Effects of different storage conditions on the metabolite and microbial profiles of white rice (*Oryza sativa* L.)

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Abstract Microbial populations in white rice (*Oryza sativa* L.) samples stored for 6 months in open or closed conditions were studied and their metabolite profiles analyzed using GC/MS to elucidate the relationship between storage and rice quality. Rice samples stored in open conditions at 25 °C were contaminated by *Aspergillus tritici*, *Cladosporium cladosporioides*, and *Penicillium* sp., whereas the control stored in closed conditions at 5 °C was mainly contaminated by *Hyphopichia burtonii* and *A. tritici*. These differences resulted in significantly different metabolite profiles. Increased mold population decreased the levels of fresh rice flavor-associated volatile metabolites and primary energy sources, but increased the levels of metabolites associated with lipid oxidation, polyols, and energy production. Thus, rice quality, especially flavor, could be significantly influenced by the increased mold population caused by open storage at 25 °C; volatile metabolites and polyols are potential indicators of rice quality.

Keywords GC/MS · Metabolomics · Microbial contamination · Rice · Storage

Introduction

Rice (*Oryza sativa* L.), one of the most important food resources in the world (Bhattacharjee et al., 2002), is the main agricultural commodity with the fourth-highest worldwide production in 2000–2013. Particularly, 90% of the world's rice production is consumed in Asia (FAO, 2000). However, recent changes in food consumption patterns have led to a decrease in rice consumption, resulting in significantly increased amounts of harvested and stored rice (Oh et al., 2007). Unfortunately, most rice is stored in silos at room temperature instead of at low temperature due to economic reasons. During a large storage period, rice quality will gradually deteriorate, being affected by several factors such as time, temperature, moisture content, and microbial contamination. Many studies on rice quality during storage showed that rice aging could lead to significant changes in the physico-chemical interactions between rice components such as protein, starch, and lipid and in enzymatic reactions (Park et al., 2012; Sowbhagya and Bhattacharya, 2001).

Fungi contamination is the main factor for rice deterioration (Sinha et al., 1969), reducing profitability and effectiveness of rice utilization (Sibanda et al., 1997). Unfortunately, microbial contamination is inevitable under inappropriate storage conditions (Oh et al., 2007). In particular, mycotoxins originated from fungi contamination may induce food poisoning with liver damage, kidney trouble, dyspnea, and hypogonadism (Fung and Clark, 2004). Therefore, many methods involving morphology, biochemistry, immunology, microbiology, and molecular

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biology have been used to identify microorganisms. However, these methods are either difficult to handle, have a narrow identification range, or take a long time to be performed (Goodacre, 2007).

Recently, metabolomics was used to investigate the quality change in grains and the relevant biomarkers of microbial contamination during storage (Cevallos-Cevallos et al., 2009). For example, ergosterol produced by fungal species were evaluated in wheat and oat grains as an indicator of fungal growth (Börjesson et al., 1992), and the causal link between metabolites and the quality of wheat, triticale, rye, oats, and barley was investigated (Buško et al., 2010). However, to our knowledge, metabolite profiles of white rice with microbial contamination have not been investigated, although those of germinating rice seeds and different rice varieties have been reported (Kusano et al., 2007; Oikawa et al., 2008; Shu et al., 2008).

Therefore, in this study, microbial populations and metabolite profiles of white rice samples stored in open or closed storage conditions at 25 °C or 5 °C were analyzed in an effort to reveal the changes in rice quality and its relationship to storage conditions.

Materials and methods

Rice samples

Rice (*Chuchung* cultivar) produced in Icheon, Korea, in 2014 was polished to be used in this study. White rice samples were divided into two groups. One group was packed in a closed plastic bag and stored at 5 °C (control) ($n = 5$), whereas the other was packed in an open plastic bag and stored at 25 °C (naturally microbial contaminated rice, NMCR) ($n = 5$). Both samples were stored under each condition for 6 months. After that, both samples were ground by a blender for metabolomic analysis and the non-ground samples were used for microbial analysis.

