested that LAF, malolactic fermentation, and glutamine deamidation occurred simultaneously in LAF-amazake. *L. sakei* UONUMA strains produced these vitamins. Moreover, it was surprising that acetylcholine, a well-known neurotransmitter, was newly generated in LAF-amazake. Here, we have succeeded in reforming the flavor of *koji* amazake and obtained these metabolic data on the two beverages. The present study could provide useful basic information for promoting functional analyses of *koji* amazake and LAF-amazake for human health.

[**Key words:** Amazake; *Koji*; *Lactobacillus sakei*; Lactic acid fermentation; Metabolomics]

Amazake is a traditional sweet Japanese beverage. There are two types depending on the preparation method: (i) made from rice *koji* (*koji* amazake, in Japanese); and (ii) Japanese sake lees (*sakekasu* amazake). In particular, *koji* amazake has been consumed for over a thousand years, and its first appearance was in the second oldest book of Japanese history "The Chronicles of Japan (*Nihon Shoki*)". *Koji* amazake is manufactured from rice *koji*, which is made by *Aspergillus oryzae* growing on steamed rice, water, and cocked rice in some cases. The glucose concentration of *koji* amazake reaches approximately 20% because the starch in rice *koji* is saccharized by enzymes such as α-amylase and glucoamylase produced by *A. oryzae*. The content of sugars, amino acids, and organic acids in *koji* amazake has been previously reported (1–3). However, little is yet known about other ingredients in *koji* amazake. In functional analyses, Oura et al. (4) reported anti-obesity, anti-hypertension, and anti-amnesic effects of *sakekasu* amazake that might be caused by dietary fibers and peptides. A recent study has shown that intake of *koji* amazake as a late evening drink improved the subjective symptoms of patients with liver cirrhosis (5). This report concluded that branched-chain amino acids in *koji* amazake influence the local immune system of the liver in cirrhotic patients. Thus, the beneficial effects of amazake on human health have been widely studied; however, the functional substances are still unclear. Therefore, it is also important to investigate metabolites of amazake to explore the functional substances influencing human health.

In addition, we attempted to reform the flavor and ingredients of *koji* amazake by lactic acid fermentation using *Lactobacillus sakei* UONUMA strains, which were isolated from Japanese pickles in snow caverns in the Uonuma region, Niigata, Japan (6). Lactic acid bacteria and its fermentation products were focused on, including their beneficial effects on health such as improving lipid metabolism (7), decreasing blood pressure (8), and immunomodulation (9). *L. sakei* is a psychrotrophic lactic acid bacteria that is important, not only for sake brewing, but also for production of dry sausage and meat/fish products. Furthermore, *L. sakei* has biotechnological potential for biopreservation (10) and immunomodulation. Masuda et al. (11,12) reported that the *L. sakei* LK-117 strain induced a sustained increase in IL-12p40 production (11) and reduced the development of atopic dermatitis-like skin lesions in a manner independent of IgE plasma levels in the NC/Nga mouse (12).

The aim of the present study was to identify the metabolites of *koji* amazake and lactic acid fermentation amazake (LAF-amazake) using a metabolomics approach as a screening method. These metabolic data would be helpful in promoting functional analyses of *koji* amazake and LAF-amazake in human health.

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MATERIALS AND METHODS

Bacterial strains, characterization The bacterial strains used in this study were *L. sakei* UONUMA-1, -2 and -3, originally isolated from a naturally fermented Japanese pickle (*nozawana-zuke*) in snow caverns. Bacterial identification was performed by 16S rRNA gene amplification. After harvesting, bacterial cells were extracted using an InstaGene Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified using the universal bacterial primers 10F (5′-AGTTTGATCCTGGCTC-3′) and 1540R (5′-AAGGAGGTGATCCAGCC-3′) designed by Sato et al. (13) with TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan). PCR conditions were as follows: a denaturing step at 95°C for 3 min, amplification for 35 cycles with denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 90 s, and then a final extension step at 72°C for 3 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The DNA sequences of the PCR products were determined using the Fasmac DNA Sequence Service (FASMAC Co., Ltd., Atsugi, Japan). The 16S rRNA gene sequence was compared with those available in the public databases of the DNA Databank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/index-j.html). Sugar fermentation patterns of *L. sakei* UONUMA isolates were determined using the API 50 CH system (bioMerieux, Marcy l'Etoile, France).

