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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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Diethylene glycol (PubChem CID: 8117)
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Oxalic acid (PubChem CID: 971)
Ferulic acid (PubChem CID: 445858)
Linoleic acid (PubChem CID: 5280450)
Tricin-7-O-rutinoside (PubChem CID: 44258273)
LysoPC 16:0 (PubChem CID: 460602)
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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

data, and the National Institute of Standards and Technology (NIST) database (version 2.0, 2011; FairCom, Gaithersburg, MD).

#### 2.4.2. UHPLC-LTQ-IT-MS/MS analysis

UHPLC-LTQ-IT-MS/MS analysis for the *koji* sample extracts was performed using a Thermo Fisher Scientific LTQ ion trap mass spectrometer equipped with an electrospray interface (Thermo Fisher Scientific, San Jose, CA) and Dionex UltiMate 3000 RS autosampler, RS column compartment, RS diode array detector, and RS pump (Dionex Corporation, Sunnyvale, CA). A Thermo Scientific Syncronis C18 UHPLC column (100 mm × 2.1 mm i.d.; 1.7 μm particle size) separated a 10-μL sample extract. The mobile phase consisted of water (solvent **A**) with 0.1% formic acid (*v/v*) and acetonitrile (solvent **B**) with 0.1% formic acid (*v/v*) at a flow rate of 0.3 mL/min. The solvent gradient program was as follows: 10% **B** for 1 min, increased to 100% **B** for 14 min, maintained for 3 min, and decreased to 10% **B** in 1 min, and maintained at 10% **B** for the final 3 min. The photodiode array detection wavelength was 200–600 nm. MS ion trapping was performed in full-scan, positive and negative ion modes over a range of *m/z* 100–1500 for soybean *koji* extracts and *m/z* 100–1000 for wheat and rice *koji* extracts. The MS operating parameters were as follows: capillary temperature, 275 °C; source voltage, ± 5 kV; and capillary voltage, 39 V. Three biological replicates were analyzed for each sample. Discriminant metabolites were tentatively identified by comparing their retention time, molecular weight, mass fragment patterns, UV absorbance, in-house library, and published literature data.

### 2.5. Data processing and multivariate statistical analysis

The raw data files from GC-TOF-MS and UHPLC-LTQ-IT-MS/MS analyses were converted into computable document form (.cdf) file formats using LECO Chroma TOF and Thermo Xcalibur (version 2.1, Thermo Fisher Scientific) software, respectively. The converted data files were accomplished using the Metalign software package (http://www.metalign.nl) to acquire a data matrix of retention times (min), normalized peak intensities, and accurate masses (*m/z*). The resulting data were exported to Excel format (Microsoft, Redmond, WA) and multivariate statistical analyses were performed by SIMCA-P$^+$ 12.0 software (version 12.0; Umetrics, Umeå, Sweden), to determine the metabolomic data variance among different *koji* samples. We performed both unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA) for the metabolomic datasets.

### 2.6. Evaluation of bioactivities (assays for antioxidant activity, total phenolic and flavonoid contents)

An ABTS assay was performed as described by Lee et al. (2016) with some modifications. Briefly, the ABTS stock solution (7 mM) was mixed with 2.45 mM potassium persulfate buffer and incubated in a water bath at 60 °C for 20 min, and then stored at room temperature for 12 h in the dark. The resulting solution was diluted with water until the absorbance value reached 0.7 ± 0.02 at 750 nm. An antioxidant assay was performed by adding 180 μL ABTS solution to 20 μL of each *koji* sample extract (10 mg/mL) in a 96-well plate, and the reaction was incubated for 6 min at 37 °C in the dark. The resulting sample absorbance was recorded using a spectrophotometer (Spectronic Genesys 6; Thermo Electron, Madison, WI) at 734 nm. A Trolox aliquot was used to draw the standard curve, and the data were expressed as Trolox equivalents of concentration (mM).

Total flavonoid content (TFC) and total phenolic content (TPC) were determined as described previously by Lee et al. (2016) with slight modifications. To determine total flavonoid (TF) contents, 20 μL of *koji* sample extracts (10 mg/mL) were added to 180 μL of 90% diethylene glycol and 20 μL of 1 N NaOH, followed by 60 min incubation at room temperature in the dark. The resulting sample absorbance values were measured at 405 nm. A naringin aliquot was used to draw a standard curve, and TFC was expressed as the naringin equivalent (mM) of total flavonoid concentration. All assays were conducted using three biological replicates for each *koji* sample extract.

To measure TPC, 20 μL of each *koji* sample extract (10 mg/mL) were added to 100 μL of 0.2 N Folin-Ciocalteu's phenol reagent in a 96-well plate, and the reaction was incubated for 6 min at room temperature in the dark. Next, 80 μL of 7.5% $NaCO_3$ were added, followed by 60 min incubation at room temperature. The reaction absorbance was recorded at 750 nm. TPC was calculated from a gallic acid standard curve and the data were expressed as gallic acid equivalents (ppm) of total phenol concentration.

### 2.7. Evaluation of physicochemical characteristics (assays for amino type nitrogen, pH, reducing sugar contents, and titratable acidity)

Evaluation of physicochemical characteristics was performed as described by Lee, Suh, Jung, and Lee (2016) with some modifications; *koji* sample (5 g) was mixed with 50 mL of distilled water, homogenized on a rotary incubator at 100 rpm and 30 °C for 1 h, and centrifuged at 2370*g* and 4 °C for 10 min. Subsequently, the supernatants were collected and filtered using 0.2-μm PTFE filters prior to assays for pH, titratable acidity, amino-type nitrogen, and reducing sugar contents.

Sample pH was determined by a pH meter (Orion 3 Star pH benchtop, Thermo Fisher Scientific, Inc.). Titratable acidity and amino-type nitrogen contents were determined using the formol titration method, as described previously by Kim, Han, and Kim (2010). Total acidity was calculated by titrating the *koji* sample solutions with 0.1 N sodium hydroxide (pH 8.4). The consumed quantity of sodium hydroxide solution ($V_a$) was converted into percent acetic acid using the following formula:

$$Titratable\ acidity(\%) = [(0.006 \times Va \times D \times F)/S] \times 100$$

Here, 0.006 is the conversion factor for acetic acid, $V_a$ is the consumption volume for sodium hydroxide (mL), and $D$, $F$, and $S$ are the dilution rate (1), factor of the 0.1 N sodium hydroxide solution (1.002), and amount of sample (1.5 g), respectively.