Microbial analysis

Samples (1 g) were homogenized in 9 mL of saline solution using a stomacher (Seward Lab System, Norfolk, UK). An aliquot of 1 mL was spread onto petri dish media containing potato dextrose agar (PDA) with 10% tartaric acid or Cooke Rose Bengal agar with antimicrobial vial A and incubated at 25 °C for 5–7 days and 3 days, respectively. Twenty-four and twenty single colonies from control and NMCR groups, respectively, were picked for subsequent culturing. Identification of each microorganism was conducted by Macrogen (Seoul, Korea). The primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3')

were used for PCR. The PCR reaction was performed with 20 ng of genomic DNA as template in a 30- μ L reaction mixture using EF-Taq (SolGent, Daejeon, Korea) as follows: activation of Taq DNA polymerase at 95 °C for 1 min, 1 min each at 55 °C and 72 °C, and 10 min at 72 °C. The amplification products were purified using a multiscreen filter plate (Millipore, Bedford, MA, USA). Sequencing was performed using the PRISM BigDye Terminator v3.1 Cycle sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA, USA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice, and then analyzed using an ABI Prism 3730XL DNA analyzer (Applied Biosystems).

Acidity

The ground samples were extracted with a solvent mixture of ether and ethanol (1:2). After shaking, phenolphthalein solution was added to the sample mixture and titrated with 0.1 N potassium hydroxide.

Sample preparation for metabolomic analysis

For volatile metabolite analysis, ground rice samples (10 g) with 2,4,6-trimethylpyridine as an internal standard were placed into a vial with a septum cap and heated at 80 °C for 1 h. After heating, a solid-phase microextraction (SPME) fiber (50/30 μ m DVB/CAR/PDMS Stableflex, Sigma-Aldrich, Saint Louis, MO, USA) was inserted into the vial cap to absorb volatile compounds in the head space at 80 °C for 20 min. The absorbed volatile compounds were then analyzed using gas chromatography (GC) (Shimadzu, Tokyo, Japan) ($n = 4$).

For non-volatile metabolite analysis, ground rice samples (50 mg) were homogenized using a bullet blender (Next Advance, Troy, NY, USA) for 10 min with 500 μ L of 50% methanol containing dicyclohexyl phthalate as an internal standard. After centrifugation at 12,000 rpm for 15 min, supernatants were completely dried using a CentriVap vacuum concentrator (LABCONCO, Kansas City, MO, USA). Residues were redissolved in a mixture of ethoxyamine hydrochloride and pyridine and incubated at 70 °C for 90 min. The methoxylated samples were derivatized with 70 μ L of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 40 °C for 50 min. The derivatized samples were analyzed using a GC/mass spectrometer (MS) ($n = 5$).

Analysis of metabolites using GC/MS

The extracts of volatile and non-volatile metabolites were analyzed using a Shimadzu GC-2010 plus (Shimadzu, Tokyo, Japan). The volatile metabolites absorbed in the SPME fiber and derivatized samples were injected into a DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, Restek, PA, USA) with split ratios of 1:8 and 1:50, respectively. For volatile metabolites, the injector temperature was set to 270 °C, the oven temperature program was initiated at 50 °C for 2 min, increased at a rate of 3 °C/min to 180 °C, increased at a rate of 10 °C/min to 300 °C, and held for 3 min. For non-volatile metabolites, the injector temperature was set to 200 °C and the oven temperature program was initiated at 70 °C for 2 min, increased at a rate of 10 °C/min to 320 °C, and held for 5 min. Helium was used as the carrier gas at 0.95 mL/min. The GC column effluent was detected using a Shimadzu GCMS-TQ 8030 mass spectrometer. A 70 eV was used for ionization. The ion source and interface temperatures were 230 °C and 280 °C, respectively. Data monitoring was performed in the full scan mode (m/z 45–550). The scan event time and velocity were 0.3 s and 2000 amu/sec, respectively. For quality control, a mixture of all non-volatile samples was injected after every 5 samples.

Data processing

Data sets obtained using a GC/MS were processed with Analyzer Pro software (Spectralworks, UK). All GC/MS peaks were acquired using an area threshold of 3000, height threshold of 1, signal to noise of 1, width threshold of 0.01, scan windows of 3, and smoothing of 3. Acquired data were aligned with retention time window of 0.1 ± 0.05 min and normalized with the internal standard. Retention indices (RIs) of metabolites were calculated using a series of C8–C40 *n*-alkanes. Metabolites were identified using calculated RIs, GC/MS databases (NIST 11 and Wiley 9 mass spectral libraries), and authentic standards.