Identification of *L. sakei* and *Lactobacillus curvatus* species was performed by the PCR using the pairs of primers that can be used to specifically detect, respectively (14). In detection of *L. sakei*, the extracted genes were amplified using the primers 5′ sak-cur (5′-GCTGGATCACCTCCTTTC-3′) and 3′ sak (5′-ATGAAACTATTAAATTGGTAC-3′) with KOD FX (Toyobo Co. Ltd., Osaka, Japan). In detection of *L. curvatus*, the extracted genes were amplified using the primers 5′ sak-cur and 3′ cur (5′-TTGGTACTATTTAATTCTTAG-3′). PCR conditions were as follows: a denaturing step at 94°C for 3 min, amplification for 35 cycles with denaturation at 94°C for 15 s annealing at 53°C for 30 s and extension at 68°C for 2 min, and then a final extension step at 68°C for 7 min. The PCR product was verified with ethidium bromide-stained agarose gels. The optical types of lactic acid produced by *L. sakei* UONUMA isolates were determined by the method described by Okada et al. (15).

Preparation of *koji* amazake and LAF-amazake The *koji* amazake used was obtained as a commercial product (Hakkaisan Brewery Co., Ltd., Minamiuonuma, Japan). It was sterilized at 85°C for 30 min. The LAF-amazake sample was prepared by mixing 1×10^6 cfu/g of *L. sakei* UONUMA strain mixture and *koji* amazake, which was then incubated at 25°C for 24 h. The koji amazake for analysis was also incubated at 25°C for 24 h.

