Process specific differential metabolomes for industrial *gochujang* types (pepper paste) manufactured using white rice, brown rice, and wheat

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**A R T I C L E   I N F O**

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Chemical compounds studied in this article:
Valine (PubChem CID: 6287)
LysoPC (18:2) (PubChem CID: 11005824)
Serine (PubChem CID: 5951)
myo-Inositol (PubChem CID: 5280961)
Daidzin (PubChem CID: 107971)
Inositate (PubChem CID: 6306)
Soyasaponin I (PubChem CID: 122097)
Capsaicin (PubChem CID: 5184943)
Dihydrocapsaicin (PubChem CID: 107982)

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**A B S T R A C T**

The metabolic perplexes for *gochujang* (GCJ) fermentative bioprocess, a traditional Korean pepper paste, has largely remain equivocal for preparative conditions and raw material (RM) additives exacerbating its commercial standardization. Herein, we outlined a differential non-targeted metabolite profiling for three GCJ (white rice-WR; brown rice-BR; wheat-WT) under varying processing steps (P1 – fermentation; P2 – *meju* addition; P3 – ripening; and P4 – red pepper addition). We correlated the process specific metabolomes with corresponding physicochemical factors, enzymatic phenotypes, and bioactivities for GCJ-types. The P1 was characterized by a uniform increase in the levels of RM-derived lysoPCs. In contrast, P2 was observed with proportionally higher levels of *meju*-released isoflavones and soyasaponins in WR-GCJ, followed by BR and WT-GCJ. The P3 involved a cumulative increase in primary metabolites in all GCJ samples except lower organic acid contents in WT-GCJ. The pepper derived flavonoids and alkaloids were selectively increased while P4 in all GCJ-types.

1. **Introduction**

Fermented foods have traditionally been the part and parcel of ethnic dietary culture ubiquitously in human societies with their own peculiar characteristics, owing to the artisanal production methods evolved in different geo-sociological regions (Cytko\'vi\'c et al., 2015). In recent years, an overwhelming number of studies have explicitly highlighted the various functional perspectives of fermented foods despite their nutritional aspects viz., anti-obesity, antitumor, and anticancer (Cho et al., 2013). *Gochujang*, a toothsome piquant red pepper soybean paste, is a traditional Korean fermented condiment blended with glutinous grains (rice or wheat), pulverized fermented soybeans (*meju*), and pepper powder (Shin, Kim, Choi, Lim, & Lim, 1996). In recent years, a number of reports have particularly highlighted the health effects of *gochujang* viz., anti-obesity, anti-cancer, or anti-metastatic, owing to an array of functional metabolites derived from capsaicin, soy isoflavones, and soyasaponins (Kim et al., 2005; Park, Kong, Jung, & Rhee, 2001; Shin et al., 2016). Buoyed by the globalization of the Korean fermented foods, *gochujang* have gained the consumers attention worldwide. The fact is evident by the recorded steep consumption and production statistics for commercial *gochujang* with an overall annual trade and exports of worth over $24 million and $28 million, respectively (Korea Agro-Fisheries & Food Trade

\[^{1}\] These authors contributed equally to this work.
Subjected to a bioprocess (Oh, Jang, Woo, Kim, & Lee, 2016). In nutritional contents, quality biomarkers, and health effects of foods spectroscopic methods (Zhou, Xiao, Tuli, & Ressom, 2012). Primarily carried out using hyphenated mass spectrometry or tissue, or an organism under varying physiological conditions, is changes in low molecular weight (<1800 Da) metabolites in a cell, aroma, nutritional, and functional aspects could potentially pave the way for standardization of its optimal fermentative production.

Metalomics, which encompasses a holistic analysis of changes in low molecular weight (~1800 Da) metabolites in a cell, tissue, or an organism under varying physiological conditions, is primarily carried out using hyphenated mass spectrometry or spectroscopic methods (Zhou, Xiao, Tuli, & Ressom, 2012). Quintessentially, the food metalomics involves the analyses of nutritional contents, quality biomarkers, and health effects of foods subjected to a bioprocess (Oh, Jang, Woo, Kim, & Lee, 2016). In recent times, a variety of traditional fermented foods viz., cheonggukjang (Oh et al., 2016), doenjang (Lee, Lee, et al. (2014)), and the soy fermented blends of Cudrania tricuspidata or Lonicera caerulea (Suh et al., 2016) etc. have been studied for their metabolomic implications in conjunction with quality parameters (Kim et al., 2010).