Statistical analysis

Processed GC/MS data sets were analyzed with multivariate statistics using SIMCA-P⁺ version 12.0.1 (Umetrics, Umea, Sweden). Comparison of data between control rice and NMCR was visualized using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The quality of PLS-DA models was evaluated using three parameters (R^2_X , R^2_Y , and Q^2_Y) and validated by permutation tests ($n = 200$). Normalized GC/MS intensities of metabolites were statistically analyzed using one-way analysis of variance (ANOVA) with

Independent Sample *t* test ($p < 0.05$) using SPSS 21.0.0.0 (IBM, New York, NY, USA). Identified metabolites with significant differences ($p < 0.05$) were also visualized in a heat map. The heat map was drawn using R with ggplot2 representing the z-score transformed data of rice metabolites in a green–red color scale, with red indicating a decrease and green indicating an increase in metabolite levels.

Results and discussion

Microbial analysis of rice subjected to different storage conditions

Microbial contamination of white rice samples stored in closed bags at 5 °C (control) and in open bags at 25 °C (NMCR) was investigated (Fig. 1). Few fungi were detected in both samples, and their microbial profiles depended on their respective storage conditions. Three molds (*Aspergillus tritici*, *Cladosporium cladosporioides*, and *Penicillium* sp.) were detected in NMCR, whereas one yeast (*Hyphopichia burtonii*) and one mold (*A. tritici*) were found in the control. *H. burtonii*, a dominant microbial species in control rice, was not detected in NMCR, whereas the proportion of *A. tritici* population increased from 12.5% in the control to 80% in NMCR. Moreover, *Penicillium* sp. and *C. cladosporioides*, which were not detected in the control, corresponded to 15% and 5%, respectively, of the population in NMCR. These results indicate that environmental conditions such as temperature, water activity, and open or closed storage influence microbial growth, and the contaminating microorganisms compete with each other for survival. *H. burtonii*, which naturally grows on starch (Deschuyffeleer et al., 2011), did not grow in NMCR in the open condition at 25 °C because the presence of *Aspergillus*, *Penicillium*, and *Cladosporium*, which are the most common fungi in outdoor and/or indoor air (Shelton et al., 2002), inhibited its growth.

Metabolite analysis of rice subjected to different storage conditions

Volatile and non-volatile rice metabolite profiles were analyzed using GC/MS (Fig. 2). A total of 228 volatile and 47 non-volatile metabolites were detected, and all data sets were statistically analyzed using PLS-DA. The control and NMCR samples were significantly separated from each other in the PLS-DA score plots with good quality parameters (R^2_X , R^2_Y , and $Q^2 > 0.851$); (Fig. 3A, D) and statistical acceptability ($p < 0.05$; permuted $R^2 < 0.4$ and $Q^2 < -0.1$); (ig. 3B, E). The S-plots showed that many rice metabolites were located in the negative sections and

Fig. 1 Microbial populations in white rice subjected to different storage conditions. The proportions of kimchi bacteria were determined using a culture-dependent identification method using 16S rRNA sequencing. Thirty single colonies per group were picked for culturing

Fig. 2 Representative gas chromatograms of volatile (A) and non-volatile metabolites (B) from white rice subjected to different storage conditions. Volatile metabolites in both rice samples collected by headspace-SPME and non-volatile metabolites extracted by 50%

methanol and derivatized by methoxyamine hydrochloride in pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide were analyzed using gas chromatography-mass spectrometry with a DB-5MS capillary column. Identified metabolites are numbered

Fig. 3 Partial least squares discriminant analysis (PLS-DA) score plots of rice volatile (A) and non-volatile metabolites (D) analyzed using gas chromatography-mass spectrometry. The qualification of

the PLS-DA models was evaluated with R^2X , R^2Y , Q^2 , and P value, and validated by cross validation with a permutation test ($n = 200$) (B, E). Identified metabolites are numbered in the S-plots (C, F)

their intensities significantly increased in the NMCR samples compared to the control, whereas metabolite intensities in the positive sections of the S-plots decreased (Fig. 3C, F). The change in profile of these metabolites contributed to the separation of both samples on the PLS-DA score plots.