Amino-type nitrogen contents were determined by adding 20 mL of formaldehyde (36%, pH 8.4) to *koji* sample solutions. The sample solutions were re-titrated after 1 min to pH 8.4, using 0.1 N sodium hydroxide solutions ($V_a$). The milligram percentage of amino-type nitrogen contents was expressed using the following formula:

$$Amino\ type\ nitrogen(\%\ mg) = [(V_a \times 1.4 \times D \times F)/S] \times 100$$

Here, $V_a$ is the consumed volume of NaOH solution (mL) and 1.4 means the nitrogen equivalent amount of the 0.1 N NaOH solution (1 mL).

The sugar contents in *koji* sample extracts (200 μL) were determined using a portable refractometer for sugar measurements (Hanna Instruments, Inc., Padova, Italy). All assays were conducted using three biological replicates for each *koji* sample.

### 2.8. Determination of enzymatic activities

Enzyme activity assays were performed for β-glucosidase, α-amylase, and protease levels in different *koji* samples, using a method adapted from Lee et al. (2016). First, 10 g of each *koji* sample were extracted with 90 mL of distilled water in a shaking incubator at 120 rpm and 30 °C for 1 h. Next, the mixtures were centrifuged at 5000 rpm and 4 °C for 5 min and the supernatants were passed through 0.2-μm PTFE filters prior to their use as a crude enzyme source in the assays. All assays were conducted for three biological replicates representing each *koji* sample extract at different fermentation time points.

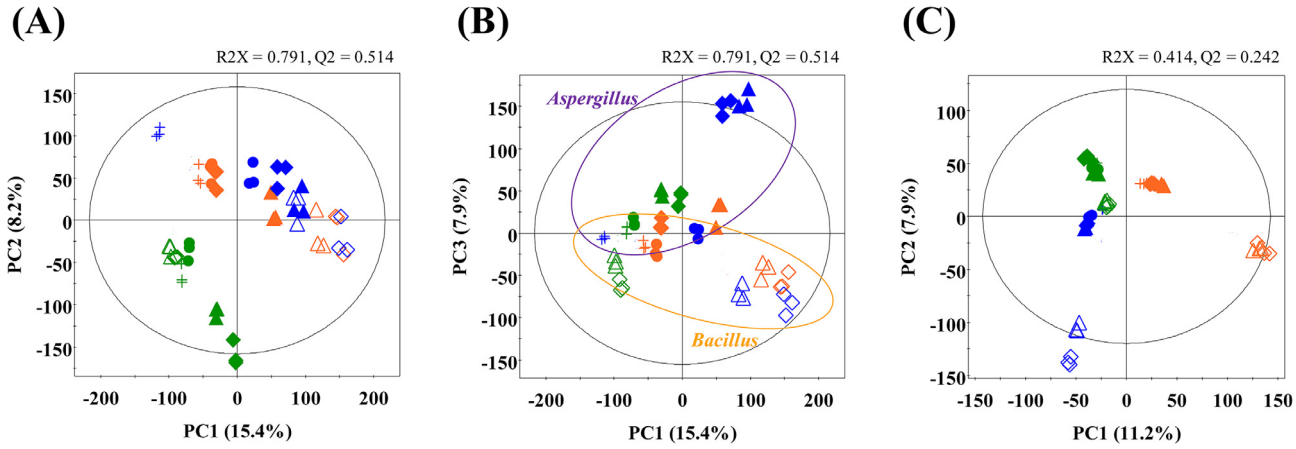


**Fig. 1.** Principal component analysis (PCA) score plot for **(1. A, B)** soybean, wheat, and rice *koji* fermented with *A. oryzae* or *B. amyloliquefaciens* based on GC-TOF-MS and **(1. C)** UHPLC-LTQ-IT-MS/MS datasets. (┼, steamed substrate, 0 h; filled symbols, fermented with *A. oryzae;* unfilled symbols, fermented with *B. amyloliquefaciens;* green symbol, soybean; orange symbol, wheat; blue symbol; rice; ●, ○, 12 h; ▲, △, 24 h; ◆, ◇, *koji,* 36 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 3. Results

### 3.1. Time-correlated metabolite profiling

Time-correlated metabolite profiling was performed for extracts from six different *koji* types made with different substrates (soybean, wheat, and rice) and microbial inocula (*A. oryzae* and *B. amyloliquefaciens*) using GC-TOF-MS and UHPLC-LTQ-IT-MS/MS analyses. The PCA score plot based on GC-TOF-MS datasets indicated a marked variance among *koji* samples across PC1 (15.4%) and PC2 (8.2%) based on different substrates (soybean, wheat, and rice), while PC3 (7.9%) highlighted the variance based on different inocula (*A. oryzae* and *B. amyloliquefaciens*), in a time-correlated scale (Fig. 1A, B). The corresponding PLS-DA score plots displayed similar patterns as PCA (Fig. S1). Significantly discriminant metabolite variables for different *koji* types were selected using corresponding PLS-DA models based on the variable importance in projection (VIP) > 0.7 values and *p*-value < 0.05. A total of 61 metabolites were selected for soybean, wheat, and rice *koji* fermentation using two different microbial strains, and the data for their relative abundance were presented as the $log_{10}$ expression of the corresponding peak areas in GC-TOF-MS datasets (Table S1). The list of discriminant metabolites included 13 sugars and sugar alcohols, 14 organic acids, 18 amino acids, 5 fatty acids, 8 nucleosides, 2 phenolic acids, and 1 vitamin among different *koji* samples.

