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Evaluating the Headspace Volatolome, Primary Metabolites, and Aroma Characteristics of *Koji* Fermented with *Bacillus amyloliquefaciens* and *Aspergillus oryzae* S

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Production of good *Koji* primarily depends upon the selection of substrate materials and fermentative microflora, which together influence the characteristic flavor and aroma. Herein, we performed comparative metabolomic analyses of volatile organic compounds (VOCs) and primary metabolites for *Koji* samples fermented individually with *Bacillus amyloliquefaciens* and *Aspergillus oryzae*. The VOCs and primary metabolites were analyzed using headspace solid phase microextraction (HS-SPME) followed by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). In particular, alcohols, ketones, and furans were mainly detected in *Bacillus*-fermented *Koji* (*Bacillus Koji*, BK), potentially due to the increased levels of lipid oxidation. A cheesy and rancid flavor was characteristic of *Bacillus Koji*, which is attributable to high content of typical 'off-flavor' compounds. Furthermore, the umami taste engendered by 2-methoxyphenol, (E,E)-2,4-decadienal, and glutamic acid was primarily detected in *Bacillus Koji*. Alternatively, malty flavor compounds (2-methylpropanal, 2-methylbutanal, 3-methylbutanal) and sweet flavor compounds (monosaccharides and maltol) were relatively abundant in *Aspergillus*-fermented *Koji* (*Aspergillus Koji*, AK). Hence, we argue that the VOC profile of *Koji* is largely determined by the rational choice of inocula, which modifies the primary metabolomes in *Koji* substrates, potentially shaping its volatolome as well as the aroma characteristics.

Keywords: *Koji* fermentation, *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, mass spectrometry, volatolome, metabolome

Introduction

Fermentation is a traditional method that makes use of the metabolic activities of microorganisms to improve the content of food and beverages with biomolecules associated with flavor, aroma, as well as organoleptic and nutritional properties [1]. The quintessential *Koji* gourmet utilizes solid-state fermentation of various substrates materials such as soybeans, wheat, or rice, with a single microbial inocula [2]. During the course of fermentative growth, inoculum such as bacteria or mold species decompose the substrate, resulting in a modified food product [3]. Undoubtedly, *Koji* represents an important starting ingredient in the manufacturing of a variety of fermented foods and beverages including soybean paste (*doenjang*, *cheonggukjang*, *miso*), soy sauce (*kanjang*), red pepper paste (*gochujang*) and rice wine (*Makgeolli*) [4].

The composition of volatile organic compounds (VOCs) in *Koji* affects its unique fragrance and aroma and determines its quality characteristics as a raw material component of *Koji* fermented foods. Moreover, the VOC composition of *Koji* is considered an important criterion

toward its consumer acceptability, necessitating the comprehensive analysis of the *Koji* volatolome. Previously, a variety of VOCs from rice *Koji* fermented with *A. oryzae* were reported including alcohols, aldehydes, ketones, and ester compounds [5]. Furthermore, the effects of various fermentative microflora on the flavor compounds in wheat *Koji* have been evaluated using a permutation of mold inocula viz., *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Cephalosporium* species [6]. Similarly, multiple classes of VOC compounds have been reported for *Bacillus-Koji doenjang*, using HS-SPME-GC-MS methods including esters, alcohols, acids, aldehydes, ketones, sulfur compounds, pyrazines, and volatile phenols [5].

In recent years, an unprecedented improvement in high throughput instrumentation has enabled multiple sampling, enhanced sensitivity, and greater precision. This, coupled with improved data mining algorithms, has brought forth the development of more conventional approaches for VOC analysis. These methods, including gas chromatography-olfaction (GC-O), simultaneous dissolvent extract (SDE), and head-space solid-phase micro-extract gas chromatography mass spectrum (HS-SPME-GC-MS), have been extremely useful in the analysis of VOCs [7]. SPME is a no-solvent method to extract VOCs from solids, liquids, or even gasses. It is considered a highly reproducible method that has been employed in food aroma and perfumery studies [8, 9]. Importantly, SPME has the ability to extract VOCs from fermented foods, including soy sauce, soy paste, and related beverages [5, 7, 10, 11]. Metabolomics has also been used to evaluate the nutritional quality of foods, plants, or fermentation products [12–14]. Recently, we have reported the time-correlated dynamic metabolomes and VOC profiles for the acetous fermentative production stages of vinegar employing the GC-TOF-MS and HS-SPME-GC-MS analyses [15].

A previous study has comprehensively delineated the discriminant metabolomes for rice *Koji* fermented with two different inocula *i.e.*, *A. oryzae* and *B. amyloliquefaciens*, highlighting the differential effects of fermentative microflora on *Koji* metabolomes [4]. Furthermore, the alterations in respective metabolomes were correlated with corresponding antioxidant levels and enzymatic activities that are considered vital for *Koji* quality characteristics. However, there have been no previous studies probing the VOC compositions of *Koji* fermented with different microflora that intertwine the subtle primary metabolomes with VOC profiles and aroma characteristics.