Of the many metabolites contributing to the separation, 13 volatile (including aldehydes, terpenoids, octenol, 2-pentylfuran, pentanoic acid, styrene, anethole, 2-ethylhexyl acrylate, and butylated hydroxytoluene (BHT)) and 28 non-volatile metabolites (including polyols, sugars, organic acids, fatty acids, alanine, and methylphenylethanolamine) were identified (Table 1), and are also shown in the GC chromatograms (Fig. 2) and S-plots. In particular, all identified non-volatile metabolites and the volatile metabolites hexanal, sabinene, nonanal, and BHT had a VIP value > 0.92 , which indicates that these compounds contributed significantly to the differences between both groups.

Rice metabolomic pathway and relative abundance of identified metabolites

To visualize the changes in metabolite levels according to storage condition, a heat map was plotted in a green–red color scale using the z-score transformed raw data of metabolites (Fig. 4). The levels of heptanal, hexanal, octanol, 2-pentylfuran, nonanal, pentanoic acid, phosphate, fumaric acid, mannose, succinic acid, arabitol, sorbitol, citric acid, threitol, inositol, malic acid, fructose, methylphosphate, and halostachine increased in NMCR, whereas the levels of other metabolites decreased.

Based on the identified metabolites, we propose a rice metabolomic pathway associated with microbial contamination, shown with the corresponding normalized intensities in Fig. 5. Microorganisms generally break down the primary metabolites such as carbohydrates, lipids, and proteins into small metabolites. After these metabolic changes, many by-products associated with nutritional quality are produced. Our data show that an increase in

Table 1 Identification of major metabolites contributing to the separation between control and NMCR samples on PLS-DA score plots

No.	Metabolites	RT ^a (min)	RI ^b	<i>p</i> value ^c	VIP ^d	Identified ^e
<i>Volatile metabolites</i>						
1	Heptanal	2.98	764	1.70E−03	0.22	RI, MS, Std
2	Hexanal	4.10	778	1.58E−02	1.48	RI, MS, Std
3	Styrene	6.57	798	5.20E−03	0.52	RI, MS, Std
4	Pinene	8.07	887	2.00E−04	0.60	RI, MS, Std
5	Sabinene	9.84	896	1.00E−03	1.26	RI, MS, Std
6	Octenol	9.96	896	1.00E−04	0.83	RI, MS, Std
7	2-pentylfuran	10.39	898	3.00E−04	0.93	RI, MS
8	Nonanal	15.72	1089	2.40E−03	1.53	RI, MS, Std
9	Anethole	20.20	1100	1.00E−04	0.53	RI, MS
10	2-ethylhexyl acrylate	21.67	1193	3.32E−06	0.55	RI, MS
11	Longifolene	29.98	1300	4.76E−02	0.30	RI, MS
12	Butylated hydroxytoluene	34.08	1495	2.70E−03	2.10	RI, MS, Std
13	Pentanoic acid	37.60	1499	1.00E−03	0.73	RI, MS, Std
<i>Non-volatile metabolites</i>						
14	Alanine	6.98	840	3.46E−06	1.34	RI, MS, Std
15	Halostachine	7.22	911	1.67E−02	0.92	RI, MS
16	Methylphosphate	8.19	916	2.80E−03	1.10	RI, MS
17	Phosphoric acid	9.67	1012	2.00E−04	1.29	RI, MS, Std
18	Glycerol	9.70	1012	4.20E−03	1.30	RI, MS, Std
19	Succinic acid	10.35	1106	2.90E−03	1.05	RI, MS, Std
20	Fumaric acid	10.89	1108	3.20E−03	1.14	RI, MS, Std
21	Malic acid	12.78	1208	2.20E−03	1.16	RI, MS, Std
22	Threitol	13.02	1209	4.10E−03	1.12	RI, MS
23	Arabitol	15.67	1503	8.00E−04	1.17	RI, MS, Std
24	Citric acid	16.83	1602	1.42E−06	1.27	RI, MS, Std
25	Fructose	17.39	1604	6.60E−03	0.98	RI, MS, Std
26	Mannose	17.60	1604	2.00E−04	1.14	RI, MS, Std
27	Sorbitol	18.02	1702	4.00E−04	1.20	RI, MS, Std
28	Inositol	19.71	1804	6.54E−08	1.30	RI, MS, Std
29	Linoleic acid	20.94	2002	1.00E−04	1.45	RI, MS, Std
30	Oleic acid	21.00	2002	1.63E−05	1.35	RI, MS, Std
31	Sucrose	24.49	2401	1.00E−04	1.29	RI, MS, Std

^aRT retention time^bRI retention index^cVIP variable importance in the projection^d*p* Values were analyzed by *t* test^eThe metabolites were identified by standards, mass spectrum, and/or RI values

mold population decreased the content of primary energy sources such as sucrose, fatty acids, and alanine, but increased lipid oxidation, polyol production, and energy production (Fig. 5).

