博士論文

Studies on the sake brewing properties of the sake rice *Koshitanrei*

（酒造好適米・越淡麗の醸造特性に関する研究）

市 川　絵 梨

広島大学大学院先端物質科学研究科

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主論文

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Introduction

Alcoholic beverages have been made from the major agricultural products of each country. Sake is the traditional Japanese alcoholic beverage brewed from raw materials, rice, and water (Yoshizawa, 1999). In this process, 2 microorganisms, the *koji*-fungus *Asperugillus oryzae* and the sake yeast *Saccharomyces cerevisiae,* are utilized. The starch of rice is decomposed to glucose by enzymes produced by *A. oryzae*. Then, the glucose is converted to alcohol by the metabolism of *S. cerevisiae*. In the sake mash (main mash, *moromi*), 2 bioconversions, saccharification from rice starch to glucose and fermentation from glucose to alcohol, take place simultaneously. This unique process is known as parallel fermentation and a key mechanism to achieve high alcohol production. This sake brewing process, which achieves high alcohol production, is a unique process in the sake brewing.

In Japan, the production quantity of sake has decreased since its peak in 1973. One of the reasons is that the consumer began to select other alcoholic beverages (beer, wine, liqueurs, etc.) as a result of diversification of lifestyles. In order to improve the quality of sake and develop new products, many sake companies have been studying the raw materials, *A. oryzae*, *S. cerevisiae*, as well as new brewing technologies, etc. As a result, various types of sake have been developed.

The main raw material for sake brewing is the rice, and the characteristic features of rice are one of most important factors determining the quality of the final product, sake. In the sake brewing process, brown rice is polished to white rice, steamed, and then used in 2 ways: for *rice-koji* making and directly added to sake mash (as *kake-mai*). Various rice cultivars have been used for sake brewing, and it is empirically known that the characteristics of rice cultivars affect the aroma/ taste of the sake. Therefore, the selection of the rice cultivar is one of the most important factors to produce high-quality sake.

On the other hand, in sake-brewing process, rice-*koji* making is one of the important steps, because it is a determinant of the aroma/ taste components of sake, being the supplier of rice-degrading enzymes and essential vitamins for yeast fermentation. In the *koji*-making process, *A. oryzae* grows on steamed rice and produces a number of enzymes, such as saccharification enzymes and proteolytic enzymes, as well as others. The activity of each enzyme is affected by rice cultivar and *koji* starters (Takahashi *et al*., 2008; Yanagiuchi *et al*., 1993). The difference in the levels of enzyme activities and other components such as vitamins directly or indirectly affects the aroma/ taste component of sake. Thus, the selection of *koji* starter for rice cultivar is important.

In general, 2 kinds of rice (*Oryza sativa* L), sake rice and cooking rice, have been used for sake brewing. Officially, sake rice has been defined as *shuzo-koteki-mai* in the Agricultural Products Inspection Act. The grain size of sake rice is larger than that of cooking rice, has a white core (called *shinpaku*), and a low protein content. Breeding of sake rice cultivars has been carried out in many areas of Japan (Maeshige and Kobayashi, 2000), and approximately 100 types of sake rice have been developed for use in sake brewing. Among them, *Yamadanishiki* (YAM, developed in Hyogo Prefecture), *Gohyakumangoku* (GOM, in Niigata Prefecture), and *Miyamanishiki* (MYN, in Nagano Prefecture) have been the major sake rice varieties widely used for high-quality sake brewing.

In Niigata Prefecture, the originally developed sake rice GOM has been used widely. GOM has contributed to high-quality sake having a clear and dry taste ("*Tanrei*", one of the perceivable attributes in the refined sake), although the hardness of its grains is insufficient for high polishing (especially to less than the 50% polishing ratio for *Daiginjo-shu*), due to the shape and size of its white core (Anzawa *et al*., 2013). The white core of GOM is larger than that of the sake rice YAM, which has been used widely for *Daiginjo-shu* brewing in Japan.

In 2004, to improve the polishing property of GOM, the sake rice *Koshitanrei* (KOS) was originally developed in Niigata Prefecture by genetically crossing GOM with YAM (Kobayashi *et al*., 2006). Recently, KOS has been used widely for high-quality sake brewing in Niigata Prefecture. However, comprehensive analysis of the components/metabolites in sake prepared from KOS has not been reported yet.

The metabolomics technique is an analysis method examining a number of intravital metabolites. Recently, this type of analysis has been applied in various fields with diversification (Putri *et al*., 2013a; Putri *et al*., 2013b). Using these techniques for the analysis of sake, Sugimoto *et al.* (2010) and Mimura *et al.* (2014) reported the relationship between the sake component/metabolites and the taste of sake. Recently, studies examining the relationship between sake-making parameters and sake metabolites (Tamada *et al*., 2017; Yazawa *et al*., 2019) have appeared. Although, a number of methods for analyzing sake compounds/metabolites have been established/developed, the metabolites of sake made from KOS have not been analyzed yet. Furthermore, the effect of the *koji* starter on the characteristics of KOS rice *koji* and sake metabolites also has not been examined, especially by the use of metabolomics techniques.

In this doctoral thesis, I studied the brewing characteristics of sake rice KOS, especially those for the production of high-quality sake. In Chapter I, in order to clarify the characteristic components/ metabolites of sake made from KOS, I examined the effect of *kake*-rice (rice for *kake*-mai) and total rice (rice for both *kake*-mai and *koji*) variation on the sake components/metabolites by using 3 different rice cultivars: KOS, YAM, and GOM. In Chapter II, in order to investigate the effect of the *koji* starter on the rice-*koji* characteristics and sake metabolites, I performed combined examination using 3 different *koji* starters and sake rice KOS to assess their effect on the sake components/metabolites.

CHAPTER I

Analysis of metabolites in Japanese alcoholic beverage sake made from the sake rice *Koshitanrei*.

1.1. Abstract

In sake brewing, the steamed rice is used in 2 ways, added to sake-mash (as *kake-mai*) and making *koji*. The rice is an important determinant for the quality of sake, as the metabolites in sake affect its taste/aroma. The sake rice *Koshitanrei* (KOS) was developed in Niigata Prefecture by genetically crossing 2 sake rice, *Gohyakumangoku* and *Yamadanishiki*. However, the metabolites in sake from KOS have not been analyzed. Here, to investigate the characteristic metabolites in sake from KOS, I performed 2 types of small-scale sake-fermentation tests changing only the rice used for *kake-mai* or total rice (both *kake-mai* and *koji*) by these 3 rice cultivars and examined the effect of KOS on sake metabolites by the metabolome analysis method using UPLC-QTOF-MS. I identified the peaks/metabolites, whose intensity in sake from KOS was higher/lower than those from the other cultivars. The brewing properties of KOS were partially characterized by this analysis.

1.2. Introduction

The Japanese traditional alcoholic beverage sake is made from rice and water by a unique process using 2 microorganisms, a fungus (*Aspergillus oryzae*, called *koji*-mold) and yeast (*Saccharomyces cerevisiae*) (Yoshizawa, 1999). The main raw material for sake brewing is the rice, and the characteristic features of this rice is one of the important factors determining the quality of the final product, sake. In sake brewing, steamed rice is used in 2 ways, directly added to sake mash (as *kake-mai*) and used to make *koji*. The taste and aroma of sake are related to a variety of components. Various rice cultivars have been used for sake brewing in order to characterize the specific taste of the sake. Therefore, the selection of the rice cultivar is one of the important concerns for brewing high-quality sake.