Considering the importance of fermentative microflora for the *Koji* volatolome, we aim toward comprehensive characterization of the headspace VOCs and primary metabolite profiles for varying *Koji* (soybean and wheat) samples fermented individually with *B. amyloliquefaciens* or *A. oryzae*. Furthermore, the *Koji* metabolome and volatolome were evaluated to correlate the varying fermentative microflora and aroma characteristics for *Koji* end products.

Materials and Methods

Chemicals and Reagents

HPLC-grade methanol, acetonitrile and water were purchased from Fisher Scientific (USA). L-Borneol and sodium chloride were purchased from Sigma-Aldrich (USA).

Microbial Cultures and Koji (Soybean, Wheat) Fermentation

As shown in Table 1, the varying fermentative microflora *i.e.*, *Bacillus amyloliquefaciens* (KCCM 11718P) and *Aspergillus oryzae* (KCCM 11300P) were procured from the CJ *Cheil-Jedang* Co., Ltd. (Korea). Two different substrates, soybean (Glycine max) and wheat (*Triticum aestivum*), were employed for *Koji* preparation. To make *Koji*, 1 kg of substrate was soaked in water for 30 min, and then drained. The soaked substrates were sterilized by autoclaving for 15 min. The steamed substrate (50 g) was inoculated with *A. oryzae* and incubated at 37°C for 5 days toward the preparation of the *Koji* pre-inoculum. The pre-inoculum substrate was mixed with the main steamed substrate (0.2%, w/w), and subjected to fermentation 37°C for 36 h. The bacterial strain *B. amyloliquefaciens* was pre-incubated in 200 ml of nutrient broth (pH 7.0) and incubated at 37°C at 200 rpm for 24 h. The cultured broth, serving as the inoculum, was added to the steamed substrate (2.0%, v/w) and fermented at 37°C for 36 h. Hence, the four different combinations of *Koji* including: a. soybean *Koji* fermented with

Table 1. The information of *Koji* samples.

Inoculum	Substrate	Time (h)	Label	Symbol
-	Soybean	0	RS	●
	Wheat	0	RW	○
B. amyloliquefaciens KCCM 11718P	Soybean	12	BS1	■
	Soybean	24	BS2	▼
	Soybean	36	BS3	▲
	Wheat	12	BW1	□
	Wheat	24	BW2	▽
	Wheat	36	BW3	△
A. oryzae KCCM 11300P	Soybean	12	AS1	■
	Soybean	24	AS2	▼
	Soybean	36	AS3	▲
	Wheat	12	AW1	□
	Wheat	24	AW2	▽
	Wheat	36	AW3	△

B. amyloliquefaciens (BS); b. wheat *Koji* fermented with *B. amyloliquefaciens* (BW); c. soybean *Koji* fermented *with A. oryzae* (AS); and d. wheat *Koji* fermented with *A. oryzae* (AW) we maintained throughout the experiment (Table 1). The samples were harvested at 12 h intervals (up to 36 h) and were immediately stored at deep freezing conditions (−80°C) until analyses. The numbers following the letters on the labels are written in order of the different harvest time while fermentation. The harvested *Koji* samples following 12, 24, and 36 h of fermentation were indicated as 1, 2, and 3 (Table 1). Three biological replicates were maintained for each sample harvested at aforementioned time periods.

Koji Sample Extraction

Extraction of headspace volatile organic compounds (VOCs). SPME was used to obtain volatile compounds in *Koji* samples. Before volatile extraction by SPME, samples were ground using a mortar with liquid nitrogen. Then, each sample (2.5 g) was mixed with 4 ml of saturated sodium chloride solution to inhibit enzyme activities during the extraction. The sample mixture and 2 µl of L-borneol (200 µg/l in methanol), an internal standard compound, were added into a 20 ml of amber SPME vial with a silicon/teflon septum (Supelco, Bellefonte, PA, USA). The mixtures were maintained at 40°C for 30 min and 75 µm carboxen/polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB) coated SPME fiber was exposed to the headspace at 40°C for 30 min. The desorption procedure was performed at 230°C in a GC injector for 5 min.

Extraction for primary metabolites. The freeze-dried *Koji* samples were ground using a mortar. Extraction of *Koji* powder (3 g) was conducted by adding 80% aqueous methanol (30 ml) and norvaline as an internal standard followed by sonication (10 min) and agitation (200 rpm for 24 h) in a rotary shaker. The sample mixtures were centrifuged (at 2,370 g for 10 min at 4°C) and the supernatants were filtered using a 0.22 µm polytetrafluoroethylene (PTFE) Millex®GP filter (Merck Millipore, Billerica, MA, USA). The sample extracts were completely dried using a speed-vacuum concentrator (Biotron, Korea) and the weighed to estimate the extraction yield. The sample derivatization reaction consisting of two stages, oximation and silylation, was carried out using the methods described previously [4].

Intrumentation

HS-SPME-GC-MS analysis. GC-MS analysis was conducted using 7890A GC system coupled to 5975C mass detector (Agilent technologies, USA) equipped with a DB-WAX column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness, J&W Scientific, USA). Oven temperature was initially maintained at 40°C for 6 min, and then raised to 200°C at a rate of 4°C/min before holding at 200°C for 5 min. The flow rate of helium was 0.8 ml/min and mass spectra was obtained with a mass scan range of 35−350 atomic mass units at a rate of 4.5 scans/sec with 70 eV of ionization energy in EI mode.

