Food Chemistry 258 (2018) 25–34

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

NMR- and GC/MS-based metabolomic characterization of *sunki*, an unsalted fermented pickle of turnip leaves

Satoru Tomita[a,*], Toshihide Nakamura[a], Sanae Okada[b]

[a] Food Research Institute, National Agriculture and Food Research Organization (NARO), 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan
[b] Kiso Town Resource Institute, 2326-6 Fukushima, Kisomachi, Kiso, Nagano 397-8588, Japan

ARTICLE INFO

Keywords:
Metabolomics
Fermented foods
Lactic acid bacteria
Lactobacillus
Brassica

ABSTRACT

This study revealed the compositional characteristics of *sunki*, a traditional, unsalted, lactic acid-fermented pickle produced using turnip leaf in Kiso district, Japan. Comprehensive compositional analysis by two metabolomic approaches based on NMR and solid-phase microextraction-GC/MS methods was used to determine its chemical composition by annotating 54 water-soluble and 62 volatile compounds. Principal component analysis showed that samples had different compositions, depending on the agricultural processing factory and production year. This variation potentially resulted from the differences in the lactic acid bacterial community produced during the spontaneous fermentation of *sunki* and in the initial nutritional composition of the turnip leaf. Partial least squares regression revealed that the acetic acid level showed a strong positive correlation with pH ($R = 0.810$), in contrast to the negative correlations of lactic acid and ethanol levels ($R = -0.533$ and -0.547). This indicated the crucial impact of acetic acid-related metabolism on acidification during *sunki* fermentation.

1. Introduction

Japan has a warm and humid climate, and a wide variety of fermented foods and beverages have been developed and inherited over the centuries. Nowadays, fermented food/beverages of Japan, including soy sauce, *miso* (fermented soy bean paste), and *sake* (Japanese rice wine), are recognized worldwide (Tamang, Watanabe, & Holzapfel, 2016). *Sunki* is a unique, traditional fermented pickle, which is only produced in the Kiso district, an inland mountainous region of Nagano Prefecture, Japan. It is made by fermenting red turnip (*Brassica rapa* L.) leaves with lactic acid bacteria (LAB) (Tamang et al., 2016). In general, salt plays a central role in pickle fermentation, as it suppresses the growth of undesirable spoilage microorganisms and promotes the release of water and nutritional compounds from the cytoplasm of vegetable plants (Henney, 2010). *Sunki* is unique in that the fermentation process occurs under unsalted conditions, in contrast to most vegetable pickles that are produced using salt. Even today, *sunki* is produced every year at the beginning of winter. In recent years, the demand for *sunki* is increasing because of the increasing awareness among the consumers about the need to reduce sodium intake and because of the health-promoting effects of LAB. This increasing demand is also making an important contribution to the growth of the rural economy.

While *sunki* is highly valuable as part of the Japanese food culture, as a biological resource, and as a product stimulating the rural economy, academic research into its fermented food properties is highly limited. However, several studies have investigated the chemical composition of *sunki*. Itabashi et al. have analyzed the levels of three organic acids (lactic acid [LacA], acetic acid [HOAc], and malic acid [MalA]), amino acids, and volatile compounds (Itabashi, 1982; Itabashi, Kawawa, & Miyao, 1990) in *sunki* samples, and provided initial information for investigating the compositional characteristics of *sunki*. However, only two or three samples were analyzed in these studies, which is insufficient for establishing the chemical composition of *sunki*. It is likely that *sunki* exhibits a large compositional variety due to the following reasons: i) it is produced by spontaneous fermentation of the first-generation batch of the year and subsequently subcultured by adding a portion of this batch to the next processing batch; ii) it is produced on a small scale in homes and many small agricultural processing factories; and, iii) it is produced using three different local turnip varieties, depending on the region of the Kiso district. Therefore, to provide a compositional overview of *sunki* as a fermented food, the chemical characteristics of a larger set of samples from different places need to be investigated through a comprehensive analysis. Moreover, it is also important to survey the metabolites responsible for the pH value, because it is a practicable marker for assessing the progress of the desired fermentation of *sunki*.