In general, 2 kinds of rice (*Oryza sativa* L), sake rice and cooking rice, have been used for sake brewing. The grain size of sake rice is larger than that of cooking rice, and sake rice has a white core (called *shinpaku*). Breeding of the sake rice cultivars has been done in many areas of Japan (Maeshige and Kobayashi, 2000). In Niigata Prefecture, the originally developed sake rice *Gohyakumangoku* (GOM) has been used widely. GOM has contributed to high-quality sake having a clear taste, although the hardness of its grains is insufficient for high polishing (especially to less than the 50% polishing ratio for *Daiginjo-shu*). This disadvantage of GOM is due to the shape and size of its white core (Anzawa *et al.*,. 2013). The white core of GOM is larger than that of the sake rice *Yamadanishiki* (YAM), which was developed in Hyogo Prefecture and has been used widely for *Daiginjo-*

shu brewing in Japan.

To improve the polishing property of GOM, the sake rice *Koshitanrei* (KOS) was originally developed in Niigata Prefecture by genetically crossing GOM with YAM (Kobayashi *et al.*, 2006). Recently, KOS has been used widely for high-quality sake brewing in Niigata Prefecture. Previously, Kobayashi *et al*. (Kobayashi *et al.*, 2006) characterized KOS, based on the results of examining the brewing properties of KOS in a sake brewing test using 120 kg (total rice, 70% polishing ratio). Further, Anzawa *et al*. (Anzawa *et al.*,. 2013) examined the polishing/brewing properties of KOS using a wide range of polished rice and reported the differences in the general properties of the sake between that made from KOS and that from GOM. However, comprehensive analysis of the components/metabolites in sake from KOS has not been reported yet.

The metabolomics technique is an analysis method examining cyclopaedically/co-instantaneously a number of intravital metabolites. Recently, this type of analysis has been applied in various fields with diversification (Putri *et al.*, 2013a; Putri *et al.*, 2013b). Previously, in the analysis of sake done by using the metabolomics technique CE-TOF MS, Sugimoto *et al*. (Sugimoto *et al.*, 2010) reported a correlation between sensory evaluation scores of sake and metabolome profiles, as well as a difference in the charged metabolites between pasteurized and unpasteurized sake during storage (Sugimoto *et al.*, 2012). In the sake analysis using GC/MS, Mimura *et al.* (Mimura *et al.*, 2014) examined the relationship between the features of sake and the component profile; and Tamada *et al*. (Tamada *et al.*, 2017) reported the characteristics of sake according to the sake-making parameters (sake yeast, rice cultivars) by GC/MS and analyzed the correlation between the component profiles of GC/MS and the intensity of "Oshi-aji" (Tamada *et al.*, 2018). Further, in the sake analysis using 2-dimensional GC with TOF-MS, Takahashi *et al*. (Takahashi *et al.*, 2016) examined the correlation between sake components and organoleptic properties; and Tokuoka *et al*. (Tokuoka *et al.*, 2017) used HILIC-TOF-MS to analyze the oligosaccharide composition of sake. Recently, Yazawa *et al*. (Yazawa *et al.*, 2019) conducted sake metabolome analysis using UPLC-QTOF-MS (ultra-performance liquid chromatography/time-of-flight mass spectrometry) and examined the relationship between sake-making parameters and sake metabolites. Although, as described above, a number of methods for analyzing sake compounds/metabolites have been established/developed, the metabolites of sake made from KOS have not been examined by using these methods.

In this study, in order to clarify the characteristic components/metabolites of sake made from KOS, I performed 2 types of small-scale sake fermentation tests using 3 rice cultivars: KOS, YAM, and GOM. At first, to investigate the effect of *kake*-rice (rice for *kake-mai*) variation on the sake components/metabolites, I performed a small-scale fermentation test changing only the type of rice cultivar used for *kake*-rice. Then, to investigate the effect of total rice variation (rice for both *kake-mai* and *koji*) on the sake components/metabolites, I performed another small-scale fermentation test using the same 3 rice cultivars. I analyzed the sake metabolome data obtained from the above 2 types of small-scale sake fermentation tests by using the recently developed sake metabolome analysis method; (Yazawa *et al.*, 2019) and by it I identified the specific peaks/metabolites, whose intensity in sake from KOS was higher/lower than those from the other 2 cultivars, YAM and GOM. The results demonstrated that certain components/metabolites in sake from KOS were different from those found for YAM or GOM and that the brewing properties of KOS could be at least partially characterized by performing this analysis.

1.3. Materials and Methods

1.3.1. Rice cultivars

Rice cultivars used for the small-scale sake fermentation test in which only the *kake*-rice (rice for *kake-mai*), referred to as the "*kake*-rice sake-making test," was changed were as follow (Fig. 1(a), Nos. 1-6): YAM, GOM, and KOS. The rice cultivar used as *koji*-rice (rice for *koji*) in this test was *Yamadanishiki* (YAM), harvested in 2015 (grow in Hiroshima Prefecture); and the 3 rice cultivars used as *kake-mai*, YAM (grow in Hiroshima Prefecture), *Gohyakumangoku* (GOM, grow in Niigata Prefecture), and *Koshitanrei* (KOS, grow in Niigata Prefecture), were harvested in 2014, 2016, and 2016, respectively. Rice cultivars used for the small-scale sake fermentation test in which both the *kake*-rice and *koji*-rice were changed (referred as "*kake*+*koji*-rice sake-making test") were as follow (Fig. 1(a), Nos. 7-12): YAM (grow in Hiroshima Prefecture), GOM (grow in Niigata Prefecture), and KOS (grow in Niigata Prefecture), all harvested in 2016. The rice grains were polished to polishing ratios of 50% and 70% by using a milling machine (RP-5 and NF-26FA, Shin-Nakano Industry, Japan).

1.3.2. A model sake sample

A model sake sample was prepared as a mixture of 68 standard compounds (model sake, marked "1" in Table S1 (Chapter I Table S1.xlsx)) in 15% ethanol. Then, the prepared sake

was aliquoted into 1-mL samples in Eppendorf tubes and stored at −80 °C.

1.3.3. Small-scale koji making

Rice with a polishing ratio of 50% or 70% was soaked in tap water to 130% water absorption and then steamed for 50 min. The steamed rice was dried to 130% water absorption, and cooled down to room temperature. Conidia spores (1 x 10^6 conidia/g *koji*-rice) of *Aspergillus oryzae* (Fig.1A, *koji* starter, *Byakuya*, Hishiroku-Moyashi, Japan) were inoculated to the steamed rice, and the inoculated rice was incubated in an incubator (KCL2000, EYRA, Japan) for 46 h, during which time the temperature and moisture were regulated. Initially, the temperature in the incubator was maintained at 32°C for 22 h, and then it was gradually increased as follows: to 34 °C for 8 h, to 37 °C for 3 h, to 40 °C for 3 h, and to 42 °C for 10 h. Humidity in the incubator was initially maintained at 90% for 22 h and then gradually decreased as follows: to 80% for 8 h, to 70% for 3 h, and to 60% for 13 h. After 46 h following inoculation, the humidity was controlled at less than 90%, and the *koji* was cooled down to slightly less than room temperature. The *koji* was stored at -30°C until used for sake making.