Analysis methods for sugars, organic acids, amino acids, soluble vitamins, ergothioneine, acetylcholine, and choline For the separation of sugars, soluble vitamins, ergothioneine (EGT), acetylcholine (ACh), choline (Ch), and betaine (BT), ultra-performance liquid chromatography (UPLC) analysis was performed with an ACQUITY H-Class UPLC system equipped with an ACQUITY QDa detector and/or a Waters 2998 Photodiode Array (PDA) detector (Waters Corporation, Milford, MA, USA). Sugar separation was performed using a Waters UPLC BHE Amide column (1.7 μm, 3.0 × 150 mm). Solvent A was water, B was acetonitrile, C was acetone, and D was 4% ammonia water. The quaternary mobile phase gradient for analysis was as follows: initial, 0% A, 47.5% B, 47.5% C, 5% D (0–7.5 min); 12% A, 41.5% B, 41.5% C, 5% D (7.5–23 min); 15% A, 40% B, 40% C, 5% D (23–33 min); 35% A, 30% B, 30% C, 5% D (33–43 min); 0% A, 47.5% B, 47.5% C, 5% D (43–43.5 min); 0% A, 47.5% B, 47.5% C, 5% D (43.5–54.5 min); 0% A, 47.5% B, 47.5% C, 5% D (54.5–54.9 min). The flow-rate was 0.25 mL/min from 0 to 43 min and 0.8 mL/min from 43 to 54.5 min and kept at 0.25 mL/min for a period of 54.9 min. Other parameters adopted were as follows: column temperature, 40°C; sample temperature, 10°C; injection volume 5 μL. Mass spectrometry (MS) conditions adopted were as follows: ionization mode, electrospray ionization (ESI)-negative; acquisition, selected ion recording (SIR); capillary voltage, 0.8 kV; cone voltage, 15 V; probe temperature, 450°C; mass range, 150–1250 *m/z*. For soluble vitamin separation, a Waters UPLC HSS T3 column (1.8 μm, 2.1 × 150 mm) was used. Solvent A was 10 mM ammonium formate containing 0.1% formic acid in water, and B was 10 mM ammonium formate containing 0.1% formic acid in methanol. The duality mobile phase gradient for analysis was as follows: initial, 99% A, 1% B (0–4.65 min); 95% A, 5% B (4.65–7.65 min); 80% A, 20% B (7.65–10.65 min); 2% A, 98% B (10.65–13.65 min); 99% A, 1% B (13.65–26.65 min). The flow-rate was 0.3 mL/min. Other parameters adopted were as follows: column temperature, 30°C; sample temperature, 10°C; injection volume 5 μL. ACQUITY UPLC PDA detector conditions adopted were as follows: detection and UV spectra, 270 nm; and scanning, 210–400 nm. ACQUITY QDa detector conditions adopted were as follows: ionization mode, ESI-positive; acquisition, SIR; capillary voltage, 0.8 kV; cone voltage, 15 V; probe temperature, 600°C; mass range, 50–800 *m/z*. For sample preparation, *koji* amazake and LAF-amazake were centrifuged at 18,400 ×*g* for 5 min at room temperature and supernatants were diluted 10-fold with water. The diluted samples were analyzed by UPLC. For EGT separation, a Waters ACQUITY UPLC HSS C18 column (1.8 μm, 2.1 × 150 mm) was used. Solvent A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile. The mobile phase gradient for analysis was as follows: initial, 99% A, 1% B (0–1.50 min); 30% A, 70% B (1.50–2.40 min); 99% A, 1% B (2.40–2.50 min); 99% A, 1% B (2.50–6.00 min). The flow-rate was 0.45 mL/min. Other parameters adopted were as follows: column temperature, 30°C; sample temperature, 6°C; injection volume 5 μL. MS conditions adopted were as follows: ionization mode, ESI-positive; acquisition, SIR; capillary voltage, 0.6 kV; cone voltage, 10 V; probe temperature, 600°C; mass range, 50–600 Da. For ACh, Ch, and BT separation, a Waters CORTECS UPLC HILIC column (1.6 μm, 2.1 × 100 mm) was used. Solvent A was 100 mM ammonium formate (pH 3.0), and B was acetonitrile. The mobile phase gradient for analysis was as follows: initial, 10% A, 90% B (0–0.75 min); 40% A, 60% B (0.75–1.00 min); 70% A, 30% B (1.00–1.25 min); 70% A, 30% B (1.25–1.90 min); 10% A, 90% B (1.90–2.00 min); 10% A, 90% B (2.00–2.50 min). The flow-rate was 0.5 mL/min. Other parameters adopted were as follows: column temperature, 45°C; sample temperature, 6°C; injection volume, 5 μL. MS conditions adopted were as follows: ionization mode, ESI-positive; acquisition, SIR; capillary voltage, 0.8 kV; cone voltage, 10 V; probe temperature, 450°C; mass range, 50–600 *m/z*.

For the separation of organic acids, the HPLC analysis was performed with an Alliance 2695 separation module and a 2998 PDA detector with Empower 3 software for data acquisition (Waters). Organic acid separation was achieved using a Waters Atlantis T3 column (3 μm, 4.6 × 150 mm). Solvent A was 20 mM phosphate buffer (pH 2.5) and B was acetonitrile. The duality mobile phase gradient for analysis was as follows: initial, 100% A, 0% B (0–4.65 min); 95% A, 5% B (4.65–7.65 min); 80% A, 20% B (7.65–10.65 min); 2% A, 98% B (10.65–13.65 min); 99% A, 1% B (13.65–26.65 min). The flow-rate was 1.0 mL/min. Other parameters adopted were as follows: column temperature, 35°C; sample temperature, 5°C; injection volume 10 μL. A Sep-Pak Accell Plus QMA Cartridge (Waters) was used to separate the organic acids. After *koji* amazake and LAF-amazake were centrifuged at 18,400 ×*g* for 5 min at room temperature, supernatants were mixed with 7 mL of acetone and filtered through 0.45-μm pore sized hydrophilic fluorocarbon polymer membranes (Advantec, Tokyo, Japan). The solution was further filtered through a Sep-Pak QMA cartridge following washing with 10 mL of water three times and eluted with 3 mL of 0.1 M HCl. The eluted fraction was analyzed by HPLC.