Further, the PCA score plots based on UHPLC-LTQ-IT-MS/MS datasets indicated the metabolic variance among different *koji* samples based on three substrate types while simultaneously displaying the least variance for samples fermented with different inocula (Fig. 1C). Particularly, the datasets for wheat *koji* sample extracts were separated along PC1 (11.2%) from soybean and rice *koji* samples, while the latter (soybean and rice *koji*) samples were also separated along PC2 (7.9%). In addition, the wheat *koji* samples fermented with *A. oryzae* (WA) and *B. amyloliquefaciens* (WB) were separated along PC2 (7.9%). The pattern of metabolic datasets in the corresponding PLS-DA score plot was similar to that of the PCA (Fig. S1B). A list of significantly discriminant metabolites (VIP > 0.7 and *p*-value < 0.05) based on the PLS-DA model for UHPLC-LTQ-IT-MS/MS datasets was prepared. Overall, 59 metabolites were putatively identified, and their relative abundances were expressed as the $log_{10}$ expression of the corresponding peak area in soybean, wheat, and rice *koji* sample datasets (Table S2). Specifically, in soybean *koji* extracts, 25 discriminant secondary metabolites were identified, including 9 flavonoids (daidzin, glycitin, genistin, acetyldaidzin, acetylglycitin, acetylgenistin, daidzein, glycitein, and genistein), 7 soyasaponins (soyasaponin A2, soyasaponin I, soyasaponin II,

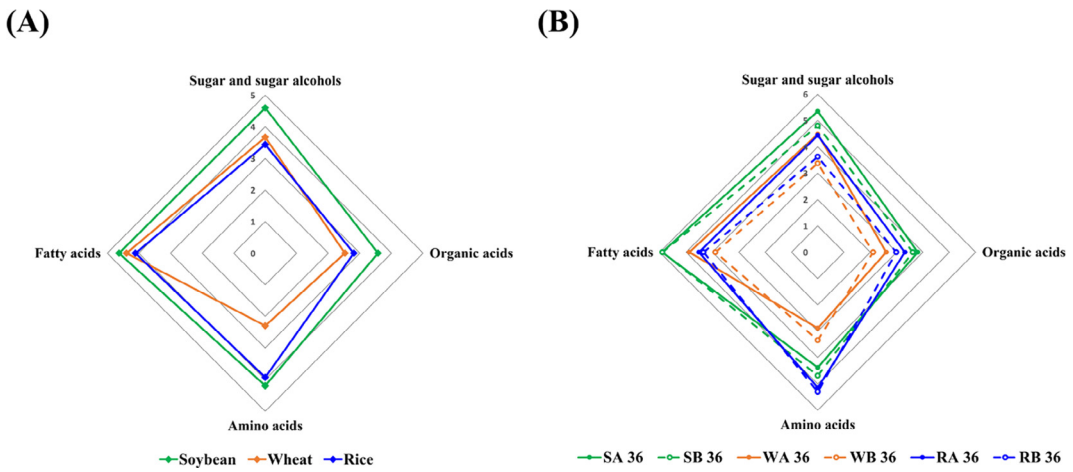


**Fig. 2.** Spider charts for primary metabolite classes outlining differential metabolic alterations according to **(A)** three different substrate types (soybean, wheat, and rice) and **(B)** two different inocula (*A. oryzae* or *B. amyloliquefaciens*). All values were averaged for *koji* types. (Data sets; green symbols – soybean; orange symbols – wheat; blue symbols – rice; t; blue symbols s symbols and -●-, fermented with *A. oryzae*; –○–, fermented with *B. amyloliquefaciens*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

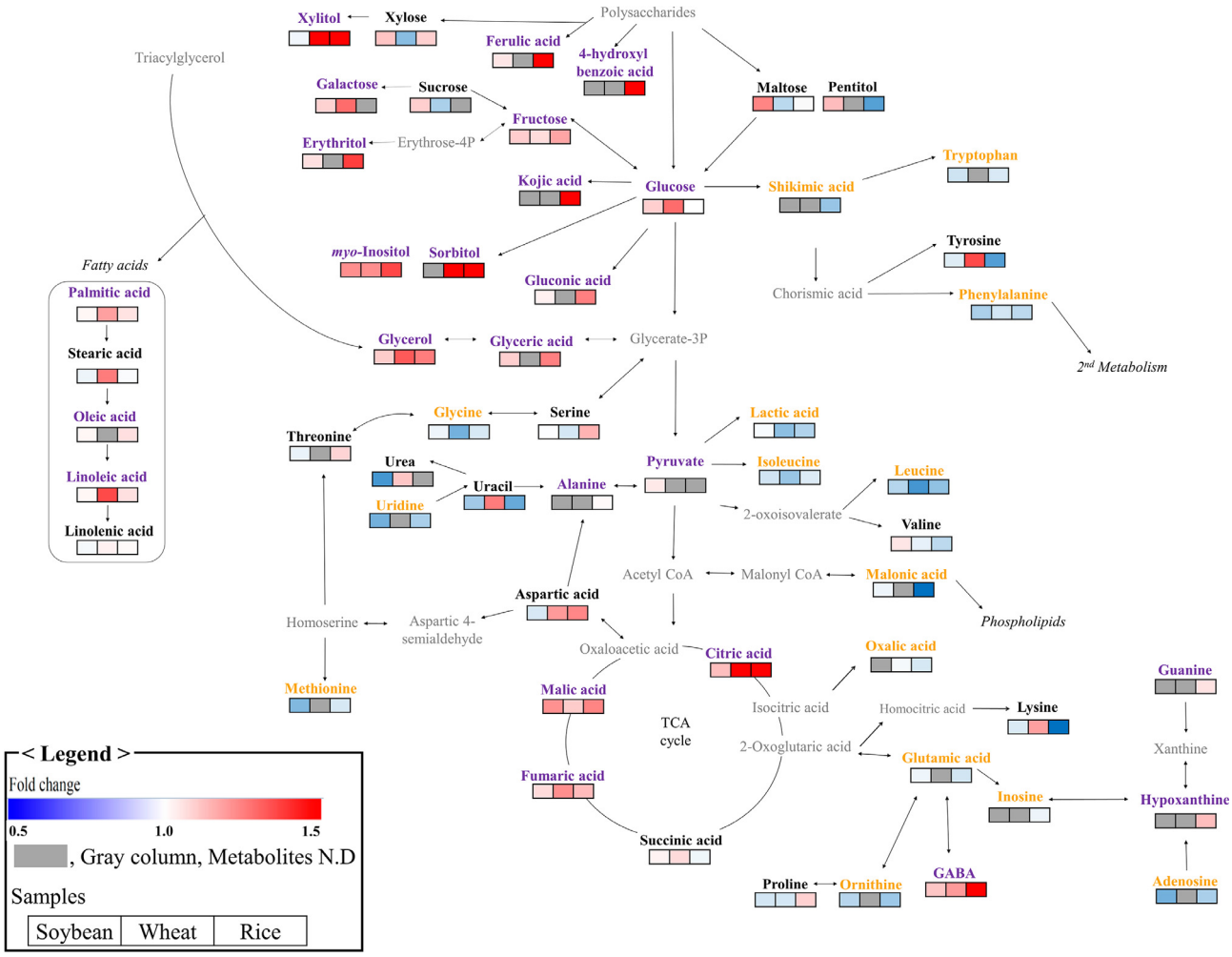


**Fig. 3.** Scheme of the primary metabolic pathway and relative metabolite contents in soybean, wheat, and rice *koji* fermented either with *A. oryzae* or *B. amyloliquefaciens*. The metabolic pathway was adopted from the KEGG database (KEGG, http://www.genome.jp/kegg). The colored squares (blue-to-red) represent the relative metabolite abundance in different *koji* samples fermented using either *A. oryzae*/*B. amyloliquefaciens* with soybean, wheat, and rice as substrates (columns from left to right) individually. Metabolites named with purple and yellow font indicate their relative higher abundance in different *koji* types fermented using *A. oryzae* and *B. amyloliquefaciens*, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