The present investigations involves the comparative mass spectrometry (MS) based metabolite profiling towards delineating the process specific metabolic alterations in a four step commercial gochujang production with varying raw materials (RM). We further made correlations among the process specific significantly discriminant metabolites and the product phenotypes viz., antioxidant activity along with total phenolic and flavonoid contents (TFC). Additionally, the physicochemical factors cum quality parameters viz., pH, titratable acidity (TA), amino-type nitrogen contents, amylase activity, and α-glucosidase activity were also evaluated to infer the effects of varying grain blends in different processing steps.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade. Acetonitrile, water, and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium carbonate, sodium hydroxide, sodium chloride, and diethylene glycol were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Trichloroacetic acid was purchased from Merck Millipore Co. (Darmstadt, Germany). Methoxyamine hydrochloride, N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), pyridine, potassium persulfate, 2,2’-azobis (3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt (ABTS), Folin–Ciocalteu’s phenol reagent, soluble starch, potassium sodium tartrate tetrahydrate, acetic acid, sodium acetate, 4-nitrophenyl-α-D-glucopyranoside, 1 N sodium hydroxide solution, formaldehyde solution, formic acid, 3,5-dinitrosalicylic acid (DNS), and the standard compounds 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), naringin, gallic acid, p-nitrophenol, norvaline, formononietin, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample preparations

The three different commercial gochujang (GCJ) types based on different grain blends i.e., white rice (WR), brown rice (BR), and wheat (WT) were procured from ‘CJ Cheiljedang Corporation’ Seoul, South Korea. Each of the GCJ types were collected from four main processes of its industrial production. As shown in Fig. 1, process 1 (P1 – fermentation) involved the fermentation of steamed raw material (RM) viz., WR, BR, or WT using Aspergillus oryzae at 35 °C for 36 h and finally resulted in koji (kJ) formation i.e., 15–25% of final products. The process 2 (P2 – meju addition) was carried out by adding meju powder (crushed soybeans fermented with A. oryzae, 1–5% of final products) to kJ to obtain a semi-finished product (SP1) before ripening. Following, the process 3 (P3 – ripening) involved the ripening of SP1 to another semi-finished product (SP2) through incubation at 30 °C for 21 days. Finally, the process 4 (P4 – red pepper addition, 10–20% of final product) was accomplished by adding the optimal proportions of red pepper powder, garlic, salt, sugar syrup, and water to obtain the desired end product for each of the commercial GCJ-types (WR-GCJ, BR-GCJ, WT-GCJ).

2.3. Sample extractions and preparation for analyses

The WR-GCJ, and BR-GCJ samples from each process were freeze-dried for 3 days, pulverized, and extracted with 80% methanol (100 mg/mL) at 25 °C through vigorous shaking for 24 h using a Twist Shaker (Biofree, Seoul, Korea). The equivalent amount of pre-treated WT-GCJ samples in 80% methanol were extracted through vigorous shaking (30 Hz/s) for 10 min using a mixer mill (Retsch MM 400, GmbH & Co, Germany). Sample extracts were performed in three biological replicates. All the sample extracts were centrifuged (5000 rpm, 4 °C, 10 min, Herth Zentrifugen, Universal 320R, Germany), and the decanted supernatants were filtered through a 0.22 μm polytetrafluoroethylene (PTFE) filter. The
filtered supernatants were concentrated using a speed vacuum concentrator (Modulspin 31, Biotron, Korea) followed by dissolution in 80% methanol. Norvaline (10 L, 0.1 mg/mL), used as an internal standard, was added to 190 μL of each dissolved extract sample, which were then dried again in a speed vacuum concentrator. The samples preparation for gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analysis involved a two-step derivatization reaction. First, the sample methoximation was carried out by dissolving dried extracts in methoxyamine–HCl (100 mg/mL in pyridine) and incubation at 30 °C for 90 min. Secondly, the methoxymated samples were silylated by adding MSTFA (100 μL) and incubating the reaction mixture at 37 °C for 30 min. The sample extracts (90 μL) for ultrahigh-performance liquid chromatography-linear trap quadrupole-electrospray ionization-ion trap mass spectrometry/mass spectrometry (UHPLC-LTQ-ESI-MS/MS) analysis, were added with for-monotenin (10 μL, 0.4 mg/mL) as an internal standard. Analyses of the sample extracts were performed in triplicate.