GC-TOF-MS analysis. The primary metabolites were analyzed on a GC-TOF-MS using an Agilent 7890A GC system (USA) with an Agilent 7693 auto-sampler and TOF Pegasus HT mass spectrometer (Leco, USA). An RTx-5MS (30 m length × 0.25 mm i.d. × 0.25 µm film thickness, J & W Scientific, USA) was used with a carrier gas, helium, at a flow rate of 1.5 ml/min. Transfer line and injector temperatures were set at 230°C and 250°C, respectively. The column temperature was constant at 75°C for 2 min, raised to 300°C at the rate of 15°C/min, and maintained as such for next 3 min. The acquisitions were recorded at the rate of 10 scans/s scanning a mass range of 50−1,000 m/z, maintaining 70 eV of ionization energy under EI mode. A 1 µl of the derivatized sample was injected at a split ratio of 5:1. Three replicates of each sample were tested.

Data Processing and Multivariate Statistical Analysis

The raw data files of GC-TOF-MS were converted to document format (*.cdf) by LECO Chroma TOF software (Version 4.44). Using the Metalign software package (http://www.metaligh.nl), retention-time correction, peak detection, and accurate masses were processed and exported resulting data to an Excel file (Microsoft, USA). The multivariate statistical analyses were conducted using SIMCA-P+ 12.0 software (Umetrics, Umea, Sweden). The patterns of metabolic variation were visualized using principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). The detected metabolites tested for significance by a one-way analysis of variance (ANOVA) using STATISTICA software (version 7.0, StatSoft Inc., USA). Primary metabolites were identified by comparing the retention times and mass fragment patterns with the National Institute of Standards and Technology (NIST) database (version 2.0, 2011, FairCom, USA) and those of standard compounds. Volatile compounds were identified by comparing their mass spectral and retention indices (RI) based on Wiley 9th edition mass spectral library (W9N08) and National Institute of Standards Technology version 08 (NIST08) libraries (Agilent Technologies, USA). The RI values were calculated using n-alkane mixture C6 to C22 alkanes as external standards. The relative peak areas of volatile compounds were quantified by comparing their peak areas to that of internal standard compound. The significantly discriminant primary metabolites were selected based on PLS-DA model at VIP>0.7 and $p < 0.05$. In comparing level of major flavor compounds, significant differences were tested by ANOVA and Duncan's multiple range tests using PASW Statistics 18 (SPAA, Inc., IL). The volatile compounds and primary metabolites levels were visualized using the heat map. Each level of volatile compounds and primary metabolites were converted as the log_{10} of peak area.

Antioxidant Activity of Koji

The estimation of antioxidant levels in *Koji* extracts were made using ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay adapted from the previously reported protocols [4]. To prepare the assay solution, 7 mM ABTS with 2.45 mM potassium persulfate was incubated under water bath 60°C for 20 min and kept for

stabilization at room temperature (28°C) for 12 h. The resulting solution was diluted using distilled water until an absorbance of 0.7 ± 0.02 at 750 nm was achieved under spectrophotometer (Spectronic Genesys 6, Thermo Electron, USA). The *Koji* sample extract (20 µl) was added upon with ABTS (180 µl) solution into a 96-well plate, and the reaction mixture was incubated under dark at 28°C for 6 min. Finally, the absorbance was recorded at 750 nm. Trolox was used as the reaction standard and the antioxidant activity data were expressed as the trolox equivalents antioxidant capacity (TEAC, mM).

Results

Time-Correlated Multivariate Analysis of Headspace Volatolome and Primary Metabolite Profiling Datasets for Koji Samples Fermented with Different Inocula

The temporal disparity in the profiles of headspace VOCs and primary metabolites in different *Koji* types were evaluated using multivariate statistical analyses of the HS-SPME-GC-MS and GC-TOF-MS datasets, respectively. As shown in Fig. 1A, the principal component analysis (PCA) score plot based on HS-SPME-GC-MS datasets indicated clustered time-correlated patterns for different *Koji* types. Notably, the VOCs profiling datasets were clustered each Koji types based on different inocula (*B. amyloliquefaciens* and *A. oryzae*) and substrates (soybean and wheat) across PC1 (25%) and PC2 (21.6%).