For the separation of amino acids, analysis was performed with a Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan). *Koji* amazake and LAF-amazake were centrifuged at 18,400 ×*g* for 5 min, and the supernatant was added to an equal part of 10% trichloroacetic acid solution. After centrifugation at 18,400 ×*g* for 5 min, supernatant was diluted five times with 0.02 M HCl and 20 μL was injected. The amino acid compositions were then obtained by automatic amino acid analysis. The amino acid analyzer-attached HITACHI HPLC 2622 PF Packed Column (4.6 mm × 60 mm) and UV detector (VIS: 570 nm, VIS: 440 nm) were used for analysis of amino acids. Wako L-8500 buffer solution PF-1, 4, and RG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used in this study. Twenty microliters of each sample was injected. Amino acid determination was performed using a Ninhydrin Reagent Set (Wako). The analytical method was performed according to the manufacturer's instructions.

Capillary electrophoresis time-of-flight mass spectrometry and liquid chromatography–time-of-flight mass spectrometry metabolomics analysis The metabolome measurements were carried out at a facility at Human Metabolome Technologies Inc. (HMT, Tsuruoka, Japan). The *koji* amazake and LAF-amazake samples (each 80 μL) for capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) were mixed with 20 μL of 1 mM internal standard solution (HMT). Then, the mixed sample was centrifugally filtered through a Millipore 5-kDa cutoff filter for 60 min at 9100 ×*g* at 4°C. The flowthrough fractions were diluted 5-fold by water. The samples (500 μL) for liquid chromatography-TOFMS (LC-TOFMS) were mixed with 1.5 mL of 6 μM methanol containing an internal standard solution (HMT). Then, the mixed samples were centrifuged for 5 min at 2300 ×*g* at 4°C. The supernatant was dried and then dissolved in 100 μL of 50% isopropanol. The prepared samples were diluted 5-fold.

CE-TOFMS was carried out using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 TOFMS, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adaptor kit, and an Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). Cationic metabolites were analyzed with a fused silica capillary (i.d. 50 mm × 80 cm) with cation electrophoresis buffer (H3301-1001, HMT) as the electrolyte. The sample was injected at a pressure of 50 mbar for 10 s. The applied voltage was set at 27 kV. ESI-MS was conducted in the positive ion mode, and the capillary voltage was set at 4000 V. The spectrometer was scanned from *m/z* 50 to 1000. Anionic metabolites were analyzed with a fused silica capillary (i.d. 50 mm × 80 cm) with anionic electrophoresis buffer (H3302-1021, HMT) as the electrolyte. The sample was injected at a pressure of 50 mbar for 25 s. The applied voltage was set at 30 kV. ESI-MS was conducted in the negative ion mode, and the capillary voltage was set at 3500 V. The spectrometer was scanned from *m/z* 50 to 1000.

LC-TOFMS was carried out using an Agilent 1200 series RRLC System SL equipped with an Agilent LC/MSD TOF (Agilent Technologies). The system was run in gradient mode using an ODS column (2 μm, 2 × 50 mm) set at 40°C. Solvent A was 0.1% formic acid containing H_2O and solvent B was 0.1% formic acid and 2 mM ammonium hydrogen carbonate containing isopropanol:acetonitrile:H_2O at 60:30:5; the flow rate was 0.3 mL/min. The gradient was set as follows: 1% B (0–0.5 min), linearly increasing to 100% B (from 13.5 min) and to 100% B at 20 min. MS analysis was carried out in both positive and negative ion ESI modes of detection. The operating parameters were: drying gas (N_2) flow rate, 10 L/min; drying gas temperature, 350°C; nebulizer pressure, 40 psi; capillary voltage, 3500 V. The mass scanning range was *m/z* 100–1700. Metabolome raw data were processed with

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