1.3.4. Small-scale sake-making test

To examine the effects of *kake*-rice and *koji*-rice on sake metabolites, I performed 2 types of small-scale sake-making tests (2 independent experiments each), "*kake*-rice sake-making test" (Fig. 1(a) Nos. 1-6) and "*kake*+*koji*-rice sake-making test" (Fig. 1(a) Nos. 7-12). In the first test, referred to as the "*kake*-rice sake-making test," the rice cultivar YAM (40% polishing ratio) was used for the *koji*-rice, and 3 rice cultivars (YAM, GOM, and KOS) each of 50% or 70% polishing ratio, were used for the *kake*-rice (Fig. 1, sample Nos.1-6, referred to as "*kake*-rice sake making test"). In the second test, designated as the "*kake*+*koji*-rice sake-making test," the total rice of both *kake*-rice and *koji*-rice were changed, and 2 polishing ratios (50% and 70%) of 3 rice cultivars (YAM, GOM, and KOS) were used for both *kake*-rice and *koji*-rice (Fig. 1, sample Nos.7-12). In the 2 types of the sake-making test, 80 g of total rice (16 g for *koji*-rice and 64 g for *kake*-rice) was used for one-step addition to the sake mash; and the sake yeast strain K701 was used. The *kake-mai* for the sake-making test was prepared as follows: The rice (64 g) was washed at 10 °C for 30 sec with tap water, drained for 15 sec, and washed for 30 sec again with more tap water. After the washing step, the rice was drained for 15 sec and then soaked to reach 130% water absorption. Next, the soaked rice was drained at 10 °C for 30 min and subsequently steamed for 50 min. The water absorption of the steamed rice was adjusted to

130%, assuming that the water content of the original white rice was 13.5%. The steamed rice was added to 103 mL of water with 1 mL (10^8 cells) of the sake yeast K701, 16 g (as polished rice) of *koji*, and 72 μL of 50% lactic acid. The sake yeast strain K701 for the sake-making test was cultured as follows: The sake yeast cells were pre-cultured overnight at 30 °C in the rice *koji* extract medium (Baume scale 10). The pre-cultured cells were then inoculated into fresh medium at 100-fold dilutions and incubated for 2 days at 30 °C in standing culture. The cultured cells were washed twice with sterilized water and thereafter added to the sake mash (final concentration, approx. 1 x 10^6 cells/mL). Sake fermentation was performed at 15 °C (in a water bath) for 20 days, and was monitored by measuring the weight reduction of the sake mash, corresponding to CO_2 evolution. After fermentation, the clear sake sample was obtained by centrifugation (5000 rpm, 10 min, 4 °C).

1.3.5. Analysis of sake

General properties (Sm, sake meter; Alc, sake alcohol; TA, total acidity; AA, amino acidity), and flavor components (EtOAc, ethyl acetate; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtCap, ethyl caproate) of the *sake* were determined by the standard method established by the National Tax Agency of Japan.

1.3.6. Enzyme activity analysis of rice koji

Enzyme activity of *koji* was analyzed by the standard method established by the National Tax Agency of Japan. The activities of 3 enzymes, i.e., α-amylase, glucoamylase, and acid carboxypeptidase, were measured by using the respective assay kits (Kikkoman, Japan) (Imai *et al.*, 1996; Shirokane *et al.*, 1996).

1.3.7. Sake metabolome analysis method

Sake samples were filtered through an Amicon Ultra 0.5 3K (Merk Millipore, Germany) and diluted 10-fold with MS-grade water. UPLC-QTOF-MS analysis was performed with a UPLC Xevo Q/TOF-MS system (Waters, Milford, USA). The method for sake metabolome analysis established by Yazawa *et al*. (Yazawa *et al.*, 2019) was followed. Each sample from 2 independent small-scale sake-making tests was analyzed 3 times.

1.3.8. Data processing of sake metabolome

Data processing by Yazawa *et al*. (Yazawa *et al.*, 2019) was basically followed. Peak detection, alignment peak data, and calculation of peak area were conducted with Peaklynx XS Rev. 1.0 software (Waters, Milford, USA). Mass range and retention time range for peak detection were determined by the error range of 14 selected peaks (precursor ions of 14 compounds) from the model sake samples (Table S1 (Chapter I Table S1.xlsx)). To

evaluate the accuracy of metabolome data, I analyzed model sake samples 7 times by using 6 to 12 samples per interval. The error range of each mass spectrum was calculated by 5-fold standard deviation of each of the 14 selected peaks. The largest value among these values was used as the error range for the peak-picking method of Peaklynx. The error range of the retention time was also calculated as the mass spectrum error. Other settings of the peak picking-method for metabolome data were as follows: initial and final retention times of monitoring, 0 to 15 min; peak width at 5% height, 4 sec; intensity threshold of peak picking, 70; mass window, 0.01 Da; and retention time window, 0.3 min. Other settings followed the manufacturer's instruction. After making a peak table, preliminary data processing was performed. Three-fold coefficient of variation (CV) was calculated from the intensities of each peak top of the selected 14 peaks, and the maximum value of 3-fold CV among 14 peaks was used as the standard value. Then, the intensity of each peak was used to calculate CV in each sake sample, and the maximum value (Max CV) was used as CV of each peak. The peak(s) whose maximum CV was greater than the standard value was eliminated further analysis. Metabolites were identified, depending on 496 standard metabolite lists (Table S1 (Chapter I Table S1.xlsx)). The threshold of mass and retention-time error for identification was 0.05 Da and 0.5 min, respectively.

1.3.9. Multiple comparison technique using metabolome data

The software JMP 13.1 (commercially available) was used throughout the analysis in this study. To examine the difference in sake metabolites by rice cultivars in the small-scale sake-making tests, Tukey's Honest Significant Difference (HDS) test was performed by using the intensity of each peak. The *P* value was calculated by using the average of the intensity of each sample, and a value of less than 0.05 was regarded as indicating a significant difference between rice cultivars.

1.4. Results and Discussion

1.4.1. Effect of Koshitanrei on the sake metabolites in kake-rice sake-making tests

To investigate the effect of *kake*-rice (rice for *kake-mai*) on sake metabolites, I performed the small-scale sake fermentation test using 3 rice cultivars (YAM, GOM, and KOS) of 2 polishing ratios (70% and 50%) as *kake*-rice and 1 rice cultivar (YAM of 40% polishing ratio) as the *koji*-rice (rice for *koji*; Fig. 1(a, b), sample Nos.1-6).

In this small-scale sake-making test, the enzyme activities of the *koji* used, fermentation rates (CO_2 decrement) during the test, and the general properties and flavor

components of the obtained sake samples are shown in Table S2A (Chapter I Table S2.xlsx), Fig. S1 (A and B) (Chapter I Fig S1.pptx), and Fig. 1(d), respectively. Fermentation rates (CO_2 decrement) of the sake-making test using KOS as the *kake*-rice tended to be slower than those obtained for the other 2 rice cultivars, YAM and GOM (Fig. S1, A and B (Chapter I Fig S1.pptx)). Sake meter (Sm) and alcohol (Alc) of the sake samples from KOS of both polishing ratios showed a lower value than those for the other 2 rice cultivars (Fig. 1(d)). In the sake sample from the rice of the 70% polishing ratio, the total acidity (Ta) and amino acidity (Aa) for KOS were higher and lower, respectively, than those for the other 2 rice cultivars (Fig. 1(d), sample Nos. 1-3). In the sake samples from the rice of the 50% polishing ratio, the concentrations of the flavor components ethyl acetate (EtOAc) and isoamyl acetate (iAmOAc) from KOS were lower than those from the other 2 rice cultivars (Fig. 1(d), sample Nos. 4-6).