In congruence to the PCA, the corresponding partial least squares discriminant analysis (PLS-DA) score plots displayed similar patterns (Fig. S1A). A total of 141 VOCs were detected in different *Koji* types (Table S1). These included 7 carboxylic acids, 22 aldehydes, 27 alcohols, 13 esters, 21 ketones, 13 aliphatic hydrocarbons, 7 aromatic hydrocarbons, 5 furans, 5 sulfur-containing compounds, 8 pyrazines, 4 phenols, 2 lactones, and 5 miscellaneous compounds among different *Koji* types. The VOCs detected from soybean *Koji* included 7 carboxylic acids, 17 aldehydes, 19 alcohols, 11 esters, 16 ketones, 13 aliphatic hydrocarbons, 7 aromatic hydrocarbons, 3 furans, 5 sulfur-containing compounds, 5 pyrazines, 4 phenols, 2 lactones, and 3 miscellaneous compounds. Similarly, among the varying VOCs detected from wheat *Koji* included 4 carboxylic acids, 19 aldehydes, 21 alcohols, 10 esters, 17 ketones, 10 aliphatic hydrocarbons, 5 aromatic hydrocarbons, 4 furans, 5 sulfur-containing compounds, 8 pyrazines, 4 phenols, 1 lactone and 4 miscellaneous compounds. Intriguingly, alcohols were identified as the most abundant

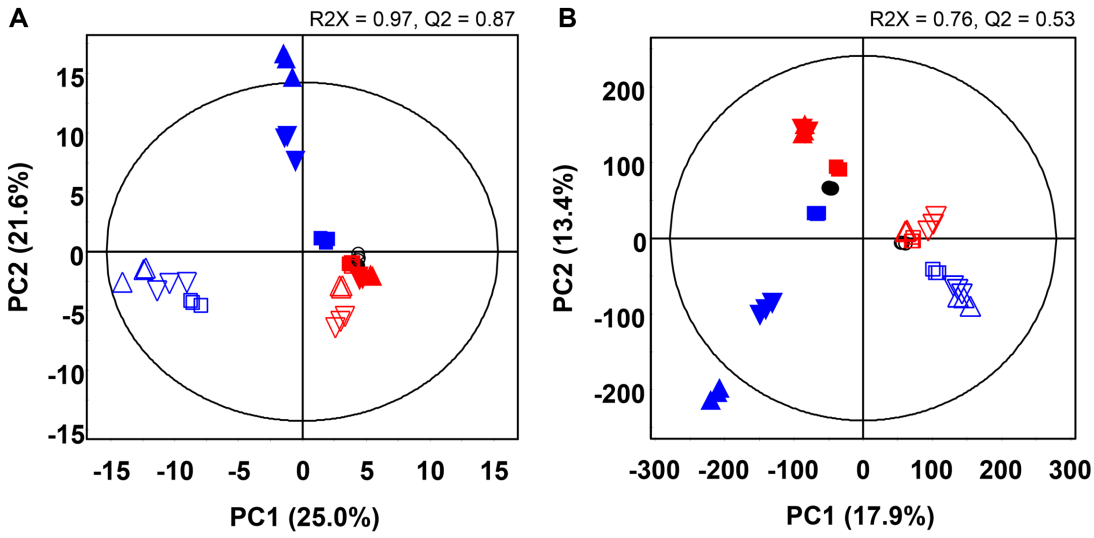

Fig. 1. Principal component analysis (PCA) plots for fermented *Koji* extracts with different combinations of substrates (soybean and wheat) and fermentative microflora (*A. oryzae* or *B. amyloliquefaciens*) during the course of fermentation based on (**A**) HS-SPME-GC-MS datasets for volatolome, and (**B**) GC-TOF-MS datasets for primary metabolites.

Here, the datasets for raw substrates are indicated - ●, RS (raw soybean); ○, RW (raw wheat). The datasets for *B. amyloliquefaciens* fermented soybean samples - ■, BS1 (harvested at 12 h); ▼, BS2 (harvested at 24 h); ▲, BS3 (harvested at 36 h); and wheat samples - □, BW1(harvested at 12 h); ▽, BW2 (harvested at 24 h); △, BW3 (harvested at 36 h). Similarly, the datasets for *A. oryzae* fermented soybean samples - ■, AS1 (harvested at 12 h); ▼, AS2 (harvested at 24 h); ▲, AS3 (harvested at 36 h); and wheat samples - □, AW1 (harvested at 12 h); ▽, AW2 (harvested at 24 h); △, AW3 (harvested at 24 h).

VOCs among the different *Koji* sample extracts, followed by ketones in soybean and aldehydes in wheat *Koji* samples. The differential variables of each different *Koji* (BS, BW, AS, AW) were selected based on the variable importance in projection (VIP) values written in the Table S1 and p-value < 0.05 obtained using the PLS-DA (Fig. S1A). The fragrance of each compound in Table S1 was created by reference [3, 5, 16−24].

Considering the variations in primary metabolite profiles based on the GC-TOF-MS datasets (Fig. 1B), the corresponding PCA score plots displayed distinct patterns based on different substrates (soybean and wheat) (PC1: 17.9%), and different inocula (*B. amyloliquefaciens* and *A. oryzae*) (PC2: 13.4%). The PLS-DA score plot also indicated a similar metabolome (Fig. S1B). Herein, we identified 13 sugars and sugar alcohols, 12 organic acids, 17 amino acids, 5 fatty acids, 3 nucleosides, and 1 phenolic acid as the primary metabolites among the different *Koji* sample extracts (Table S2). The differential variables of each different *Koji* (BS, BW, AS, AW) were selected based on the variable importance in projection (VIP) > 0.7 values and p-value < 0.05 obtained using the PLS-DA.