2.4. GC-TOF-MS analysis for WR–GCJ, BR–GCJ, and WT-GCJ sample extracts

An Agilent 7890 gas chromatography system (Agilent Technologies, Palo Alto, CA, USA), equipped with an Agilent 7693 autosampler and Pegasus® High-Throughput-TOF-MS (LECO, St. Joseph, MI, USA), was used for GC-TOF-MS analysis of GCJ sample extracts. The GC-TOF-MS analysis and operational parameters used in this study were adapted from Oh et al., 2016.

2.5. UHPLC-LTQ-ESI-IT-MS/MS analysis for WR-GCJ and BR-GCJ sample extracts

A Thermo Fischer Scientific LTQ ion trap mass spectrometer was employed with an electrospray interface (Thermo Fischer Scientific, San José, CA), DIONEX UltiMate 3000 RS Pump, RS Autosampler, RS Column Compartment, and RS Diode Array Detector (Dionex Corporation, Sunnyvale, USA). The conditions and operational parameters for UHPLC-LTQ-ESI-IT-MS/MS analysis were adjusted according to the method described by Oh et al., 2016.

2.6. UPLC-Q-TOF-MS analysis of WT-GCJ sample extracts from different processes

UPLC-Q-TOF-MS analysis was performed using a UPLC system (Waters Corp., Milford, MA, USA) combined with a Q-TOF Premier MS (Waters Micromass Technologies, Manchester, UK) system, coupled with a UV detector and an autosampler. For GCJ sample extracts representing each process in its industrial production, 5 L was injected into an ACQUITY BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm, Waters Corp.). The gradient mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. The mobile phase gradient was initially maintained at 5% B for 1 min, then raised to 100% B over 10 min at a flow rate of 0.3 mL/min, held at 100% B for 2 min, and then finally decreased to 5% B over 1 min. The total analysis run time was 14 min, including column re-equilibration to the initial conditions. The metabolites were analyzed in both positive and negative ion modes under the following operating conditions: cone voltage, 40 V; capillary voltage, 2.5 kV; mass range, m/z 100–1000; and source and desolvation temperatures of 100 and 300 °C, respectively. Capillary voltages were set at −2.3 kV and +2.5 kV in negative and positive ionization modes, respectively. All peak data were analyzed with Mass Lynx software (Version 4.1, Waters Corp.).

2.7. Determination of physiochemical factors – pH, titratable acidity, and amino-type nitrogen contents

The GCJ (5 g) sample replicates (n = 3) for each process in its industrial production were homogenized with distilled water (25 mL) and their pH was measured using a pH meter (Thermo, USA). Further, the titratable acidity was estimated by titrating the homogenized samples using 0.1 N NaOH solution to a set pH level of 8.4. The amino-type nitrogen contents were measure by adding 36% formaldehyde solution (20 mL) to the titrated samples, followed by sample re-titration to pH 8.4 using 0.1 N NaOH solution. The quantitative estimation for amino-type nitrogen contents in the samples was performed using the following formula:

\[
\text{Amino type nitrogen (%) } = \frac{(\text{Volume of 0.1 N NaOH (mL)} - \text{Volume of retitration 0.1 N NaOH (mL)}) \times 1.4 \times (\text{mg/mL}) \times 5 \times df}{\text{Homogenized samples (mg)}} \times 100
\]

All the results of quantitative analyses for three GCJ-types (WR-GCJ, BR-GCJ, WT-GCJ) representing each process (P1, P2, P3, P4) while industrial production were expressed as an average value supplemented with standard deviations.