Next, to investigate the effect of the *kake*-rice on sake metabolites, I constructed a peak table (Table S3 (Chapter I Table S3.xlsx)) containing all metabolome data of 1215 peaks, whose intensity was higher than the threshold of peak picking, as described in Materials and Methods (*Data processing of sake metabolome*). Among 1215 peaks, I identified 153 peaks (counting "number of ino(s) from one compound"), categorized 201 peaks, and among the identified 153 peaks, detected 84 compounds (counting "1" = "more than one ion from one compound") in 496 compounds (Table S1 (Chapter I Table S1.xlsx)), based on the retention time and m/z of the ion(s) (P, precursor/parent ion; fx, in-source fragment ion; derived from the compounds) in Table S1. I used this peak table (Table S3 (Chapter I Table S3.xlsx)) for further analysis. At first, to investigate the character of each sake sample, I performed principal component analysis (PCA) by using this peak table (Fig. 2(a)). As the result of PCA score plotting of the metabolome data from the "*kake*-rice sake-making test," the contribution ratios of PC1 and PC2 were 41.8% and 19.6%, respectively (Fig. 2(a)). On the PC1 axis, the sake samples from YAM and GOM of the "*kake*-rice sake-making test" (sample Nos. 1, 2, 4, and 5) were clearly separated by the polishing ratio, with the samples of 50% and 70% polishing ratio separated in a positive and negative direction, respectively. On the other hand, unlike the samples of the other 2 rice cultivars of the 70% polishing ratio (No.1 for YAM and No.2 for GOM), the sake sample from KOS of the 70% polishing ratio (No. 3) was separated to the same area (in a positive direction on the PC1 axis) as the samples of the 50% polishing ratio. These results suggested that, in this "*kake*-rice sake-making test", the components of sake samples from KOS were less affected by

the difference in the polishing ratio (70% or 50%).

Next, to explore the peak(s) affecting the classification on the axis in Fig.2(a), I performed Tukey's HSD (honest significant difference) test by using the constructed peak table (Table S3 (Chapter I Table S3.xlsx)). In this analysis, I eliminated 121 peaks (the intensity 0 in some of sample Nos.1-6). As the result of this Tukey HSD test, by focusing on the peaks in sake samples from KOS, I selected the peaks whose intensities were higher ($p < 0.05$; KOS > YAM, GOM) and lower ($p < 0.05$; KOS < YAM, GOM) than those for the others (YAM and GOM). Regarding the selected peaks, Venn diagrams of the higher peaks (KOS > YAM, GOM) and the lower peaks (KOS < YAM, GOM) are shown in Fig. 2(b) and Fig.2(c), respectively.

In the Venn diagrams of the higher peaks (KOS > YAM, GOM; Fig. 2(b)), the number of the higher peaks for the 70% polishing ratio only was 202 (I in Fig. 2(b)), the number of the higher peaks for the 50% polishing ratio only was 110 (III), and the number of the higher peaks for both polishing ratios was 107 (II). The number of the peaks affected by the 70% polishing ratio only (202/I in Fig. 2(b)) was about two-fold that of those affected by the *kake*-rice of 50% polishing ratio only (110/III).

In the Venn diagrams of the lower peaks (KOS < YAM, GOM; Fig. 2(c)), the number of the lower peaks for the 70% polishing ratio only was 143 (I in Fig. 2(c)); that for the 50% polishing ratio only, 20 (III); and that for both polishing ratios was 53 (II). Thus, the number of the peaks affected by the 70% polishing ratio only (143/I in Fig. 2(c)) was about 7-fold that of the peaks affected by the *kake*-rice of 50% polishing ratio only (20/III). These results suggested that, in the "*kake*-rice sake-making test," the difference in sake components between KOS and the others at the 70% polishing ratio was bigger than that in sake components between KOS and the others at the 50% polishing ratio.

Further, I categorized or identified the metabolites of the KOS-affected peaks by using the metabolite list (Table S1 (Chapter I Table S1.xlsx)). The results (Fig. 2(b)) showed that the numbers of the identified/categorized higher peaks for KOS were 16/25 among 202 for the 70% polishing ratio only, 27/38 among 110 for the 50% polishing ratio only, and 8/16 among 107 for both polishing ratios. On the other hand, the numbers of the identified/categorized lower peaks for KOS were 28/38 among 143 for the 70% polishing ratio only, 4/4 among 20 for the 50% polishing ratio only, and 9/9 among 53 for both polishing ratios (Fig. 2(c)). The metabolites (ID No. Compound_detected ion, in Table S1 (Chapter I Table S1.xlsx)) of the identified peaks (Fig. 2(b) and 2(c)) are indicated in Tables

1 and 2. The contents of alpha-etylglucoside (α-EG) in the sake samples from KOS of both polishing ratios were lower than those in the samples from the other 2 rice cultivars (Table 2, II, 8 peaks). It should be noted that 1 peak (α-EG_f1, Table 1, I) of α-EG for the 70% polishing ratio only showed behavior opposite to that of the other 8 peaks (Table 2, II).

1.4.2. Effect of Koshitanrei on the sake metabolites in total rice (kake+koji-rice) sake-making tests

To investigate the effect of the total rice variation on sake metabolites, I performed the small-scale sake fermentation test using 3 rice cultivars (YAM, GOM, and KOS) of 2 polishing ratios (70% and 50%) as *kake*- and *koji*-rice (Fig. 1(a, c), sample Nos. 7-12).

In this small-scale test, the enzyme activities of the used *koji*, fermentation rates (CO_2 decrement) during the test, and the general properties and flavor components of the obtained sake samples are shown in Table S2B (Chapter I Table S2.xlsx), Fig. S1 (C and D) (Chapter I Fig S1.pptx), and Fig. 1(d), respectively. In the rice of the 70% polishing ratio, the 4 enzyme activities examined in the *koji* from KOS were lower than those in *koji* from the other rice cultivars (Table S2B (Chapter I Table S2.xlsx), sample Nos. 7-9). In the rice of the 50% polishing ratio, glucoamylase of *koji* from KOS tended to be lower than those from the other rice cultivars (Table S2B (Chapter I Table S2.xlsx), sample Nos. 10-12). Fermentation rates (CO_2 decrement) in the sake fermentation test using KOS of the 2 polishing ratios were the intermediate between those for GOM and YAM (Fig. S1, C and D (Chapter I Fig S1.pptx)). In the sake sample from the rice of both polishing ratios, the sake meter (Sm) and total acidity (Ta) or amino acidity (Aa) values for KOS were higher and lower, respectively, than those from the other rice cultivars (Fig. 1(d), sample Nos. 7-12). The concentrations of flavor components ethyl acetate (EtOAc) and isoamyl acetate (iAmOAc) in sake from KOS of the 50% polishing ratio were higher than those from YAM or GOM (Fig. 1(d), sample Nos. 10-12).