Time-Correlated Variations in the Relative Abundance of VOCs and Primary Metabolites among Bacillus- and Aspergillus-Fermented *Koji* Types

The marked disparity in the relative levels of metabolites, including VOCs and primary metabolites and the temporal variation among the different *Koji* types were displayed using a heat map representation of the data (Figs. S2 and S3). Since the commercial end-product *Koji* samples are generally harvested following 36 h fermentation, we carried our experiments to 36 h evaluating the VOC and primary metabolite profiles at every 12 h intervals. In general, the relative abundance of most VOCs and primary metabolites increased during the course of fermentation.

Notwithstanding different substrate types, the VOCs detected in *Bacillus Koji* (BS3, BW3) contained higher relative amounts of carboxylic acids (2-methylpropanoic acid, 3-methylbutanoic acid, 3-methylbut-2-enoic acid, 4-methylpentanoic acid), alcohols (ethanol, 2-methylbutan-2-ol, butan-1-ol, pentan-2-ol, pent-1-en-3-ol, 4-methylpentan-2-ol, pentan-1-ol, 5-methylhexan-2-ol, heptan-2-ol, heptan-1-ol, 6-methylheptan-2-ol, 5-methylheptan-2-ol, oct-1-en-3-ol, 2,5-dimethylhexan-3-ol, 2-ethylhexan-1-ol, butane-2,3-diol, octan-1-ol, butane-1,3-diol, nonan-1-ol, (Z)-non-3-en-1-ol, phenylmethanol, 2-phenylethanol), esters (methyl acetate, methyl 2-methylpropanoate, ethyl 2-methylpropanoate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, methyl 4-methylpentanoate, ethyl hexanoate), ketones (propan-2-one, butan-2-one, butane-2,3-dione, 3-methylpentan-2-one, 5-methylhexan-2-one, 4-methylpent-3-en-2-one, heptan-2-one, 4-methylheptan-2-one, 4,6-dimethylheptan-2-one, 5-methylheptan-2-one, 3-hydroxybutan-2-one, octan-2-one, nonan-2-one, decan-2-one, 1-phenylethanone), furans (2-methylfuran, 2,3,5-trimethylfuran, 2-butylfuran, 2-pentylfuran), pyrazines (2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine), and phenols (2-methoxyphenol, phenol, 4-ethenyl-2-methoxyphenol, 4-ethenylphenol). However, the relative levels of aldehydes (propanal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2-methylpentanal, hexanal, (E)-2-methylbut-2-enal, octanal, (E)-hept-2-enal, 2-phenylacetaldehyde, 4-methylbenzaldehyde, 2-phenylbut-2-enal, (Z)-5-methyl-2-phenylhex-2-enal) were higher in *Aspergillus Koji* compared to *Bacillus Koji* (Fig. S2).

In contrast, most of the primary metabolites were detected in higher quantities in *Aspergillus Koji* (AS3, AW3). These include sugars and sugar alcohols (glycerol, erythritol, fructose, glucose, galactose, sorbitol, myo-inositol, glyceryl-glycoside), organic acids (pyruvic acid, succinic acid, glyceric acid, fumaric acid, malic acid, citric acid, gluconic acid), and fatty acids (palmitic acid, linoleic acid, oleic acid). The notable exception were amino acids (alanine, leucine, isoleucine, proline, glycine, serine, threonine, methionine, glutamic acid, phenylalanine, ornithine, lysine, tryptophan), which showed a higher relative abundance in *Bacillus Koji* compared to *Aspergillus Koji* (Fig. S3).

Aroma Quality of Volatile Compounds in *Koji*

The comparative evaluation of aroma qualities for *Bacillus Koji* and *Aspergillus Koji* were presented in Table 2. There are six main flavors associated with *Koji* including green, cheesy & rancid, malty, sweet, floral and fruity. In *Bacillus Koji*, the number of flavor compounds including 3 for 'green' ((E)-oct-2-enal, pent-1-en-3-ol, benzaldehyde), 3 for 'cheesy & rancid' (2-methylpropanoic acid, 3-methylbutanoic acid, 4-methylpentanoic acid), 4 for 'sweet' (2-butylfuran, 2-pentylfuran, butane-2,3-dione, 4-ethenylphenol) and 9 for 'fruity' (ethyl-2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, methyl 4-methylpentanoate, ethyl hexanoate, heptan-2-one, octan-2-one, nonan-2-one), were detected at relatively higher concentrations compared to *Aspergillus Koji*. In contrast, the number of malty flavor compounds (2-methylpropanal, 2-

Table 2. The relative contents of volatile compound-related flavor in soybean and wheat *Koji* after 36 h fermentation either with *A. oryzae* or *B. amyloliquefaciens*.