soyasaponin IV, soyasaponin βg, soyasaponin βa, and soyasaponin γg), and 4 lysophospholipids (lysoPC18:3, lysoPC18:2, lysoPC16:0, and lysoPE18:2). In wheat *koji* samples, a total of 16 discriminant metabolites were detected including 3 flavones (di-*C*,*C*-pentosyl-apigenin, tricin and tricin-*O*-rhamnoside-*O*-hexoside), 5 lysophospholipids (lysoPC18:3, lysoPE18:2, lysoPC18:2, lysoPC16:0, and lysoPC18:1), and 9 non-identified metabolites. Similarly, rice *koji* sample extracts were determined with 6 flavonoids (apigenin-*C*-glucosyl-*C*-arabinoside, chrysoeriol-hexoside, chrysoeriol-rutinoside, isovitexin-*O*-glucoside, and tricin), 2 fatty acids (pinellic acid and hydroxy-*oxo*-octadecenoic acid), 9 lysophospholipids (lysoPE14:0, lysoPC14:0, lysoPC18:3, lysoPC16:1, lysoPE18:2, lysoPC18:2, lysoPE16:0, lysoPC16:0, and lysoPC18:1), and 8 non-identified metabolites as significantly discriminant.

### 3.2. Metabolic disparity among 36 h fermented koji end-products

*Koji* end-products of commercial value are generally harvested at 36 h. Hence, we specifically highlighted the metabolic disparity among different *koji* sample extracts harvested at 36 h (SA36, SB36, WA36, WB36, RA36, and RB36) analyzed using GC-TOF-MS and UHPLC-LTQ-IT-MS/MS (Fig. S2A and C). Multivariate analysis based on GC-TOF-MS datasets displayed marked metabolic disparity among the 36-h *koji* samples, based on different inocula along PLS1 (23.7%) and PLS2 (20.1%) (Fig. S2A). Intriguingly, the multivariate analysis for UHPLC-LTQ-IT-MS/MS data highlighted the metabolic disparity among the *koji* samples based on different substrate materials (soybean, wheat, and rice) only across PLS1 (19.1%) and PLS2 (18.0%), regardless of the different inocula (Fig. S2C).

The corresponding four classes of primary metabolites were indicated using a spider chart outlining differential metabolic alterations for the fermentation end-products of different *koji* types (Fig. 2B). Considering the relative abundance of primary metabolites in different *koji* end-products, sugars and sugar alcohols were primarily associated with SA36 coupled with higher levels of organic acids and fatty acids. However, the highest relative abundance of amino acids was linked to RB36 sample extracts. Although the primary metabolite levels showed notable disparity, their compositions were similar in all *koji* types. In contrast, the discriminant secondary metabolites detected from different *koji* types were chemically different. For example, soyasaponins and isoflavones (acetyl-glucosides, *β*-glucosides, aglycones) were detected exclusively from soybean *koji*. On the other hand, tricin and apigenin derivatives were detected mainly from wheat and rice *koji* samples. Interestingly, lysophospholipids were commonly detected in all *koji* types with higher variability and larger proportions in rice *koji* sample extracts.

### 3.2.1. Effects of substrate materials

Comparative metabolite profiling based on GC-TOF-MS and UHPLC-LTQ-IT-MS/MS analyses for raw substrate materials (soybean, wheat, and rice) was performed to evaluate naturally-occurring metabolites in steamed but unfermented *koji* substrates. The discriminant metabolites among the three substrates were similar to those detected in the six different *koji* types fermented with two different inocula (Fig. S2B and D). Overall, 13 sugars and sugar alcohols, 14 organic acids, 18 amino acids, 5 fatty acids, 8 nucleosides, 2 phenolic acids, and 1 vitamin were detected significantly discriminant among the three substrates (Table S1). As shown in Fig. 2A, the discriminant primary metabolite classes were represented in a spider chart, with soybean showing higher levels of sugars and sugar alcohols, organic acids, fatty acids, and amino acids. Similar to the metabolite profiles of fermented samples, both the nature and relative levels of secondary metabolites were similar to those described for fermented *koji* samples (Table S2).

### 3.2.2. Effects of microbial inocula

The effects of varying microbial inocula (*A. oryzae* and *B. amyloliquefaciens*) mediated fermentative alteration of metabolite profiles in different *koji* samples were verified using the corresponding PLS-DA model based on GC-TOF-MS and UHPLC-LTQ-IT-MS/MS datasets (Fig. S3). We observed a common trend in the metabolic datasets with marked disparity between *A. oryzae* and *B. amyloliquefaciens* fermented *koji* samples, regardless of the substrates. Fig. 3 depicts the scheme of the primary metabolic pathway and relative metabolite contents in soybean, wheat, and rice *koji* fermented using the two inocula. A heat map representation for the relative abundance (fold-change levels) of primary metabolites among different *koji* types was made on a biosynthetic pathway map adapted from the KEGG database. Particularly, most sugars and sugar alcohols (glycerol, erythritol, xylitol, fructose, glucose, galactose, sorbitol, and *myo*-inositol), organic acids (citric acid, fumaric acid, gluconic acid, glyceric acid, kojic acid, malic acid, and pyruvate), fatty acids (palmitic acid, oleic acid, and linoleic acid), and γ-aminobutyric acid (GABA) were relatively higher in *koji* fermented with *A. oryzae*, regardless of the substrate type. On the other hand, shikimic acid pathway-derived aromatic amino acids (tryptophan and phenylalanine), branched chain amino acids (isoleucine and leucine), some organic acids (lactic acid, malonic acid, and oxalic acid) and nucleosides (adenosine, uridine, inosine) were relatively higher in *B. amyloliquefaciens*-derived *koji* samples regardless of the substrates types.