Next, to investigate the effect of the total rice variation on sake metabolites, as in the case of the "*kake*-rice sake-making test", I constructed a peak table (Table S3 (Chapter I Table S3.xlsx)) containing all metabolome data of 1215 peaks and used it for further analysis. At first, to investigate the character of each sake sample, I performed the PCA by using this peak table (Fig. 3(a)). The results of PCA score plotting of the metabolome data from the "*kake*+*koji*-rice sake-making test," the contribution ratios of PC1 and PC2 were 37.9% and 35.0%, respectively (Fig. 3(a)). On the PC1 axis, the sake sample Nos. 7-12 were clearly separated by the polishing ratio; and the samples of 70% polishing ratio

(Nos.7-9) and those of the 50% one (Nos.10-12) were separated in a positive and negative direction, respectively (Fig. 3(a)). On the other hand, on the PC2 axis, unlike the other 2 rice cultivars, the samples from KOS (Nos. 9 and 12) were concentrated in a negative direction (Fig. 3(a)). A comparison of the results of the "*kake*+*koji*-rice sake-making test" with those of the "*kake*-rice sake-making test" suggested that the difference in the effect of the polishing ratio on the sake components from total rice of KOS (Nos. 9 and 12, in Fig. 3(a)) was bigger than that on those from *kake*-rice of KOS (Nos. 3 and 6, in Fig. 2(a)).

Next, as was the case for the *kake*-rice test, to explore the peaks affecting the distribution on the axis in Fig.3(a), I performed Tukey's HSD test using the constructed peak table (Table S3 (Chapter I Table S3.xlsx)). In this analysis, I eliminated 92 peaks (the intensity 0 in some of sample Nos.7-12). By focusing on the peaks in sake samples from KOS, I selected the peaks whose intensities were higher ($p < 0.05$; KOS > YAM, GOM) or lower ($p < 0.05$; KOS < YAM, GOM) than those for the other rice cultivars. Using data on the selected peaks, I prepared Venn diagrams of the higher peaks (KOS > YAM, GOM) and lower peaks (KOS < YAM, GOM), Fig. 3(b) and Fig. 3(c), respectively.

In the Venn diagrams of the higher peaks (KOS > YAM, GOM; Fig. 3(b)), the number of the higher peaks for the 70% polishing ratio only was 23 (I in Fig. 3(b)); that for the 50% polishing ratio only, 237 (III); and that for both ratios, 78 (II). The number of the peaks affected by 50% polishing ratio only (237/III in Fig. 3(b)) was about 10-fold that of those affected by *kake*-rice of the 70% polishing ratio only (23/I in Fig. 3(b)). In the Venn diagrams of lower peaks (KOS < YAM, GOM; Fig. 3(c)), the number of the lower peaks for the 70% polishing ratio only was 70 (I in Fig. 3(c)); that for the 50% polishing ratio only, 118 (III); and that for both polishing ratios, 289 (II).

The number of the peaks affected by the 50% polishing ratio only (118/III in Fig. 3(c)) was 1.7-fold that of those affected by the *kake*-rice of the 70% polishing ratio only (70/I in Fig.3(c)). In the case of the sake components from KOS of the "*kake*+*koji* sake-making test," the number of peaks (higher and lower) affected by rice cultivars of the 50% polishing ratio only was higher than that of those of the 70% polishing ratio only (Fig. 3(b), I-23 *vs*. III-237; Fig. 3(c), I-70 *vs*. III-118). These results suggested that, in the "*kake*+*koji*-rice (total rice) sake-making test," the difference in the effect on sake components between KOS and the others at the 50% polishing ratio was bigger than that in the effect on sake components between KOS and others at the 70% polishing ratio. When the results of the "*kake*-rice sake-making test" were compared with those of the "*kake*+*koji*-rice sake-making

test," the number of lower peaks for KOS of both 70% and 50% polishing ratios was 5.5-fold higher in the latter than in the former (Fig. 2(c)-II/53 *vs.* Fig. 3(c)-II/289).

Further, I categorized or identified the metabolites of the KOS-affected peaks by using the metabolite list (Table S1 (Chapter I Table S1.xlsx)). As shown in the results (Fig. 3(b)), the numbers of the higher identified/categorized peaks for KOS were 1/1 among 23 for the 70% polishing ratio only, 23/26 among 237 for the 50% polishing ratio only, and 13/17 among 78 for both polishing ratios. On the other hand, the numbers of the lower identified/categorized peaks for KOS were 3/5 among 70 for the 70% polishing ratio only, 14/17 among 118 for the 50% polishing ratio only, and 64/90 among 289 for both polishing ratios (Fig. 3(c)). The metabolites (ID No. Compound_detected ion, in Table S1 (Chapter I Table S1.xlsx)) of the identified peaks (Fig. 3(b) and 3(c)) are indicated in Tables 3 and 4. The higher peaks in the sake sample from KOS were α-EG and citric acid for both 70% and 50% polishing ratios, and malic acid for the 50% polishing ratio (Table 3). On the other hand, the lower peaks in the sake sample from KOS were amino acids and dipeptides (Table 4). In the "*kake*-rice sake-making test", the α-EG concentration of the sake sample from KOS was lower than that for the other rice cultivars (Table 2, II), but in the "*kake*+*koji*-rice sake making test," the α-EG concentration of the sake sample from KOS was higher than that for the other rice cultivars (Table 3, II). It should be noted that 1 peak (α-EG_f1, Table 4, III) of α-EG for the 50% polishing ratio only showed a behavior opposite to that of the other 6 peaks (Table 3, II).

1.4.3. Taste- and aroma-related sake metabolites affected by KOS

Various components including carbohydrates, organic acids, amino acids, and inorganic ones are related to the taste and aroma of sake (Brewing Society of Japan, 1999). Therefore, I focused on the components related to the taste and aroma of sake among the identified metabolites/components affected by KOS (Tables 1-4). Previously it was reported that the amino acids arginine, alanine, glutamic acid, and aspartic acid, are related to the taste of sake (Iwano *et al.*, 2004). Among these 4 amino acids, in the 2 types of small-scale sake fermentation test ("*kake*-rice sake-making test" and "*kake*+*koji*-rice sake-making test"), 2 amino acids (glutamic acid and aspartic acid) showed characteristic behavior in the sake from KOS compared to that of those from the other 2 rice cultivars. In the "*kake*+*koji*-rice (total rice) sake-making test" using the rice of both 70% and 50% polishing ratios, the levels of these 2 amino acids, glutamic acid and aspartic acid, in the sake from KOS were lower than those in that from the other rice cultivars (Nos. 9 and 12, in Fig. 4(a) and 4(b)).

Similarly, in the "*kake*-rice sake-making test" using the rice of the 70% polishing ratio, these 2 amino acids in the sake from KOS were lower in concentration than in the sake made from the other rice cultivars (No. 3 in Fig. 4(a) and 4(b)). The clear taste of the sake from KOS may be attributed to the lower content of these 2 amino acids.