2.8. Determination of amylase and α-glucosidase activities

The amylase and α-glucosidase activities were estimated using the modified method adapted from Ghosh et al. (2015). The crude enzymes from process specific GCJ samples (10 g) were extracted with water (90 mL) under vigorous agitation using a shaking incubator (30 °C, 120 rpm, 1 h). Subsequently, the samples were centrifuged (5000 rpm, 4 °C, 10 min), and the supernatants were filtered through a 0.2 μm polytetrafluoroethylene (PTFE) filter followed by enzymatic activity analysis. The data for enzymatic activities determined for three GCJ-types (WR-GCJ, BR-GCJ, WT-GCJ) representing each process (P1, P2, P3, P4) while industrial production were expressed as an average value supplemented with standard deviations.

2.9. Determination of total polyphenol and total flavonoid contents

Total polyphenol contents (TPC) and the total flavonoids contents (TFC) assays were performed according to the Suh et al., 2016 method. In TPC assay, each sample extract (20 mL) was reacted with 0.2 N Folin–Ciocalteu reagent (100 μL) for 5 min in the dark. Then, 7.5% NaCO3 (80 μL) was added, and absorbance was measured at 750 nm using a spectrophotometer. Standard curves were made in the linear range of 7.81 ppm and 500 ppm for gallic acid equivalents. The results were expressed in ppm of gallic acid per milligram of each sample extract.
The TFC assay, each sample extract (20 μL) was mixed with 1 N NaOH (20 μL) and 90% diethylene glycol (180 μL). The reaction mixture was incubated in dark for 60 min at room temperature and the absorbance was recorded at 405 nm using a spectrophotometer. The standard curve was linear between the concentration range 6.25 ppm and 200 ppm of naringin equivalents, while the results were expressed in ppm of naringin per milligram of sample extracts. All experiments were performed in triplicate, using the same sample extracts as those in mass spectrometry analysis.

The TPC and TFC data for three GCJ types in each process were expressed as an average value with standard deviations.

2.10. Determination of antioxidant activity

The ABTS assay was performed according to the method described by Suh et al., 2016, with some modifications. The 7 mM ABTS stock solution was dissolved in 2.45 mM potassium persulfate solution, and the mixture was stored for 24 h in dark at 4°C. The solution was diluted until the absorbance reached 0.7 ± 0.02 at 750 nm, measured using a spectrophotometer (Spectronic Genesys 6; Thermo Electron, Madison, WI, USA). Each sample extract (20 μL) was reacted with diluted ABTS solution (180 μL) for 7 min at room temperature in dark, followed by absorbance measurement at 750 nm. A standard curve was made in the linear range, 0.0625 mM–2 mM, Trolox equivalents, with results were expressed in μmol of Trolox equivalents (TE) per milligram of each sample. All assays were performed in triplicate, using the same sample extracts as those in mass spectrometry analysis. The ABTS assay data for three GCJ-types in each process were expressed as an average value with standard deviations.