Since, the secondary metabolite profiles from different *koji* types varied depending on the substrate types (soybean, wheat, and rice), the effects of different inocula are not discussed in the present section (Fig. S1B). As shown in Table S2, the SA36 contents of most flavonoids, soyasaponins, and lysophospholipids were higher than those of SB36. For wheat *koji*, the average content of flavonoids and lysophospholipids in WB36 was higher than that in WA36. The average contents of flavonoids and lysophospholipid metabolites in rice *koji* were higher in RB36 than RA36.

### 3.3. Effects on associated phenotypes (bioactivities, physicochemical characteristics, and enzyme activities)

Considering the effects of different substrate types (soybean, wheat, and rice) and microbial inocula (*A. oryzae* and *B. amyloliquefaciens*) on six different *koji* combinations, we analyzed the associated bioactivities, physicochemical characteristics, and enzyme activities (Table 2). In general, enzyme activities associated with each *koji* type increased linearly during fermentation with microbial growth. Among the 36-h fermented *koji* end-products, α-amylase activity was highest in RA36 extracts (10.8 U/g of *koji*), followed by WA36 and WB36. On the other hand, the β-glucosidase and protease activities were highest for WA36 (0.19 U/g of *koji*) and SA36 (2.13 U/g of *koji*), respectively. Considering the effects of different inocula, higher α-amylase activities were

**Table 2**
A comparison heat map tabular representation of enzymatic activities, bioactivity phenotypes, and physiological characteristics for different *koji* types fermented using either *A. oryzae* or *B. amyloliquefaciens*.

| **soybean *koji*** | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | soybean | | | SA12 | | | SA24 | | | SA36 | | | SB12 | | | SB24 | | | SB36 | | |
| α-amylase activity[a] | 0.60 | ± | 0.01 | 3.28 | ± | 0.20 | 5.38 | ± | 0.15 | 3.65 | ± | 0.21 | 3.32 | ± | 0.14 | 2.87 | ± | 0.38 | 3.13 | ± | 1.09 |
| β-glucosidase activity[a] | 0.03 | ± | 0.00 | 0.03 | ± | 0.00 | 0.16 | ± | 0.00 | 0.10 | ± | 0.00 | 0.03 | ± | 0.00 | 0.04 | ± | 0.00 | 0.10 | ± | 0.00 |
| protease activity[a] | 0.01 | ± | 0.00 | 0.01 | ± | 0.01 | 0.81 | ± | 0.05 | 2.13 | ± | 0.11 | 0.03 | ± | 0.01 | 0.85 | ± | 0.04 | 1.12 | ± | 0.05 |
| ABTS[b] | 0.05 | ± | 0.00 | 0.05 | ± | 0.00 | 0.07 | ± | 0.00 | 0.08 | ± | 0.00 | 0.04 | ± | 0.00 | 0.05 | ± | 0.00 | 0.05 | ± | 0.00 |
| TPC[c] | 7.07 | ± | 0.35 | 8.24 | ± | 0.24 | 13.90 | ± | 0.56 | 12.28 | ± | 0.09 | 8.99 | ± | 0.59 | 10.85 | ± | 0.15 | 10.92 | ± | 0.56 |
| TFC[d] | 5.01 | ± | 0.37 | 5.77 | ± | 0.08 | 6.61 | ± | 0.31 | 7.66 | ± | 0.62 | 5.49 | ± | 0.22 | 5.71 | ± | 0.17 | 5.48 | ± | 0.39 |
| pH[e] | 6.86 | ± | 0.05 | 6.62 | ± | 0.06 | 5.93 | ± | 0.08 | 6.20 | ± | 0.08 | 6.80 | ± | 0.16 | 7.01 | ± | 0.06 | 6.83 | ± | 0.69 |
| titratable acidity[f] | 0.12 | ± | 0.01 | 0.22 | ± | 0.01 | 1.36 | ± | 0.04 | 0.41 | ± | 0.02 | 0.32 | ± | 0.02 | 0.39 | ± | 0.00 | 0.42 | ± | 0.15 |
| amino-nitrogen content[g] | 34914 | ± | 1590 | 56112 | ± | 2322 | 783074 | ± | 7416 | 97884 | ± | 6671 | 62347 | ± | 3179 | 126875 | ± | 3918 | 163972 | ± | 10254 |
| sugar content[h] | 0.90 | ± | 0.08 | 1.10 | ± | 0.08 | 2.63 | ± | 0.05 | 1.20 | ± | 0.08 | 1.03 | ± | 0.12 | 1.23 | ± | 0.05 | 1.17 | ± | 0.05 |
| **wheat *koji*** | | | | | | | | | | | | | | | | | | | | |
| | Wheat | | | WA12 | | | WA24 | | | WA36 | | | WB12 | | | WB24 | | | WB36 | | |
| α-amylase activity[a] | 0.56 | ± | 0.01 | 3.21 | ± | 0.14 | 9.71 | ± | 0.16 | 9.72 | ± | 0.20 | 9.04 | ± | 0.35 | 8.27 | ± | 0.92 | 7.63 | ± | 0.86 |
| β-glucosidase activity[a] | 0.03 | ± | 0.00 | 0.03 | ± | 0.00 | 0.11 | ± | 0.00 | 0.19 | ± | 0.00 | 0.06 | ± | 0.00 | 0.06 | ± | 0.00 | 0.08 | ± | 0.00 |
| protease activity[a] | 0.02 | ± | 0.01 | 0.02 | ± | 0.01 | 0.89 | ± | 0.04 | 0.82 | ± | 0.10 | 0.13 | ± | 0.04 | 0.61 | ± | 0.06 | 0.62 | ± | 0.03 |
| ABTS[b] | 0.03 | ± | 0.00 | 0.05 | ± | 0.00 | 0.06 | ± | 0.00 | 0.05 | ± | 0.00 | 0.05 | ± | 0.00 | 0.06 | ± | 0.00 | 0.06 | ± | 0.00 |
| TPC[c] | 7.03 | ± | 0.60 | 9.38 | ± | 0.64 | 13.92 | ± | 0.60 | 9.73 | ± | 0.24 | 8.45 | ± | 0.28 | 9.90 | ± | 0.35 | 11.42 | ± | 0.41 |
| TFC[d] | 4.25 | ± | 0.20 | 4.28 | ± | 0.43 | 3.22 | ± | 0.15 | 3.67 | ± | 0.26 | 3.33 | ± | 0.20 | 3.65 | ± | 0.05 | 3.43 | ± | 0.13 |
| pH[e] | 6.86 | ± | 0.05 | 6.62 | ± | 0.05 | 5.93 | ± | 0.08 | 6.20 | ± | 0.05 | 6.80 | ± | 0.11 | 7.01 | ± | 0.04 | 6.83 | ± | 0.03 |
| titratable acidity[f] | 0.08 | ± | 0.00 | 0.20 | ± | 0.00 | 0.83 | ± | 0.04 | 0.77 | ± | 0.04 | 0.38 | ± | 0.02 | 0.49 | ± | 0.02 | 0.57 | ± | 0.02 |
| amino-nitrogen content[g] | 19483 | ± | 1102 | 33511 | ± | 1543 | 440791 | ± | 13343 | 452949 | ± | 39503 | 83545 | ± | 5198 | 174259 | ± | 9174 | 211043 | ± | 441 |
| sugar content[h] | 0.67 | ± | 0.05 | 0.93 | ± | 0.05 | 2.83 | ± | 0.05 | 2.87 | ± | 0.05 | 3.03 | ± | 0.05 | 3.50 | ± | 0.00 | 3.67 | ± | 0.05 |
| **rice *koji*** | | | | | | | | | | | | | | | | | | | | |
| | Rice | | | RA12 | | | RA24 | | | RA36 | | | RB12 | | | RB24 | | | RB36 | | |
| α-amylase activity[a] | 0.39 | ± | 0.01 | 5.67 | ± | 0.07 | 10.58 | ± | 0.43 | 10.79 | ± | 0.03 | 8.96 | ± | 0.25 | 7.04 | ± | 0.55 | 6.52 | ± | 0.32 |
| β-glucosidase activity[a] | 0.00 | ± | 0.00 | 0.01 | ± | 0.00 | 0.02 | ± | 0.00 | 0.03 | ± | 0.00 | 0.03 | ± | 0.00 | 0.03 | ± | 0.00 | 0.03 | ± | 0.00 |
| protease activity[a] | 0.01 | ± | 0.01 | 0.00 | ± | 0.02 | 0.11 | ± | 0.01 | 0.22 | ± | 0.00 | 0.06 | ± | 0.00 | 0.19 | ± | 0.01 | 0.19 | ± | 0.01 |
| ABTS[b] | 0.03 | ± | 0.00 | 0.05 | ± | 0.00 | 0.04 | ± | 0.00 | 0.03 | ± | 0.00 | 0.02 | ± | 0.00 | 0.04 | ± | 0.00 | 0.05 | ± | 0.00 |
| TPC[c] | 3.42 | ± | 0.05 | 5.19 | ± | 0.42 | 5.67 | ± | 0.03 | 4.23 | ± | 0.12 | 1.86 | ± | 0.06 | 5.51 | ± | 0.01 | 6.31 | ± | 0.12 |
| TFC[d] | 0.98 | ± | 0.03 | 1.02 | ± | 0.08 | 0.73 | ± | 0.06 | 0.60 | ± | 0.08 | 0.98 | ± | 0.09 | 1.68 | ± | 0.07 | 1.98 | ± | 0.12 |
| pH[e] | 6.36 | ± | 0.52 | 3.93 | ± | 0.50 | 4.27 | ± | 0.36 | 4.30 | ± | 0.04 | 5.57 | ± | 0.27 | 6.39 | ± | 0.03 | 6.15 | ± | 0.00 |
| titratable acidity[f] | 0.03 | ± | 0.01 | 0.38 | ± | 0.20 | 0.62 | ± | 0.27 | 0.51 | ± | 0.01 | 0.11 | ± | 0.00 | 0.18 | ± | 0.00 | 0.21 | ± | 0.00 |
| amino-nitrogen content[g] | 6546 | ± | 1010 | 23224 | ± | 3247 | 130616 | ± | 3422 | 129058 | ± | 9352 | 19873 | ± | 701 | 86974 | ± | 1403 | 88844 | ± | 0.00 |
| sugar content[h] | 0.10 | ± | 0.00 | 0.63 | ± | 0.05 | 2.40 | ± | 0.08 | 2.23 | ± | 0.05 | 2.40 | ± | 0.08 | 3.57 | ± | 0.05 | 3.70 | ± | 0.00 |