It has been reported that the major component of "*hineka*", one of the unpalatable flavors in sake, is dimethyl tri-sulfide (DMTS) (Isogai *et al.*, 2006) and that the production of 1,2-dihydroxy-5-(methylsulfinyl)prentan-3-one (DMTS-P1), the precursor of DMTS, is related to the methionine salvage pathway genes in *Saccharomyces cerevisiae* (Wakabayashi *et al.*, 2013). Therefore, since sulfur-containing amino acids are related to the methionine salvage pathway, I next focused on methionine and cysteine to determine if they would be affected by KOS (Fig. 4 (c) and 4(d)). The content of methionine in the sake from KOS in the 2 types of small-scale sake fermentation test ("*kake*-rice sake-making test" and "*kake*+*koji*-rice sake-making test") tended to be lower than that in sake from the other rice cultivars (Nos.3, 6, 9, and 12 in Fig. 4(c)). Also, the cysteine content in the sake from KOS in the "*kake*+*koji*-rice sake-making test" was lower than that in the sake made from the other rice cultivars (Nos.9 and 12 in Fig. 4(d)). These results suggested that the lower contents of sulfur-containing amino acids in the sake from KOS would prevent deterioration of the sake's quality.

Next, as carbohydrate is also related to the taste of sake, I focused on the α-EG content in the sake from KOS. In the "*kake*-rice sake- making test," the content of α-EG, responsible for the bitterness following the sweet taste (Oka and Sato, 1976), in the sake from KOS at either the 70% or 50 % polishing level was lower than that in the sake made from the other rice cultivars (Nos.3 and 6 in Fig. 4(e)). In contrast, in the "*kake*+*koji*-rice sake-making test" at both 70% and 50% polishing ratios, the α-EG contents in sake from KOS were higher than those from the other rice cultivars (Nos.9 and 12 in Fig. 4(e)). These results suggest that the α-EG content of sake from KOS was influenced by the *koji* and support the PCA results, which showed that the use of KOS as the *koji*-rice caused a greater diversity of the sake peaks/metabolites (compare between "sample Nos. 9 and 12 in Fig. 3(a)" and "sample Nos. 3 and 6 in Fig. 2(a)").

In the PCA results from the "*kake*-rice sake-making test" (Fig. 2(a)), the sake samples from KOS of the 70% polishing ratio (sample No. 3) were located near the sake samples from the other rice cultivars of the 50% polishing ratio (sample Nos.4-6). Further, in the PCA results obtained from the "*kake*+*koji*-rice sake-making test" (Fig. 3(a)), the sake

samples from KOS of both 70% and 50% polishing ratios (No. 9 and 12) were separated in a negative direction on the PC2 axis, unlike those from the other rice cultivars, which were separated in a positive direction on this axis. These results were also supported by cluster analysis (data not shown).

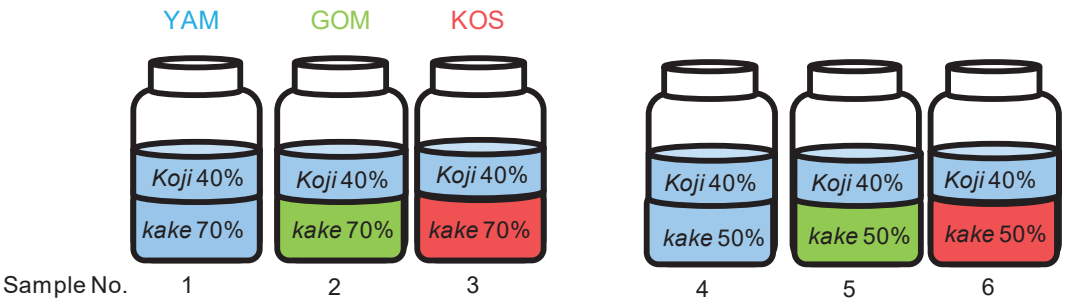
Currently, although the number of identified peaks was just a few, I detected many unknown peaks affected by KOS in this study. Indeed, 3 unknown peaks (RT, m/z: 1.254, 62.0608; 2.539, 287.1495; 1.252, 431.1168) were detected only in the sake from KOS (intensity 0 in the sake from others) in the total rice sake-making tests (sample Nos. 7-12). For future clarification of the characteristics of KOS, it will be important to identify unknown peaks affected by KOS.

(a)

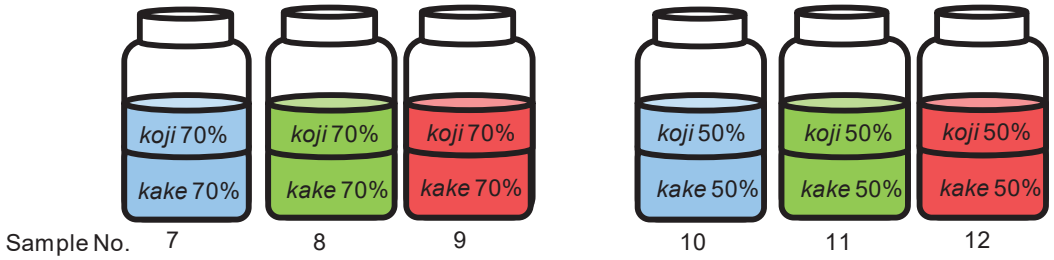
Sample No.	Rice cultivar (Polishing ratio %) *kake*-rice	*koji*-rice	*Koji* starter	Yeast strain
1	YAM (70)	YAM (40)	*Byakuya*	K701
2	GOM (70)			
3	KOS (70)			
4	YAM (50)			
5	GOM (50)			
6	KOS (50)			
7	YAM (70)	YAM (70)	*Byakuya*	K701
8	GOM (70)	GOM (70)		
9	KOS (70)	KOS (70)		
10	YAM (50)	YAM (50)		
11	GOM (50)	GOM (50)		
12	KOS (50)	KOS (50)		

YAM (*Yamadanishiki*), GOM (*Gohyakumangoku*), KOS (*Koshitanrei*)
Total rice 80g

(b)



(c)



(d)

Sample No.	General properties Sm	Alc (%)	Ta (ml)	Aa (%)	Flavor components (ppm) EtOAc	iAmOAc	iAmOH	EtOCap
1	-6	19.0	2.5	2.6	110	7.4	206	1.7
2	-4	19.1	2.7	2.5	149	10.7	199	1.8
3	-13	17.6	3.3	2.0	120	7.4	170	1.2
4	-16	18.1	3.1	1.7	138	11.0	171	1.7
5	-9	17.8	3.3	1.7	130	10.0	196	1.3
6	-24	16.0	3.3	1.7	97	5.5	149	0.9
7	-19	18.3	3.9	2.7	89	3.9	143	1.1
8	-9	20.8	3.4	2.7	127	6.9	169	1.5
9	-7	19.9	3.1	2.0	112	5.1	143	1.4
10	-34	16.4	3.9	2.1	75	3.9	132	1.0
11	-23	17.6	3.7	2.0	93	4.5	144	0.9
12	-19	18.0	3.4	1.4	109	7.6	149	1.5

Fig. 1. Combination of sake-making parameters and properties of sake samples analyzed in the small-scale sake fermentation test.