Green flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
(E)-Oct-2-enal	N.D.	1.31 ± 0.05	N.D.	1.26 ± 0.05
Pent-1-en-3-ol	1.08 ± 0.04	1.11 ± 0.03	N.D.	N.D.
Benzaldehyde	2.50 ± 0.10	2.17 ± 0.03	2.24 ± 0.03	2.16 ± 0.04
Acetaldehyde	N.D.	1.78 ± 0.07	2.04 ± 0.12	1.70 ± 0.05
Cheesy, Rancid flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
2-Methylpropanoic acid	2.81 ± 0.15	2.36 ± 0.01	1.24 ± 0.04	1.29 ± 0.02
3-Methylbutanoic acid	2.76 ± 0.11	3.22 ± 0.01	1.79 ± 0.08	2.31 ± 0.01
4-Methylpentanoic acid	1.82 ± 0.12	N.D.	N.D.	N.D.
Acetic acid	N.D.	2.27 ± 0.11	2.08 ± 0.06	1.35 ± 0.05
Butanoic acid	0.93 ± 0.10	1.56 ± 0.12	N.D.	1.57 ± 0.10
3-Methylbutan-1-ol	2.25 ± 0.02	2.10 ± 0.05	1.50 ± 0.08	2.37 ± 0.00
Malty flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
2-Methylpropanal	N.D.	0.89 ± 0.10	2.30 ± 0.03	1.86 ± 0.02
2-Methylbutanal	1.14 ± 0.07	1.72 ± 0.14	3.03 ± 0.07	2.13 ± 0.03
3-Methylbutanal	1.12 ± 0.05	2.02 ± 0.15	3.55 ± 0.06	3.10 ± 0.01
Sweet flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
2-Butylfuran	N.D.	0.85 ± 0.05	N.D.	N.D.
2-Pentylfuran	2.08 ± 0.19	2.40 ± 0.03	2.06 ± 0.12	0.79 ± 0.08
Butane-2,3-dione	2.05 ± 0.06	2.57 ± 0.04	1.77 ± 0.11	1.73 ± 0.03
4-Ethenylphenol	2.04 ± 0.04	0.64 ± 0.08	N.D.	N.D.
Hexanal	2.07 ± 0.14	2.42 ± 0.07	2.33 ± 0.13	2.82 ± 0.04
2-Ethylfuran	1.40 ± 0.04	N.D.	1.46 ± 0.07	N.D.
Maltol	1.41 ± 0.07	N.D.	1.97 ± 0.05	N.D.
3-Methylbut-2-enal	N.D.	N.D.	1.05 ± 0.07	1.00 ± 0.06
Floral flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
2-Phenylacetaldehyde	1.09 ± 0.01	1.30 ± 0.06	1.64 ± 0.08	1.36 ± 0.01
2-Phenylbut-2-enal	N.D.	N.D.	2.00 ± 0.06	0.88 ± 0.01
2-Phenylethanol	0.73 ± 0.06	1.33 ± 0.14	N.D.	0.36 ± 0.09
Phenol	1.42 ± 0.02	1.51 ± 0.07	N.D.	0.60 ± 0.04

Table 2. Continued.

Fruity flavor				
Compounds	*B. amyloliquefacience*		*A. oryzae*	
	Soybean *Koji*	Wheat *Koji*	Soybean *Koji*	Wheat *Koji*
Ethyl 2-methylpropanoate	1.37 ± 0.05	0.85 ± 0.19	0.57 ± 0.02	N.D.
Ethyl 2-methylbutanoate	1.67 ± 0.07	N.D.	N.D.	N.D.
Ethyl 3-methylbutanoate	N.D.	N.D.	N.D.	1.39 ± 0.11
3-Methylbutyl acetate	N.D.	0.95 ± 0.05	N.D.	0.73 ± 0.07
Methyl 4-methylpentanoate	1.10 ± 0.04	N.D.	N.D.	N.D.
Ethyl hexanoate	2.15 ± 0.04	1.80 ± 0.11	N.D.	0.59 ± 0.07
Heptan-2-one	2.43 ± 0.07	2.34 ± 0.15	0.86 ± 0.10	0.88 ± 0.08
Octan-2-one	1.13 ± 0.09	2.47 ± 0.10	0.69 ± 0.13	N.D.
Nonan-2-one	2.04 ± 0.01	1.62 ± 0.04	N.D.	N.D.
(E)-Hept-2-enal	N.D.	N.D.	N.D.	1.75 ± 0.08
2-Phenylethyl acetate	N.D.	N.D.	0.35 ± 0.06	N.D.
Ethyl acetate	1.60 ± 0.11	2.96 ± 0.04	2.49 ± 0.13	2.83 ± 0.13

[a]The value means log (peak area) ± STD.

[b]N.D. means not detected.

methylbutanal, 3-methylbutanal) were relatively abundant in *Aspergillus Koji* compared to *Bacillus Koji*. However, the number of floral flavor compounds in both the *Bacillus* and *Aspergillus* fermented *Koji* types were commensurate to their respective headspace abundance irrespective of the varying substrates.

Discussion

The present study is designed to explore the intricate relationship between subtle primary metabolites and the flavor-imparting VOCs in different types of *Koji* fermented with different inocula. The corresponding metabolic pathways indicated common trends, regardless of the varying substrates, between *Koji* sample extracts fermented either with *B. amyloliquefaciens* or *A. oryzae* (Fig. 2).