[a] Expressed as unit / g of *Koji*
[b] Expressed as TEAC (mM / g of *Koji*)
[c] Expressed as GE (mM / g of *Koji*)
[d] Expressed as NE (mM / g of *Koji*)
[e] Expressed as pH
[f] Expressed as %acid
[g] Expressed as mg%
[h] Expressed as Brix
* The heat map color (blue-to-red) represents the relative fold change normalized values for each of the biochemical phenotypes.

observed for *A. oryzae*-fermented *koji* samples between 24 and 36 h, despite the higher initial enzymatic activities for *B. amyloliquefaciens*-fermented *koji* at the initial stage (12 h). For the remaining two enzymes, *β*-glucosidase and protease, the observed activities increased linearly until 36 h in all *koji* samples.

Regarding the biochemical phenotypes, ABTS antioxidant activity (0.08 mM/g of *koji*) and TFC (7.66 mM/g of *koji*) were highest at 36 h, while TPC (13.9 mM/g of *koji*) peaked at 24 h in soybean *koji* samples fermented using *A. oryzae*. Examination of the physicochemical characteristics for different *koji* types revealed a marginal but considerable decrease in pH and reciprocal increase in titratable acidity levels for all samples during fermentation (0–36 h). Similarly, amino-type nitrogen and sugar contents increased during fermentation, with the corresponding highest values determined for SA24 and RB36, respectively.

## 4. Discussion

Traditional *koji* manufacturing primarily involves substrates including soybean, wheat, or rice fermented using *A. oryzae* or *B. amyloliquefaciens*. However, the optimal combination of substrate materials and microbial inoculum coupled with their cumulative effects on *koji* end-products are largely unknown. We employed a comparative and systematic MS-based metabolomic approach for evaluating the effects of different substrates and microbial inocula combinations on *koji* end-products and correlated their direct effects on associated biochemical phenotypes determining their commercial quality.