* Corresponding author.
E-mail address: satorutomita@affrc.go.jp (S. Tomita).

https://doi.org/10.1016/j.foodchem.2018.03.038
Received 30 June 2017; Received in revised form 7 March 2018; Accepted 10 March 2018
Available online 12 March 2018

In recent years, the dramatic progress in instrumental analysis has enabled the development of metabolomic approaches, which combine comprehensive compositional analysis by high-throughput analytical instruments and multivariate statistical analysis to extract the compositional differences in large datasets (metabolite profile). The metabolomic approach has been globally applied in studies on representative fermented foods and beverages containing LAB, such as yoghurt, cheese, wine, soy sauce, *miso*, *sake*, and *kimchi*, providing comprehensive information on their compositional characteristics (Hong, 2011; Lu, Hu, Miyakawa, & Tanokura, 2016; Ochi, Naito, Iwatsuki, Bamba, & Fukusaki, 2012; Park et al., 2016; Shiga et al., 2014; Sugimoto et al., 2012; Yoshida, Yamazaki, Ozawa, Mizukoshi, & Miyano, 2009). Moreover, these studies successfully highlighted some of the metabolites involved in the important microbial activities of LAB, including malolactic fermentation for wine production (Hong, 2011), proteolytic activity during yoghurt production (Settachaimongkon et al., 2014), and promotion of ripening during cheese production (Ochi et al., 2013).

In the present study, we applied metabolomics using two different non-targeted analytical techniques to the investigation of the chemical characteristics of *sunki* samples. The analysis was based on the comprehensive metabolite profiles of water-soluble and volatile flavor compounds obtained by nuclear magnetic resonance (NMR) spectroscopy and headspace solid-phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS), respectively. Herein, we describe the detailed chemical composition of *sunki* and provide an overview of its compositional variety, based on the data obtained using samples from eight agricultural processing factories over two years.

2. Materials and methods

2.1. Sampling of sunki pickle

Sunki samples produced in November-December in 2015 and 2016 were provided from eight agricultural product-processing factories (A–H) in Kiso district, Japan, and were stored in a freezer at −20 °C until use. Samples used in this study, and their pH and Na^+ concentration are listed in Table S1. The pH and Na^+ concentration were determined using a LAQUA F-72 and a LAQUAtwin Na-11 (Horiba, Kyoto, Japan), respectively. Liquid part of *sunki* pickle was used for the compositional analysis because of its advantages of high homogeneity and ease of analytical sample preparation as compared to lyophilized sample. A preliminary spectral comparison confirmed that the signal patterns of ^{1}H NMR spectra were sufficiently consistent between the samples of liquid part and lyophilized *sunki* (Fig. S1). The samples were used for NMR and SPME-GC/MS analyses in a single analytical replicate.

2.2. NMR analysis

Water-soluble compounds of *sunki* pickle were analyzed by NMR spectroscopy. To prepare an analytical sample of pickle liquid, 140 µL of the clear supernatant was diluted with 560 µL of 125 mM potassium phosphate buffer (KPi), consisting of K_2HPO_4/KH_2PO_4 (pH 7.0) in deuterium oxide (D_2O; 99.9% D; Cambridge Isotope Laboratories, Andover MA, USA). After centrifugation, the supernatant was transferred to a 5 mm O.D. NMR tube (Norell, Landisville, NJ). Pickled leaf samples were lyophilized for one week and then ground into fine powder. Water-soluble compounds in leaf samples were extracted by suspending 10 mg of the dried powder in 700 µL of 100 mM KPi in D_2O followed by vortexing for 5 min at 25 °C. After centrifugation, the supernatant was transferred to an NMR tube through a simple surgical cotton filter to remove the suspended debris. For these NMR samples, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS-d_6; Cambridge Isotope Laboratories) was used as an internal standard at a concentration of 1.0 mM. Proton (^{1}H), carbon (^{13}C), and 2D NMR spectra were recorded on an Avance-500 spectrometer (Bruker BioSpin, Karlsruhe, Germany), using previously described acquisition parameters and conditions (Tomita et al., 2015). Metabolite annotation was facilitated by analyzing the NMR spectra measured using an Avance-800 spectrometer (Bruker BioSpin) and by referring to public NMR spectral databases (SpinAssign program in the PRIMe web service, http://prime.psc.riken.jp; Human Metabolomics Database, http://www.hmdb.ca; Biological Magnetic Resonance Data Bank, http://www.bmrb.wisc.edu).