2.11. Data processing and multivariate statistical analysis

GC-TOF-MS raw data files were converted to computable document form (.cdf) using LECO chroma TOF™ software, and UPLC-Q-TOF-MS data were converted into a NetCDF files (.cdf) using Mass Lynx DataBridge (Version 4.1, Waters Corp.). Additionally, the accurate masses and elemental compositions were calculated using the Mass Lynx software. UHPLC-Q-TQ-ESI-MS/MS raw data files were converted using Thermo Xcalibur software (Version 2.1; Thermo Fisher Scientific Inc., USA). Acquired .cdf formatted data were subjected to preprocessing alignment using the MetAlign software package (http://www.metalign.nl). After alignment, the resulting peak list was obtained as a .txt file, which was exported into Microsoft Excel (Microsoft, Redmond, WA, USA). The Excel file included the corrected peak retention times (min), peak areas, and corresponding mass (m/z) data for further analysis. The pre-processed data were subjected to multivariate statistical analysis using SIMCA-P® 12.0 software (Version 12.0, Umerics, Umea, Sweden) and the trends of metabolite variations (primary and secondary metabolites) in GCJ-types from industrial processing steps were determined using principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA). Selected metabolites with variable importance in the projection (VIP) values of >0.7 and a p-value < 0.05 were considered significantly discriminant among the samples. For TPC, ABTS, and TFC, differences were tested by analysis of variance (ANOVA) and Duncan’s multiple range tests, using PASW Statistica 18 (SPSS Inc., Chicago, IL, USA). A heat map was produced using MeV software version 4.8 [multiple array viewer, TM4: Microarray Software Suite. Available online: http://www.tm4.org/ (accessed on September 25, 2014)].
are listed in Table 1. Additionally, the discriminative metabolites identified through various hyphenated MS-based analytical methods are shown in Supplementary Tables S1–S6. In process 1, RM turned koji through A. oryzae mediated fermentation followed by the generation of primary metabolites viz., amino acids (valine, isoleucine, serine, threonine, etc.), organic acids (acetic acid, etc.), fatty acids (propanoic acid, etc.), and sugars & sugar alcohols (myo-inositol, sucrose, etc.). In case of WR-GCJ and BR-GCJ, espe-

Fig. 2. The levels of physicochemical factors; (A) pH levels, (B) titratable acidity, (C) amino-type nitrogen, along with secreted enzymes; (D) amylase activity, (E) α-glucosidase activity for GCJ-types for different production processes. All the data were presented as the mean value (n = 3) with standard deviations (±SD) for GCJ-types [WR-GCJ, BR-GCJ, WT-GCJ] corresponding to different processes (P1, P2, P3, P4) in industrial productions. The various processes involved are indicated as, Process 1 (P1): fermentation; Process 2 (P2): addition of meju powder (meju Addition); Process 3 (P3): ripening of semi-finished products (Ripening); Process 4 (P4): addition of red pepper powder and etc. (red pepper addition).

Fig. 3. The PCA score plots derived from GC–TOF–MS (A, B, and C), UHPLC–LTQ–ESI–IT–MS/MS (D and E), and UPLC-Q-TOF-MS (F) data sets for different processes symbolized as: P1 (fermentation: +), P2 (meju addition: ⭕), P3 (ripening: ▲), and P4 (red pepper addition: ▼). The plots representing different processes for GCJ types are indicated with different colors; WR-GCJ (+, ◦, ▲, ■), BR-GCJ (+, ◦, ▲, ■), and WT-GCJ (+, ◦, ▲, ■).
Fig. 4. Heat map representations for changes in the relative levels of significantly discriminant primary and secondary metabolites among (A) WR-GCJ, (B) BR-GCJ, and (C) WT-GCJ, according to the processes while industrial GCJ production. The results of in vitro assays for (D) antioxidant activity test (ABTS), (E) total polyphenol contents (TPC), and (F) total flavonoid contents (TFC) were presented as the mean values (±SD) for GCJ-types (WR-GCJ, BR-GCJ, WT-GCJ) corresponding to different processes (P1, P2, P3, P4) in industrial productions.

Table 1
The alteration of primary and secondary metabolites in gochujang (fermented red pepper paste) according to production process.