The observed metabolic disparity among the six different *koji* types can be attributed to the following choices; (a) three different substrate materials (soybean, wheat, and rice), and (b) two different microbial inocula (*A. oryzae* and *B. amyloliquefaciens*). Among the different *koji* types, the levels of significantly discriminant primary metabolites were relatively higher in soybean, excluding the effects of microbial inocula (Fig. 2A). However, considering the effects of microbial inocula and substrate types together, the relative abundance of amino acids was higher in rice *koji* (RA36 and RB36) fermented using either inocula (Fig. 2B). Further, the soybean *koji* was characterized by higher flavonoid contents followed by wheat and rice *koji* throughout the fermentative bioprocess. Different flavonoid biosynthetic mechanisms in each substrate material may have resulted in the observed disparity. Isoflavonoid biosynthesis is closely linked to the phenylpropanoid pathway, while raw soybean naturally contains abundant relative enzymes, such as phenylalanine ammonia-lyase, chalcone synthase, and flavanone 3-hydroxylase to produce higher flavonoids (Yu & McGonigle, 2005). Further, the isoflavone aglycone contents, such as daidzein, genistein, and glycitein, were temporally increased during fermentation of soybean *koji* (Table S2). In agreement with a previous report by Kaya et al. (2008) describing isoflavone glycoside hydrolysis to isoflavone aglycones through *β*-glucosidase activity, we observed a positive correlation between isoflavone glycosides levels and *β*-glucosidase activity during *koji* fermentation (Fig. 4). The contents of isoflavones, which are antioxidant in nature, showed positive correlations in the ABTS activity assay (Fig. 4). Previously, Lam, Liu, and Lo (2015) identified a chain of metabolite successions (naringenin followed by apigenin, luteolin, and chrysoeriol) leading to tricin biosynthesis. Thus, the observed decrease in apigenin and chrysoeriol levels in rice *koji* may have contributed to the tricin biosynthesis during fermentation. The dietary consumption of food-derived antioxidants like flavonoids has been suggested to engender umpteen health effects through mitigating free radical damage (Juan & Chou, 2010). Hence, the antioxidant levels in *koji* can have numerous quality implications and can signify its nutritional quality.

The metabolic composition of *koji* samples was greatly altered during the course of fermentation concomitant to the release of free and assimilable metabolites vital for the organoleptic and functional properties of *koji* end-products (Tables S1 and S2). Despite three different substrates, materials were independently used for different *koji* types, the primary metabolite contents varied largely according to the varying inocula used in the study. Previously, the quality characteristics of *koji* were largely evaluated based on its primary metabolite contents as well as the levels of secretory hydrolytic enzymes (amylase, glucosidase, and protease), which alter these metabolite levels during microbial fermentation (Zhu & Tramper, 2013). Biochemically, the extracellular secretion of *α*-amylases results in the hydrolysis of the '*α*-1,4-glycosidic' linkages of starch and polysaccharides in substrate materials into simple sugars like glucose or maltose (Saker, 2017). Similarly, *β*-glucosidase catalyzes the hydrolysis of alkyl and aryl *β*-glucosides, short oligosaccharides, and disaccharide glucosides (Gaensly, Agustini, da Silva, Picheth, & Bonfim, 2015). Hence, the higher extracellular accumulation of sugar hydrolytic enzymes may have caused the linear increase in metabolites related to carbohydrate metabolism, regardless of the substrate types in different *koji* fermentations. The release of simple sugars is vital for rapid colonization of microbial inocula, which enhances the fermentative bioprocess (Solis-Pereira, Favela-Torres, Viniegra-González, & Gutiérrez-Rojas, 1993). This is substantiated by the observed higher relative abundance of sugars in *A. oryzae*-fermented *koji* (AK) with higher *α*-amylase and *β*-glucosidase activities, compared to *B. amyloliquefaciens*-fermented *koji* (BK) (Table 2 and Fig. 4A). Further, the reportedly higher NADPH-consuming reaction in *A. oryzae* might be associated with the observed higher abundance of sugar alcohols (Fernandes & Murray, 2010). The characteristic mild-sweet savor in *koji* fermented foods is largely attributed to the fermentative release of simple sugars, especially monosaccharides, including glucose, galactose, and fructose.

The varying organic acid levels for different *koji* types were mainly affected by different microbial inocula, with most organic acids notably abundant in AK than BK (Fig. 3B). Fungi produce organic acids in abundance turning the niche environment acidic and thus gain a competitive advantage over other competitive microorganisms (Benoit-Gelber et al., 2017). In the present study, most organic acids, including commercially important citric and gluconic acid were relatively higher in AK (Table S1). In contrast, lactic acid levels were relatively higher in BK because of the widely characterized lactate dehydrogenase activity in *Bacillus* species (Dennis & Kaplan, 1960). Among fungi, lactate production is usually compromised because of the respective pathway shunt to fumarate (Dave & Punekar, 2015). Considering the physicochemical implications of organic acid secretion, the pH decrease during the course of *koji* fermentation, coupled with an increase in titratable acidity, suggests a strong correlation between the organic acid abundance, pH levels, and associated titratable acidity (Table 2). The organic acid levels in food products are often considered vital for determining their physicochemical properties, characteristic tang, shelf life, and overall commercial quality (Baek, et al., 2010). Previously, Lee et al. (2016) reported an increase in organic acid contents and proportional decrease in pH values during *koji* fermentation. Similarly, reciprocal trends between titratable acidity and pH levels were reported by others (Jang et al., 2017).

The different *koji* types examined in the study displayed a uniform increase in free amino acid levels, followed by a concomitant increase in amino type nitrogen contents during the course of fermentation (Table S1). Particularly, the aromatic and branched-chain amino acids were higher in BK samples, which may be associated with higher branched-chain aminotransferases activity, catalyzing the transamination reaction to generate branched-chain amino acids. Bacteria contain a single branched chain aminotransferase (Hutson, 2001). Further, we observed a positive correlation between the relative abundance of amino acids and protease activity (Fig. 4). Hence, an increase in amino acid levels in each *koji* sample might have resulted from the degradation of crude proteins in substrates during fermentation (Dai et al., 2017). In *koji* fermented food products, the amino acid compositions determine their characteristic taste and flavor. In particular, the glutamic acid and aspartic acid proportions in fermented foods are often correlated to their characteristic umami taste. Similarly, alanine,

glycine, lysine, and serine engender mild sweetness, while leucine and isoleucine generate bitter taste in fermented end-products (Jang et al., 2017).