(a), Sake-making parameters, such as rice cultivar (YAM, *Yamadanishiki*; GOM, *Gohyakumangoku*; and KOS, *Koshitanrei*) and polishing ratio (%) of *kake*- and *koji*-rice, *koji* starter, and yeast strain, are indicated. (b), Three rice cultivars (YAM/blue, GOM/green, and KOS/red) of 2 polishing ratios (70% and 50%) for *kake*-rice and one common rice cultivar (YAM, 40% polishing ratio) for *koji*-rice were used in this test (total rice, 80 g; 16 g for *koji*-rice and 64 g for *kake*-rice). This test (sample Nos. 1-6) was referred to as the "*kake*-rice sake-making test" in this study. (c), Three rice cultivars (YAM, GOM, and KOS) of 2 polishing ratios (70% and 50%) were used for both *kake*- and *koji*-rice in this small-scale sake fermentation test (total rice, 80 g; 16 g for *koji*-rice and 64 g for *kake*-rice). This test (sample Nos. 7-12) was referred to as the "*kake*+*koji*-rice sake-making test". (d), General properties and flavor components of sake samples. General properties (Sm, sake meter; Alc, alcohol content; TA, total acidity; AA, amino acidity) and flavor components (EtOAc, ethyl acetate; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtCap, ethyl caproate) of sake samples were analyzed by the standard method established by the National Tax Agency of Japan. All data are indicated as the averages for 2 independent tests.

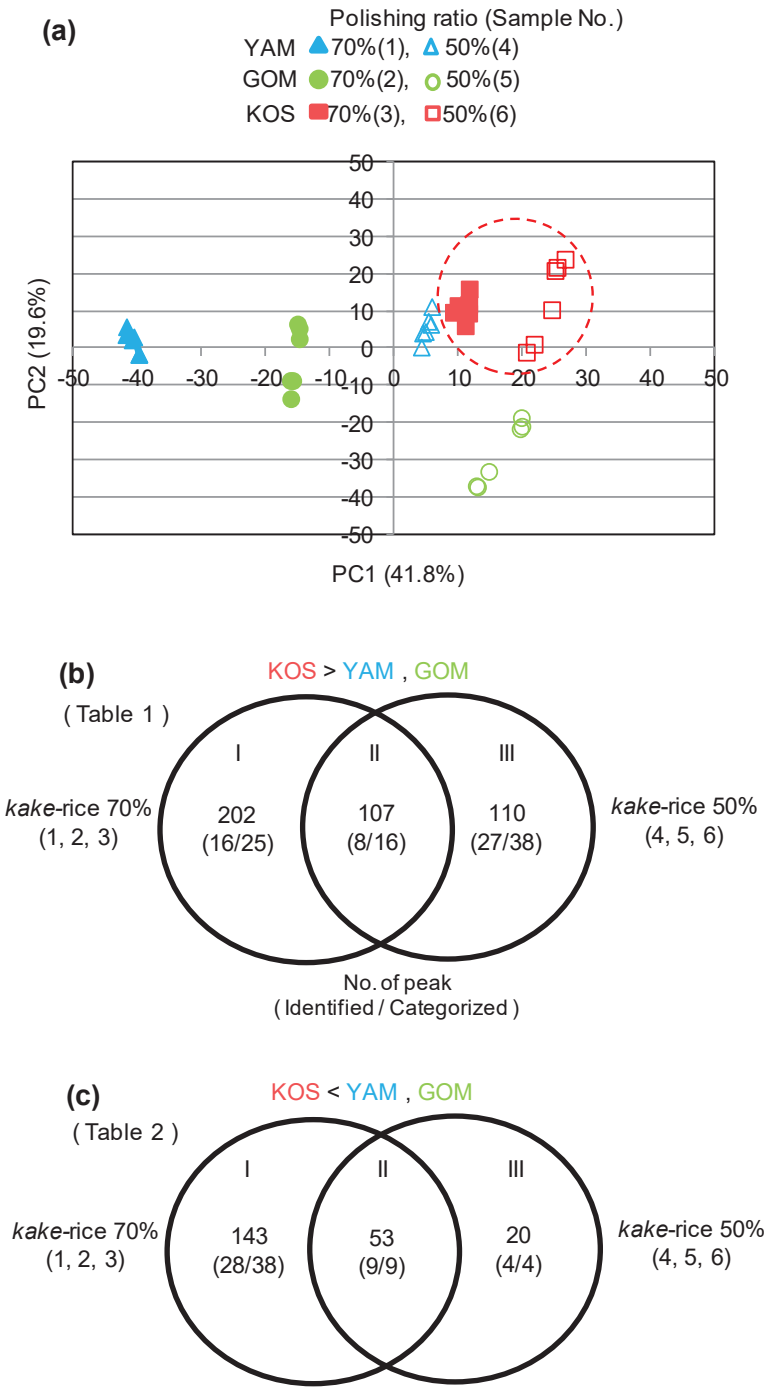


Fig. 2. Analysis of sake metabolites in the *kake*-rice sake-making test.

(a), PCA score plots of metabolome data (3 times analyses of each sample) from 2 independent *kake*-rice sake-making tests (sample Nos. 1-6 in Fig. 1). Blue-triangle, green-circle, and red-square marks indicate 3 rice cultivars, YAM, GOM, and KOS, respectively. Filled and open marks indicate sample Nos. 1-3 (*kake*-rice of 70% polishing ratio) and

sample Nos. 4-6 (*kake*-rice of 50% polishing ratio), respectively. The dotted circle indicates the located area for the samples from KOS. (b), Venn diagrams of the peaks (KOS > YAM, GOM), whose intensity in sake made from KOS (*kake*-rice of 70% and 50% polishing ratios) was higher ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (*kake*-rice of 70% and 50% polishing ratios). The numbers in the Venn diagrams indicate the numbers of peaks (number of the identified/categorized peaks). The metabolites (ID No. Components) of the identified peaks are shown in Table 1. (c), Venn diagrams of the markers (KOS < YAM, GOM), whose intensity in sake made from KOS (*kake*-rice of 70% and 50% polishing ratios) was lower ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (*kake*-rice of 70% and 50% polishing ratio). The metabolites (ID No. Components) of the identified peaks are shown in Table 2.

Table 1. The metabolites (ID No. component) of the identified peaks in Fig. 2B (KOS > YAM, GOM).

Category	KOS > YAM, GOM		
	I	II	III
nitrogen compound	258 Agmatine_f1		
	385 L-DOPA _P		
			224 L-Cysteine_f1
			232 L-Histidine_(2; P, f1)
			233 L-Phenylalanine_f1
			234 L-Arginine_f1
			236 L-Tyrosine_(4; P, f1, f3, f4)
			257 beta-Phenylethylamine_(2; P, f1)
dipeptide	470 Ala-Phe_P		
	474 Leu-Val_P		
	519 Gln-Tyr_P		
	523 Ala-Gln_P		
	526 Val-Phe _P		
	564 Glu-Ala_P		
	567 Phe-Asp_P		
		503 Gln-Gln_P	
		533 Asp-Glu_P	
		572 Glu-Glu_P	
			483 Gly-Thr_(2; P, f2)
			484 Gly-Trp_P
			490 Gly-Phe_P
			491 Gly-Pro_P
			494 Gly-Tyr_P
			525 Val-Gln_P
			527 Leu-Phe_P
			530 Pro-Gln_P
			536 Gln-Leu_(2; P, f1)
carbohydrate	12 alpha-Ethylglucoside_f1		
		21 Raffinose_f2	
		367 Melibiose_f16	
			21 Raffinose_(2; f3, f10)
organic acid	195 n-butyric acid_f4		
	209 Succinic acid_(3; P, f1, f2)		
		179 Gluconolactone_f4	
			157 Vanillic acid_P
vitamine		318 Inositol_f5	
ester		67 Ethyl 3-hydroxybutyrate_f7	
			463 Ethyl-glucopyranoside_f6
carbohydrate alcohol	2 Erythritol_P		
nucleic acid			273 Inosine_f3
other	456 Phosphoric acid_P		

The intensity of the peaks in sake made from KOS (kake-rice of 70% and 50% polishing ratio) was higher ($p < 0.05$) than that of it for the sake made from the other 2 rice cultivars, YAM and GOM (kake-rice of 70% and 50% polishing ratio). Identified peaks (ID No. Compound_detected ion, Table S1) at 70% polishing ratio only (I), at both 70% and 50% polishing ratios (II), and at 50% polishing ratio only (III) are indicated. The detected ions are indicated as P, precursor/parent ion; fx, in-source fragment ion; more than one (No. of detected ions; kind of ions).