<table>
<thead>
<tr>
<th>Process</th>
<th>Increase</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (Fermentation)</td>
<td>• Amino acids (Valine, Isoleucine, Serine, Threonine, etc)</td>
<td>–</td>
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<tr>
<td></td>
<td>• Organic acids (Acetic acid, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Fatty acid (Propanoic acid, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Sugars &amp; sugar alcohols (myo-Inositol, Sucrose, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Lyso PCs</td>
<td></td>
</tr>
<tr>
<td>P2 (meju Addition)</td>
<td>• Isoflavones (Daidzin, Genistin, Daidzein, etc)</td>
<td>• Amino acids (Isolucine, etc.)</td>
</tr>
<tr>
<td></td>
<td>• Soyasaponins (I, II, III, IV, V)</td>
<td>• Organic acid (Citric acid, etc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Fatty acid (Propanoic acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sugars &amp; sugar alcohols (Xylitol, Sucrose, etc.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Lyso PCs</td>
</tr>
<tr>
<td>P3 (Ripening)</td>
<td>• Amino acids (Alanine, Isoleucine, Glycine, Aspartic acid, Serine, pyroglutaric acid, Valine, Proline, Threonine, GABA, Glutamic acid, etc.)</td>
<td>• Lyso PCs</td>
</tr>
<tr>
<td></td>
<td>• Organic acid (Lactic acid, Acetic acid, Malic acid, Citric acid, Succinic acid, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Sugars &amp; sugar alcohols (Xylose, Glucose, Glucopyranoside, myo-Inositol, etc.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Isoflavones (Genistin, Daidzin, Daidzein, Glycitein, Genistein)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Soyasaponins (I, II, III, IV)</td>
<td></td>
</tr>
<tr>
<td>P4 (red pepper Addition)</td>
<td>• Organic acid (Citric acid, etc.)</td>
<td>• Amino acids (Alanine, Valine, Threonine, Aspartic acid, etc.)</td>
</tr>
<tr>
<td></td>
<td>• Sugars &amp; sugar alcohols (Maltose, etc.)</td>
<td>• Fatty acid (Propanoic acid, etc.)</td>
</tr>
<tr>
<td></td>
<td>• Flavonoids (Luteolin 7-o-apiosyl-glucoside, Apin, Apigenin-glucoside, etc.)</td>
<td>• Sugars &amp; sugar alcohols (myo-Inositol, etc.)</td>
</tr>
<tr>
<td></td>
<td>• Alkaloids (Capsaicin, dihydrocapsaicin)</td>
<td>• Soyasaponins (I, II, III, IV, V)</td>
</tr>
</tbody>
</table>

The criteria for selection is shown as in common metabolite which increased or decreased in two or more red pepper paste among WR, BR, and WT.
cially, the levels of lysoPCs were significantly increased while P1 (late RM fermentation) and P2 (early meju addition) (Fig. 4A and B). In contrast, the lysoPCs levels were not selectively spiked for WT-GCJ while the fermentation phases (Fig. 4C). The process P2 (meju addition) was followed by the specific rise in the levels of soybean-originated metabolites viz., isoflavones (daidzin, genistin, daidzein, glycitein, genistein) for all GCJ-types, and soyasaponins (V, I, II, III, IV) except WT-GCJ. The largest numbers of metabolites were altered during P3 (ripening process) with a soybean-originated metabolites increased briskly. Additionally, the primary metabolites levels viz., amino acids (alanine, isoleucine, glycine, aspartic acid, serine, pyroglutamic acid, valine, proline, threonine, GABA, glutamic acid, etc.), organic acids (lactic acid, acetic acid, malic acid, citric acid, succinic acid, etc.), and sugars & sugar alcohols (xylose, glucose, glucopyranose, and myoinositol, etc.) increased varyingly during P3 for all GCJ-types. However, lysoPCs levels were significantly fell in all cases. In P4, the final step in GCJ production (red pepper addition), the contents of red pepper-originated flavonoids and alkaloids, including luteolin 7-O-apiosyl glucoside, apin, apigenin glucoside, capsaicin, and dihydrocapsaicin were selectively increased, while those for isoflavones and soyasaponins were decreased. The relative levels of metabolite classes viz., amino acid, organic acid, fatty acid, sugar & sugar alcohols, lysoPC, soyasaponin, isoflavone, flavonoid, and alkaloid were further represented using the box and whisker plots (Fig. 5). The trends for metabolite classes in GCJ-types from different processes were found in agreement to those observed in heat map analysis.