Considering the fatty acid contents, palmitic acid, linoleic acid, and oleic acid levels were linearly increased, with respective higher abundances in AK samples compared to BK, independent of the varying substrate materials. Biochemically, the free fatty acids are commonly derived from the secreted lipase activity, hydrolyzing the substrate derived molecules *viz.*, triacylglycerols, diglycerides, monoacylglycerols, and phospholipids (Gambacorta et al., 2009). Herein, the observed higher relative abundance of free fatty acids in AK might be correlated to the suitable extracellular environment (pH 6–7) manifested, while *koji* fermentation allowing the optimal bioactivity of *Aspergillus*-secreted lipases (de Oliveira Carvalho, Contesini, Bizaco, & Alves Macedo, 2005). In contrast, the lipases from *B. amyloliquefaciens* function optimally between the alkaline pH range 10–11, and hence might not be sufficiently active (Saengsanga, Siripornadulsil, & Siripornadulsil, 2016). Herein, we conjecture that the marked disparity in relative levels of free fatty acids in different *koji* types (AK > BK) might have been caused by the near neutral pH range (6–7) prevailing during *koji* fermentation, optimally favorable for *A. oryzae*-secreted lipases. In particular, linoleic acid and linolenic acid, belonging to the class of essential fatty acids (EFAs), are considered vital dietary nutrients (Cunnane, & Anderson, 1997).

## 5. Conclusions

The present study revealed the metabolic repertoire of varying *koji*

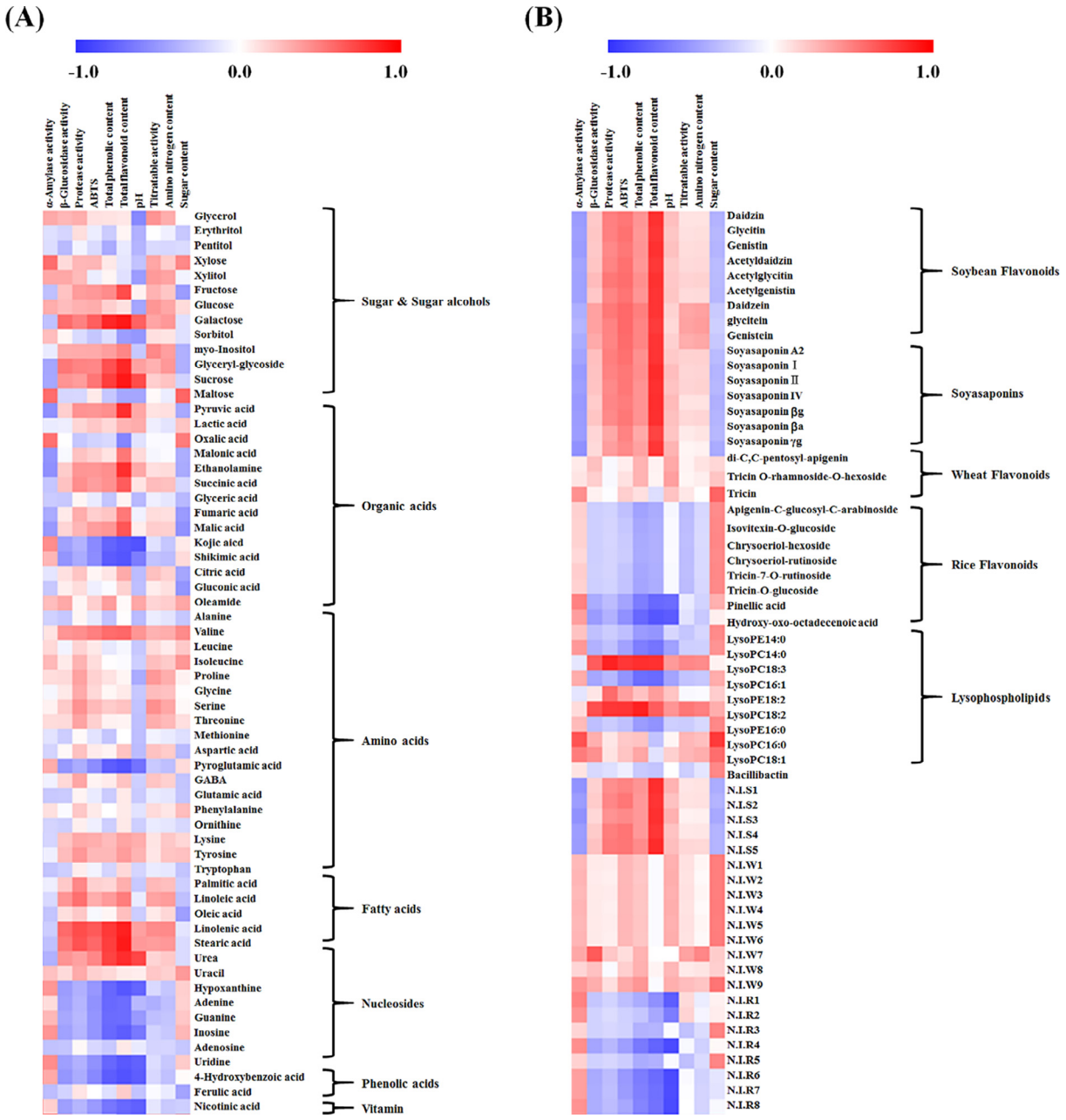


**Fig. 4.** Heat map representations for significantly discriminant **(A)** primary metabolites, and **(B)** secondary metabolites, showing correlations with biochemical phenotypes *viz.*, enzyme activities (α-amylase, β-glucosidase, and protease), antioxidant activity (ABTS), total phenolic content (TP), total flavonoid content (TF), pH, titratable acidity, amino type nitrogen, and sugar contents. Each square indicates the Pearson's correlation coefficient values ($r$). The blue color represents a negative correlation ($-1 < r < 0$) and red color represents a positive correlation ($0 < r < 1$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

types (soybean, rice, and wheat), fermented using either *A. oryzae* or *B. amyloliquefaciens* inocula. Interestingly, primary metabolites compositions, which largely determine the organoleptic properties of *koji* end-products (36 h), varied according to different substrate materials and microbial inocula. In contrast, secondary metabolites constituting the functional components of *koji* were affected by substrate types alone, with soybean *koji* naturally rich in soyasaponins and isoflavonoids, hence displaying higher antioxidant activities. Similarly, tricin compounds were detected as the main flavonoids in rice and wheat *koji* samples, regardless of the microbial inocula used for carrying out the substrate fermentation. Overall, *A. oryzae*-fermented *koji* was rich in sugar and sugar alcohols, organic acids, and fatty acids, while *B. amyloliquefaciens*-inoculated samples displayed higher amino acid contents for *koji* end-products. The systematic metabolomic approach adopted in the present study showed promising applications toward production optimization and quality control analyses of *koji* products and associated bioprocesses.

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