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Untargeted metabolite profiling for *koji*-fermentative bioprocess unravels the effects of varying substrate types and microbial inocula

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Oxalic acid (PubChem CID: 971)
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ABSTRACT

Untargeted metabolomics unraveled the effects of varying substrates (soybean, wheat, and rice) and inocula (*Aspergillus oryzae* and *Bacillus amyloliquefaciens*) on metabolite compositions of *koji*, a starter ingredient in various Asian fermented foods. Multivariate analyses of the hyphenated mass spectrometry datasets for different *koji* extracts highlighted 61 significantly discriminant primary metabolites (sugars and sugar alcohols, organic acids, amino acids, fatty acids, nucleosides, phenolic acids, and vitamins) according to varying substrates and inocula combinations. However, 59 significantly discriminant secondary metabolites were evident for *koji*-types with varying substrates only, *viz.*, soybean (flavonoids, soyasaponins, and lysophospholipids), wheat (flavones and lysophospholipids), and rice (flavonoids, fatty acids derivatives, and lysophospholipids). Independently, the substrates influenced primary metabolite compositions in *koji* (soybean > wheat, rice). The inocula choice of *A. oryzae* engendered higher carbohydrates, organic acids, and lipid derivative levels commensurate with high *α*-amylase and *β*-glucosidase activities, while *B. amyloliquefaciens* affected higher amino acids levels, in respective *koji* types.

1. Introduction

Koji-derived fermented foods and beverages are diet staples in East-Asian countries. A quintessential *koji* preparation involves partially cooked cereal (rice/wheat/barley) or soybean fermentation with *Aspergillus oryzae* or *Bacillus* species for a relatively short period of 2–3 days (Lee, Lee, Jang, Shin, Moon & Lee, 2016). Traditionally, *koji* is employed as an indispensable starter ingredient to prepare foods and beverages, such as *miso* (Japanese soybean paste), *sake* (Japanese rice wine), *doenjang* (Korean soybean paste), *gochujang* (Korean pepper paste), and *kanjang* (Korean soy sauce), among many others (Lee et al., 2016; Zhu & Tramper, 2013). In general, *koji* is prepared by either traditional artisan or optimized industrial processes. Since traditional *koji* fermentation relies largely on spontaneously colonized or *nuruk* (rice or barley straw) transferred microbial inocula, its quality control and consumer acceptance criteria are often subjected to scrutiny. In contrast, industrial *koji* fermentation involves substrate inoculation with a well-characterized inoculum under controlled incubation conditions. In either of the modes, the *koji* fermentation is biochemically characterized by the secretory hydrolysis of partially cooked substrate materials, releasing simple nutrients in assimilable forms maneuvering the end product metabolite compositions (Kim et al., 2010).

Owing to different socio-geographical traditions, the typical *koji* gourmet uses various substrates, including rice, sorghum, wheat, corn, and barley. In particular, barley malt is used in the process of making beer in the West, while soybean and rice *koji* are employed as starter ingredients for making sake, soy sauce, soy paste, and certain vinegar types in the Orient (Zhu & Tramper, 2013; Yu et al., 2012). Among the various substrate materials used for *koji* preparation, soybean has high contents of free sugars, lipids, minerals, vitamins, isoflavones, flavonoids, saponins, proteins, and peptides. Particularly, soy isoflavones have been reported to mitigate cancer post-menopausal osteoporosis and cardiovascular ailments (Setchell & Cassidy, 1999). In contrast, cereal substrates rich in carbohydrates, proteins, dietary fiber, and vitamins result in *koji* end products with high contents of functional phytochemicals (Bhanja, Kumari, & Banerjee, 2009). In general, soybean, wheat, or rice fermentation releases antioxidant components, which greatly enhance the nutritional as well as functional values of

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koji end-products (Bhanja et al., 2009; Juan & Chou, 2010; Yen, Chang, & Su, 2003).

A variety of microbial inocula are used for *koji* fermentation, including fungi (*Rhizopus*, *Penicillium*, *Monascus* and *Aspergillus* species), yeast (*Saccharomyces cerevisiae*) and bacteria (*Bacillus subtilis*, *B. natto*, *B. amyloliquefaciens*) (Zhu & Tramper, 2013; Yu et al., 2012). *A. oryzae* is a generally recognized as safe (GRAS) mold, supporting its safe applications in the fermentative production of functional metabolites, pharmaceuticals, industrial enzymes and fermented foods (Benoit-Gelber et al., 2017). Taxonomically, *A. oryzae* is classified under section *Flavi* along with *A. sojae*, *A. parasiticus*, and toxin-producing *A. flavus*. However, *A. oryzae* has long been revered as an atoxigenic species with toxin-producing genes reportedly lost or degenerated during the two millennia of domestication for indented fermentation (Matsushima, et al., 2001; Machida, Yamada, & Gomi, 2008). On the other hand, *Bacillus* species, including *B. amyloliquefaciens* and *B. subtilis,* are characterized by high growth rates, overwhelming secretion of hydrolytic enzymes, and accepted probiotic status, making them suitable candidates for food fermentation (Arguelles-Arias et al., 2009; Das, Nakhro, Chowdhury, & Kamilya, 2013). Although *Bacillus* species are known to produce biogenic amines with potential toxicity for human consumption, the safety criteria for *B. amyloliquefaciens* are well established (de Boer Sietske & Diderichsen, 1991; Alvarez & Moreno-Arribas, 2014). The microbial growth, metabolism, and succession events altogether determine the overall quality of fermentation end-products (Jeong, Jung, Lee, Jin, & Jeon, 2013). Hence, *A. oryzae* (trivially: *koji* mold) and *B. amyloliquefaciens* are most commonly used microbial inocula for fermentative manufacturing of various *koji* types.