Linoleic and oleic acids, the major fatty acids in rice (Yasumatsu and Moritaka, 1964), were degraded by the contaminating microorganisms and oxidized. However,

palmitic acid and stearic acid, the other major fatty acids in rice, were not significantly changed. The contents of linoleic acid and oleic acid decreased in the NMCR samples, whereas the levels of oxidized lipid products such as aldehydes (heptanal, hexanal, and nonanal), 2-pentylfuran, pentanoic acid, and octanol (Hatanaka, 1993; Kaminski et al., 1972; Liu et al., 2017) increased compared to those

Fig. 4 Heat maps of rice volatile (A) and non-volatile (B) metabolites analyzed using gas chromatography-mass spectrometry. Heat map color represents the z-score transformed raw data of volatile metabolites with significant difference between both rice sample groups and were plotted in a green–red color scale, in which red and green indicate decrease and increase in metabolite levels, respectively. (Color figure online)

in the control rice. In addition, these oxidized lipid by-products are strongly associated with the unpleasant odor of microbial contamination or in old rice (Buttery et al., 1998; Maga, 1984). To confirm the increase in lipid oxidation induced by mold contamination, the acidity of the control and NMCR samples was determined, with NMCR (acid value = 0.73) having a value 1.8 times higher than that of the control (acid value = 0.42). These results suggest that lipid oxidation induced by mold contamination leads to a reduced nutritional quality of rice. In particular, these oxidized lipid products increased by long-term storage and high storage temperature (Liu et al., 2017) as well as mold contamination can be used as indices to evaluate the quality of stored rice. In addition to lipid oxidation, rice carbohydrate was degraded by the contaminating microorganisms into small sugars or polyols. The contents

of inositol, arabitol, sorbitol, and threitol in the NMCR samples were 3–8 times higher than those in the control. A similar result was observed in pigmented rice (Kim et al., 2013) and wheat grains fermented at low water activity by *Aspergillus oryzae* (Ruijter et al., 2004). Several reports show that the content of polyols increases when rice and whole wheat grains are subjected to osmotic and salt stress with low water activity (Majee et al., 2004; Ruijter et al., 2004), because at low water activity, microorganisms try to prevent water loss from the cells by accumulating compatible solutes such as polyols.

Microbial contamination also led to accumulation of citric acid cycle intermediates such as citrate, succinate, fumarate, and malate in the NMCR samples. The levels of citrate, succinate, and malate in the NMCR samples were 4.4-fold, 4.5-fold, and 18.8-fold higher, respectively, than those in the control, and fumarate was observed in the NMCR but not in the control samples. Although energy production was not measured directly in this study, accumulation of citric acid cycle intermediates in the samples might lead to an increase in energy production.

Unlike the increased levels of odor metabolites induced by lipid oxidation, mold contamination led to a decrease in the levels of fresh rice flavor-associated volatile metabolites such as pinene, sabinene, anethole, 2-ethylhexyl acrylate, and longifolene (Bullard and Holguin, 1977; Maga, 1984; Monsoor and Proctor, 2004; Vairappan and Nagappan, 2014) by sixfold, 14-fold, 30-fold, 18-fold, and twofold, respectively, in the NMCR samples. In particular, anethole and 2-ethylhexyl acrylate confer sweet flavor notes. In addition to these metabolites, the levels of BHT, styrene, and glycerol also decreased by 28-fold, sixfold, twofold, respectively, after microbial contamination. Alanine was not detected in the NMCR samples, whereas phosphates and methylphosphates were newly detected and elevated by 2.8-fold, respectively, in the NMCR samples. In summary, these results show that the sensory quality of white rice significantly deteriorated after microbial contamination owing to increased amounts of unpleasant odors and decreased natural fresh flavors. In addition, the aforementioned volatile metabolites and polyols might be used as biomarkers for monitoring rice quality.

Fig. 5 Schematic representation of the rice metabolomics pathway associated with microbial contamination and the relative abundance of identified metabolites. The horizontal axis of the bars shows the

normalized intensity and significant differences with control at * $p < 0.05$ and ** $p < 0.01$

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