The biosynthetic pathway map indicates the metabolites and their relative occurrence in each of the *Koji* types (*Bacillus Koji* than *Aspergillus Koji*) and is based on the KEGG database and previously published literature [5, 10, 11, 16, 17, 25]. Considering some of the selected VOC and primary metabolite levels, we will discuss the potential biosynthetic mechanisms substantiating this observed metabolic disparity between the varying *Koji* types with two different inocula. Reportedly, 3-hydroxybutan-2-one (acetoin) synthesis in *B. subtilis* involves two enzymes, which catalyze the conversion of pyruvate to acetolactate (acetolactate synthase) and its subsequent conversion to acetoin (acetolactate decarboxylase) [26]. Hence, we suggest that the coherent activities of these enzymes may have affected the higher relative abundance of 3-hydroxybutan-2-one in *Bacillus Koji*, while the higher pyruvate levels detected in *Aspergillus Koji* may be attributed to the defective function of these pathways (Fig. 2). The higher phenylacetaldehyde levels in *Koji* samples fermented with *A. oryzae* can be correlated to the higher expression of enzymes such as aromatic amino acid decarboxylase and monoamine oxidase [27].

The analysis of flavor (green, cheesy, sweet, rancid, and fruity)-related VOCs using HS-SPME-GC-MS indicated that most of the acids, alcohols, esters, ketones, furans, pyrazines, and phenols were generally more abundant in *Bacillus* fermented *Koji*, with the exception of some sweet flavor compounds, including maltol, hexanal, and 2-ethylfuran. The engendering sweet flavor of VOCs, attributed to hexanal, 2-ethylfuran, 3-hydroxy-2-methyl-4H-pyran-4-one (maltol), 2-butylfuran, 2-pentylfuran, butane-2,3-dione, 4-ethenylphenol, and 3-methylbut-2-enal, have been previously characterized [17–19]. Since, maltol is produced through the sugar Maillard reaction [17], the higher relative levels of monosaccharide sugars in *Aspergillus Koji* produced by this reaction may have added to its sweet flavor (Fig. S3). Notably, VOCs and metabolites associated with sweet flavor, such as maltol, 2-ethylfuran,

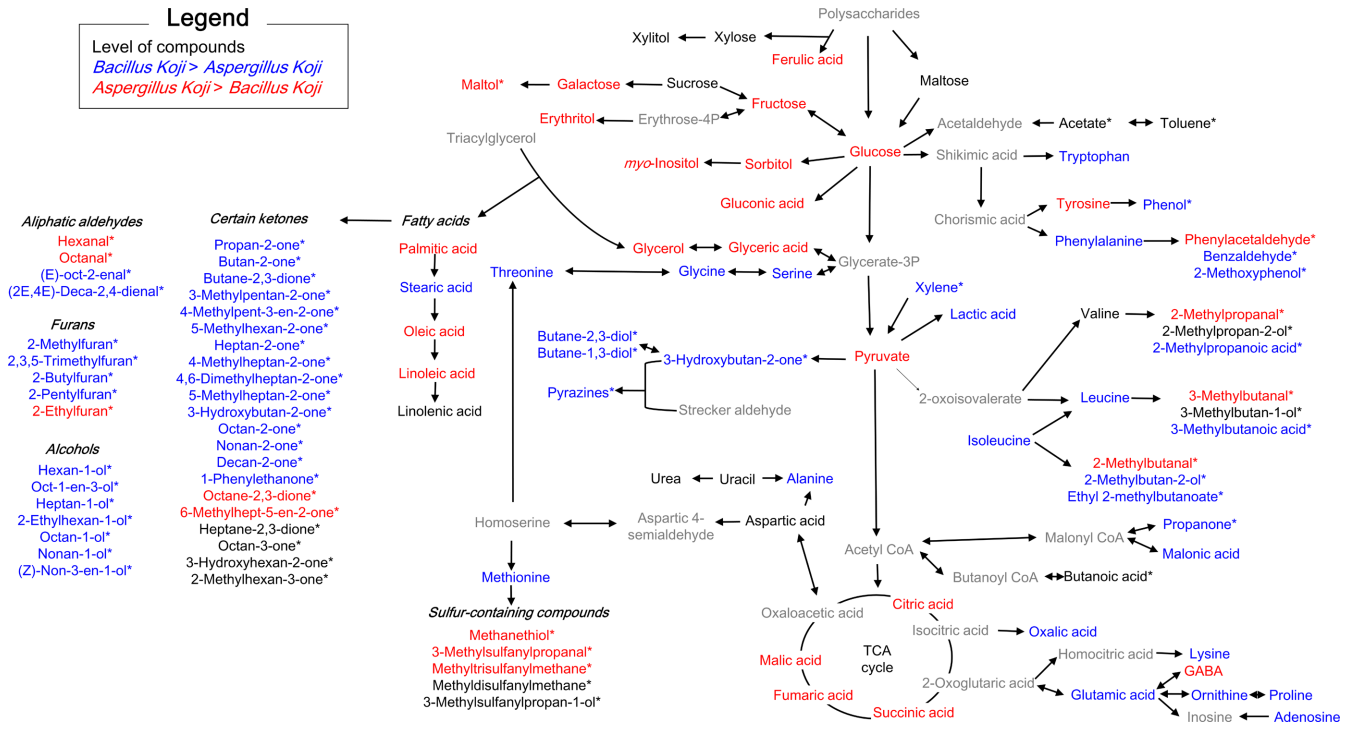

Fig. 2. Scheme of the primary metabolic pathway and volatile compound formation in soybean and wheat *Koji* fermented after 36 h with either with *A. oryzae* or *B. amyloliquefaciens*.