The mean alterations in bioactivity phenotypes while different processes of industrial manufacturing for three GCJ-types were evaluated through ABTS assay for antioxidant activity (Fig. 4D), TPC assay for total polyphenol contents (Fig. 4E), and TFC assay for total flavonoid contents (Fig. 4F). In congruence to the metabolite profiles, the observed bioactivities were also varied during the different processes of GCJ production. Specifically, the ABTS assay determined antioxidant activities were observed highest while the late P2 (meju addition) and early P3 (ripening) followed by a slight decrease in later stages. However, a marginal increase in antioxidant activities were again observed after adding red pepper powder in P4. The TPC activity was specifically spiked while P3 (ripening step), whereas the TFC activity was gradually increased from P1 to P4.

4. Discussion

In the present study, we adopted a metabolomic approach to delineate the quality characteristics for three GCJ-types (WR-GCJ, BR-GCJ, and WT-GCJ) according to the processing steps viz., P1

![Fig. 5. Box and whisker plots for nine metabolite classes outlining the differential metabolic alterations according to the processes in GCJ manufacturing. All values were averaged for GCJ-types (WR-GCJ, BR-GCJ, and WT-GCJ) according to the processes and each process was indicated; process 1 (P1): Fermentation; process 2 (P2): addition of meju powder (meju addition); process 3 (P3): ripening of semi-finished products (Ripening); process 4 (P4): addition of red pepper powder and etc. (red pepper addition).](image-url)
Coauthors have suggested that the growth of harmful bacteria is of vital importance for enhanced shelf-life of end products. The addition of lysoPCs (Fig. 5) as well as few amino acids viz., valine, isoleucine, and GABA (Itoh, Kawashima, & Chibata, 2006). In this process, a plethora of discriminative metabolites were observed depending upon the RM contents viz., WR, BR, or WT, which were significantly altered during the later processes. Table 1 summarizes the significantly altered metabolites with their relative levels increased or decreased during the different processes of GCJ production. In P1, the relative levels of various classes of discriminant metabolites viz., amino acids, organic acids, fatty acids, sugars & sugar alcohols, and lysoPCs were variably increased among the different GCJ-types (Fig. 4). The observed inception of primary and secondary metabolite synthesis during P1 was consistent with a steep fall in pH levels coupled with a gradual rise in titratable acidity and amino-type nitrogen contents for the GCJ sample extracts (Fig. 2A, B, and C). The successive increase in an average antioxidative activity during P1 (Fig. 4D) can be directly linked with an elevation in the levels of lysoPCs (Fig. 5) as well as few amino acids viz., valine, isoleucine, and GABA (Itoh, Kawashima, & Chibata, 1975, and Chang, Han, Wight, & Chait, 2006). In case of WR-GCJ and WT-GCJ, the overall levels of discriminant primary metabolites were significantly altered for the increased activity of hydrolytic enzymes secreted by Aspergillus species (Lee et al., 2016). In contrast, the levels of primary metabolites while P1 in BR-GCJ were not significantly altered owing the probable morphological cessation of hydrolytic activity by rice bran in BR substrate (Moonngarm & Saetung, 2010). As indicated in corresponding heat maps (Fig. 4) and box plots (Fig. 5), the meju powder addition to koji during P2 was followed by a significant increase in the levels of soybean-originated metabolites, such as isoflavones and soyasaponins viz., daidzin, genistin, daidzein, and soyasaponins (I, II, III, IV, V). Functionally, soyasaponin compounds are known to have a number of functional effects viz., neuroprotective, hypcholesterolemic, and anti-hepatotoxic (Hong et al., 2013; Lin, Meijer, Vermeer, & Trautwein, 2004; Yang, Dong, & Ren, 2011). Concurrently, TFC and antioxidant activity were also observed higher owing to the significant levels of soy derived metabolites in late P2 stages, as reported earlier by Lee, Lee, et al. (2014). Intriguingly, the addition of meju powder, except for WT-GCJ, was followed by a significant decrease in the levels of primary metabolites and lysoPCs. On the other hand, the onset of P2 also witnessed a steep drop in the pH levels (~6.5–4.5) owing to the release of organic acids during late P1 (fermentation). Further, the pH levels were observed varying in a narrow range (~4.4–5.0) in consecutive processes