Untargeted metabolomics coupled with phenotype analyses may greatly unravel the nutritional, functional, or consumer safety aspects of fermented foods (Lee et al., 2017a,b; Jang et al., 2017). Although a number of previous studies have characterized *koji* metabolites and its fermentative bioprocess, a comprehensive overview of the variation of untargeted metabolites in major *koji* types (soybean, rice, and wheat) fermented with different inocula (*A. oryzae* or *B. amyloliquefaciens*) seems largely uncharted. Herein, we hypothesize that untargeted mass spectrometry (MS) analytical datasets examining the temporal fermentative stages of *koji* manufacturing can be transformed into a metabolomic framework rationalizing optimal substrate and microflora selection, enabling the desired preparation of *koji* end-products. Herein, we performed an MS-based metabolomic analyses for *koji* fermentative bioprocesses with different substrates as well as microbial inocula, delineating their cumulative effects on its biochemical as well as physicochemical characteristics.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade water, acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Analytical-grade sodium dihydrogen phosphate, sodium chloride, sodium hydroxide, sodium carbonate, disodium hydrogen phosphate, and diethylene glycol were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). All remaining analytical-grade reagents and standard compounds used in the study were from Sigma-Aldrich (St. Louis, MO).

2.2. Microbial cultures and koji (soybean, wheat, and rice) fermentation

Aspergillus oryzae KCCM 11300P and *Bacillus amyloliquefaciens* KCCM 11718P were from CJ CheilJedang Corporation (Suwon, Korea). Three different raw substrates, including soybean (*Glycine max*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*), were used for different *koji*-type preparations. The detailed method employed for different *koji* preparations was based on a method previously described by Lee et al. (2016). Overall, six different *koji* types (soybean *koji*: *A. oryzae* fermented-SA and *B. amyloliquefaciens* fermented-SB; wheat *koji*: WA and WB; rice *koji*: RA and RB) were maintained in the experiment with appropriate replicates (Table 1). The samples were harvested every 12 h for each *koji* type and immediately stored at −80 °C until further analyses.

Table 1
Sample information and acronyms for designating different *koji* types fermented either with *Aspergillus oryzae* or *Bacillus amyloliquefaciens*.

Substrate	Inoculum	Symbol	Time (hr)	Sample name
Soybean	–	+	0	soybean
	A. oryzae	●	12	SA12
	A. oryzae	▲	24	SA24
	A. oryzae	◆	36	SA36
	B. amyloliquefaciens	○	12	SB12
	B. amyloliquefaciens	△	24	SB24
	B. amyloliquefaciens	◇	36	SB36
Wheat	–	+	0	wheat
	A. oryzae	●	12	WA12
	A. oryzae	▲	24	WA24
	A. oryzae	◆	36	WA36
	B. amyloliquefaciens	○	12	WB12
	B. amyloliquefaciens	△	24	WB24
	B. amyloliquefaciens	◇	36	WB36
Rice	–	+	0	rice
	A. oryzae	●	12	RA12
	A. oryzae	▲	24	RA24
	A. oryzae	◆	36	RA36
	B. amyloliquefaciens	○	12	RB12
	B. amyloliquefaciens	△	24	RB24
	B. amyloliquefaciens	◇	36	RB36
Each substrate (soybean, wheat, and rice)	*A. oryzae*	–	36	AK
	B. amyloliquefaciens	–	36	BK

2.3. Sample preparation

Harvested *koji* samples were extracted for metabolite profiling, as previously described by Lee et al. (2016). The extraction yield of each sample was calculated and samples were re-suspended in 80% methanol solution. The samples for gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) analysis were made by dissolving 10 mg of dried *koji* extracts in 100 μL of 80% methanol with added norvaline (800 ppm) as an internal standard (IS). The samples were again dried using a speed vacuum concentrator prior to a two-staged derivatization step. First, sample oximation was performed by dissolving the re-dried sample extracts with 50 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) and incubating the reaction at 30 °C for 90 min. Next, silylation was carried out by adding 50 μL of MSTFA and reaction incubation at 37 °C for 30 min. The dried sample extracts (50 mg) for ultra-high-performance liquid chromatography linear trap quadrupole ion trap tandem mass spectrometry (UHPLC-LTQ-IT-MS/MS) analysis were dissolved in 1 mL of 80% methanol with formononetin (2ppm) as an IS. The samples were syringe-filtered using a 0.2-μm polytetrafluoroethylene (PTFE) filter prior to the analysis.

2.4. Instrumentation

2.4.1. GC-TOF-MS analysis

GC-TOF-MS analysis was accomplished using an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA) coupled to a Pegasus HT TOF-MS (Leco Corporation, St. Joseph, MI) and Agilent 7693 autosampler. Sample was separated on an Rtx-5MS column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; Restek Corp., Bellefonte, PA). The operational parameters were adapted from Lee et al. (2016). Three biological replicates were analyzed for each sample. The metabolites were identified by comparing their retention times and mass fragment patterns with standard compounds, in-house library