2.3. SPME-GC/MS analysis

Volatile compounds of *sunki* pickle were investigated using a GCMS-QP2010 Ultra instrument (Shimadzu, Kyoto, Japan) equipped with an AOC-5000 autosampler (Shimadzu). A SUPELCO 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane fiber (2 cm length; Sigma-Aldrich, St. Louis, MO, USA) was used for SPME. *Sunki* pickle liquid (1 mL) was transferred to a 20-mL screwcap vial and kept at 4 °C during waiting time prior to measurement. The vial was preheated in the heating unit of the autosampler at 50 °C for 10 min, with agitation at 250 rpm. Headspace volatile compounds were first captured by exposing the SPME fiber for 20 min and then desorbed for 5 min in an injection port operated at 250 °C in splitless mode. The injected compounds were separated on an Rtx-WAX capillary column (60 m × 0.25 mm I.D. × 0.25 µm film thickness; Restek, Bellefonte, PA, USA) with a carrier gas (helium) at a flow rate of 2 mL/min. The column temperature was isothermally held at 40 °C for 5 min and then raised by 5 °C/min to 180 °C, followed by 10 °C/min to 200 °C, and held for 5 min. The MS analysis was operated in the electron ionization mode with the following parameters: ionization energy of 70 eV; ion source temperature of 230 °C; interface temperature of 250 °C; and, scan range of 33–350 *m/z*. The retention index was calibrated using the alkane standard solution C_6–C_{20}. Peak annotation was performed by comparing the mass spectra and retention index with those in the NIST 02 MS library (National Institute of Standards and Technology, Gaithersburg, MD, USA). When appropriate, the MassBank spectral database (http://www.massbank.jp) was also used.

2.4. Dataset preparation for non-targeted metabolomics

Non-targeted, NMR-based metabolomics was performed to characterize the *sunki* samples based on their water-soluble metabolite profiles. Prior to dataset preparation from the ^{1}H NMR spectra, it was confirmed that there were no crucial chemical shift fluctuations, which lead to significant impact on data interpretation. To prepare a dataset for multivariate analysis, processed ^{1}H NMR spectra were subdivided into 0.04 ppm width integral regions (buckets) in the 10.0–0.50 ppm spectral range. Twelve buckets containing the residual solvent signal, ranging from 5.16 to 4.68 ppm, were excluded from the analysis. To correct the difference in concentration between the pickle liquid samples, the buckets were normalized to the total intensity of the NMR spectrum of each sample. The generated dataset for NMR-based metabolomics comprised 226 buckets. For characterization of volatile metabolite profiles by GC/MS-based metabolomics, a dataset was prepared as described previously (Iijima et al., 2016). Briefly, GC/MS raw data were processed by GCMSsolution software (Shimadzu) and converted into AIA files. Baseline correction and peak alignment were carried out using MetAlign software (Lommen, 2009) and the convergence of *m/z* ions for a single compound was performed using AIoutput software (Tsugawa et al., 2011). The variable derived from HOAc was excluded from the dataset since it showed saturated peaks in all samples. The generated dataset was comprised 357 peaks.

2.5. Statistical analysis

To analyze the difference in metabolite profiles among the *sunki* samples, principal component analysis (PCA) was performed with the