i.e., P2 to P4 on account of the higher levels of free amino acids (Fig. 2A) (Kim & Yi, 2010). In food processing, the pH levels of microbial fermentation while P3 (ripening) largely affects bio- transformation of proteins, carbohydrates, and glycosides into simple molecules viz., amino acids, sugars, and aglycones (Lee et al., 2012). In particular, amino acids are closely connected to the unique taste, nutrition, and functional aspects of GCJ, and are generally produced by protein degradation (Kang et al., 2011). Alanine, glycine, serine, glutamic acid, and isoleucine are related to sweet, bitter, and savory tastes (Lee, Lee, et al., 2014). Among them, glutamic acid is highly correlated with umami taste (Kaneko, Kumazawa, Masuda, Henze, & Hofmann, 2006). In addition, alanine, proline, and threonine are known to contribute to the sweet taste in fermented soy-foods including GCJ (Villares, Rostagno, García-Lafuente, Guíllamón, & Martínez, 2011). During P3, lysoPCs were significantly decreased, while most of the metabolites viz., amino acids, organic acids, sugars & sugar alcohols, isoflavones, soyasaponins, and tricin were relatively increased (Fig. 5). These metabolic drifts might have contributed to the enhanced bioactivities including ABTS levels, TPC, TFC as well as the characteristic flavors and taste (umami, sweet, and savory) in GCJ. Further, we observed the rapid increase in the levels of valine while P3 in WR and WT-GCJ, which might have accelerated the microbial growth and production of fermentative enzymes (Baek et al., 2010). In addition, the levels of maltose, glucopyranose, and glucose also increased significantly during P3, which reportedly influence the mouthfeel and sweet flavor of GCJ (Kim et al., 2012). The levels of organic acid were affected by species of microbial strains used in the preparation of GCJ, which in turn affects the pH levels, taste, and aroma in end products (Baek et al., 2010). Earlier, Villares et al., 2011 and Lee, Seo, Oh, & Lee, 2014 have reported that during A. oryzae mediated soybean fermentation, acetyl glycoside and glycosides are generally transformed into aglycones, causing changes in isoflavone contents and distribution patterns during ripening. In our study, both aglycone and glycoside isoflavones were increased during ripening. Moreover, the quality indicators like titratable acidity and amino-type nitrogen contents were notably increased during P3, which were correlated to the observed alterations in free amino acids levels released through enzymatic hydrolysis of soy proteins (Kim, Oh, & Shin, 2008). The addition of red pepper powder and other ingredients in P4 was followed by the specific elevation in the levels of alkaloids (capsaicin and dihydrocapsaicin) and flavonoids (luteolin 7-O-apiosyl glucoside, luteolin-glucoside, apin, and apigenin glycosides), which effects the quintessential piquant spicy flavor in GCJ (Fig. 5). Additionally, the levels of organic acids viz., acetic acid, malic acid, and citric acid were sharply increased during P4, which greatly influence the flavor properties and shelf life of fermented foods (Fig. 4 and Fig. 5).

In conclusion, we performed a comparative evaluation for metabolite levels, bioactivities, and quality parameters for three GCJ-types while different processes of its industrial manufacturing. The GCJ samples (WR-GCJ, BR-GCJ, WT-GCJ) were examined according to the RM used (white rice, brown rice, and wheat) and the production processes. We delineated the fermentative bioprocess and the cascade of metabolomic alterations in correlation with quality parameters for industrial GCJ production. The initial fermentation of RM was primarily linked with the production of metabolites imparting sweet taste to koji (P1). Further, the
blending with meju powder (P2) and subsequent ripening (P3) processes caused the metabolites inception resulting in characteristic savory and umami taste as well as the functional bioactivities of commercial GCJ. Ultimately, adding red pepper powder and several other additives (P4) caused the production of metabolites influencing its sour and spicy taste. The quality parameters changed drastically during the production process, with exception to the pH levels. Hence, this integrated delineation of the dynamic correlations among metabolite levels, bioactivities, and quality parameters provides an appropriate model towards standardization of the industrial GCJ manufacturing using varying substrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.04.154.

References