The pathway was adopted from the KEGG database (KEGG, http://www.genome.jp/kegg) and references. *Volatile compounds; Color of compound name: Blue, higher levels in *Bacillus Koji* than *Aspergillus Koji*; Red, higher levels in *Aspergillus Koji* than *Bacillus Koji*; Black, Detected compounds regardless of inocula; Gray, Non-detected compounds in *Koji*.

hexanal, and monosaccharides, were detected in higher levels in *Aspergillus Koji* (Table 2). Maltol has been previously detected as a major VOC using the solvent-assisted flavor evaporation (SAFE) method in the headspace of fermented soybean paste *doenjang* [11].

The 'cheesy & rancid' flavor compounds including 2-methyl butanoic acid and 3-methyl butanoic acid were regarded as off-flavor VOCs in doenjang fermented with *B. amyloliquefaciens* [5]. Furthermore, VOCs including 3-methylbutanal, 2-methylbutanal, 2-methoxyphenol, and (E,E)-2,4-decadienal typically generate the umami aftertaste flavor in *miso* [3]. In addition, glutamic acid and aspartic acid are amino acids (primary metabolites) known for contributing toward umami taste in fermented foods. Among these compounds, 2-methoxyphenol, (E,E)-2,4-decadienal, and glutamic acid were mainly detected from *Bacillus Koji* regardless of substrates, and can, therefore, be correlated to the fermentative growth of *B. amyloliquefaciens* (Figs. 2 and S2).

Aliphatic aldehydes, furans, alcohols, and certain ketones are usually formed through lipid oxidation [5, 10]. In the present study, we determined a higher abundance of these VOCs from *Bacillus Koji*. Furans are formed from the auto-oxidation products of linolenic acid and sugar dehydration during the Maillard reaction [5, 17]. However, the antioxidant inhibits the oxidation of unsaturated fatty acids including linolenic acid and linoleic acid, and it can effectively limit the furan production [28]. Based on the results of the ABTS assay, *Aspergillus Koji* extracts displayed higher antioxidant activity than *Bacillus Koji* (Fig. S4). Hence, it can be assumed that the lipid oxidation occurred at higher levels in *Bacillus Koji* than in *Aspergillus Koji*, and thus, VOCs, including aliphatic aldehydes, furans, alcohols, and certain ketones, were more abundant in *Bacillus Koji*. Sulfur-containing compounds are mainly produced by the degradation of methionine during the Maillard reaction [11, 16]. Therefore, the higher abundance of Sulfur-containing compounds in *Aspergillus Koji* may be attributed to the higher free sugar contents, which promote the Maillard reaction. Although, the levels of methionine were relatively higher in *Bacillus Koji*, the lower occurrence of Maillard reaction likely resulted in the lower abundance

of organo-sulfur compounds. Hence, the marked disparity in the levels of VOCs and primary metabolites in different *Koji* types fermented with varying inocula greatly affects the end-product flavor, which is potentially vital for its consumer acceptability and commercial value.

Recapitulating the main findings, we examined 141 headspace VOCs and 51 primary metabolites in different *Koji* types. We noted higher abundance of carboxylic acids, alcohols, esters, ketones, furans, pyrazines, phenols, and amino acids in *Bacillus Koji*, regardless of the substrate (soybean or wheat). In contrast, aldehydes, sugars and sugar alcohols, organic acids, and fatty acids were at higher concentrations in *Aspergillus Koji*. A majority of the flavor-related VOCs were detected in *Bacillus Koji*, especially green, cheesy & rancid, sweet, and fruity flavors. Moreover, the aftertaste flavor compounds including glutamic acid, (E,E)-2,4-decadienal, and 2-methoxyphenol associated with umami taste were detected in higher amounts in *Bacillus Koji*. Alternatively, *Aspergillus Koji* displayed higher abundance of malty as well as selected sweet flavor VOCs (maltol, hexanal, 2-ethylfuran) and metabolites (monosaccharides). *Bacillus Koji* showed more compounds associated with sweet flavor than *Aspergillus Koji*. However, the primary metabolites, especially the monosaccharides engendering sweet taste, were relatively higher in *Aspergillus Koji*. Additionally, only two VOCs were detected in *Aspergillus Koji* and *Bacillus Koji* related to the umami taste. However, the analysis of primary metabolites also revealed that the number of umami related compounds in *Bacillus Koji* were higher than those in *Aspergillus Koji*. Further, these results highlight the need for an integrated approach correlating the VOCs and metabolites with different fermentative inocula toward optimal *Koji* making. Hence, the present study holds the potential toward engineering the flavor volatolome in fermented foods through a rational choice of fermentative microflora, thus maneuvering the consumer acceptance of fermented end-products.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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