Table 2. The metabolites (ID No. Compound_detected ion) of the identified peaks in Fig. 2(c) (KOS < YAM, GOM).

Category	KOS < YAM, GOM		
	I	II	III
nitrogen compound	218 L-Alanine_P		
	220 L-Serine_f2		
	223 L-Threonine_f2		
	226 L-Leucine_(2; P, f1)		
	227 L-Ornithine_f2		
	228 L-Aspartic acid_(3; f1-3)		
	229 L-Lysine_f1		
	230 L-Glutamic acid_P		
	231 L-Methionine_(2; f1, f2)		
	232 L-Histidine_f1		
dipeptide	502 Leu-Leu_P		
	513 Val-Val_(2; P, f1)		
	522 Ser-Val_f2		
	531 Ala-Thr_P		
	574 His-Leu_P		
		522 Ser-Val_f1	
carbohydrate		12 alpha-Ethylglucoside_(8; P, f4, f6-11)	
organic acid	174 Citramalic acid_(3; P, f6, f7)		
	206 Pyroglutamic acid_(2; P, f2)		
			190 Malic acid_(4; P, f2-4)
ester	414 N-(2-phenylethyl)acetamide_f3		
nucleic acid	263 Uracil_P		
	266 Hypoxanthine_P		

The intensity of the peaks in sake made from KOS (kake-rice of 70% and 50% polishing ratio) was lower ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (kake-rice of 70% and 50% polishing ratio). Identified compounds (ID No. Compound_detected ion, Table S1) at 70% polishing ratio only (I), at both 70% and 50% polishing ratios (II), and at 50% polishing ratio only (III) are indicated. The detected ions are indicated as Table 1.

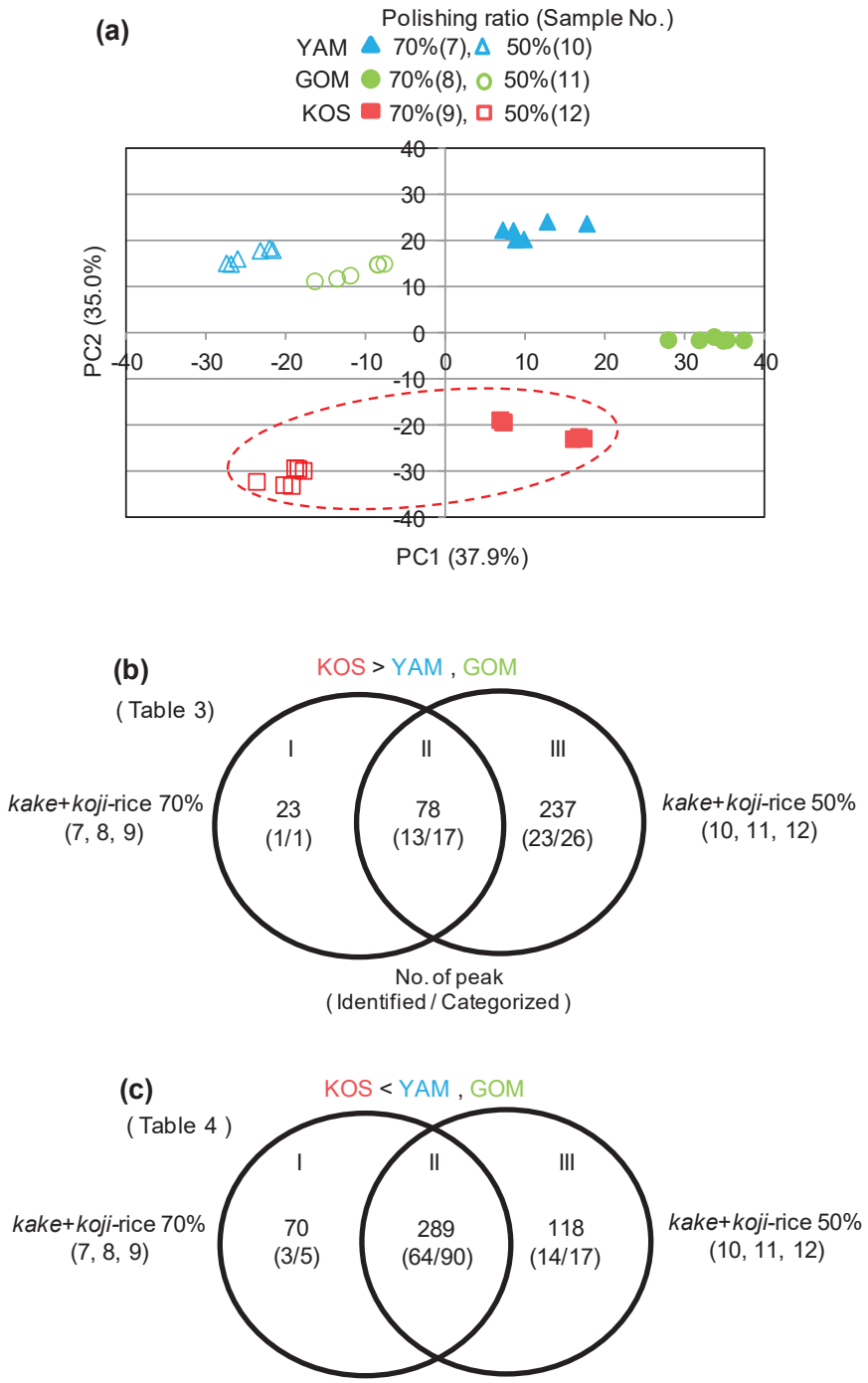


Fig. 3. Analysis of *kake+koji*-rice sake-making test.

(a), PCA score plots of metabolome data (3 times analyses of each sample) from 2 independent *kake+koji*-rice sake-making tests (sample Nos. 7-12 in Fig. 1). Blue-triangle, green-circle, and red-square marks indicate 3 rice cultivars, YAM, GOM, and KOS, respectively. Filled and open marks indicate sample Nos. 7-9 (total rice of 70% polishing

ratio) and sample Nos. 10-12 (total rice of 50% polishing ratio), respectively. The red-dotted ellipse indicates the located area for the samples from KOS. (b), Venn diagrams of the peaks (KOS > YAM, GOM), whose intensity in sake made from KOS (total rice of 70% and 50% polishing ratios) was higher ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (total rice of 70% and 50% polishing ratios). The numbers in Venn diagrams indicate the numbers of peaks (number of the identified/categorized peaks). The metabolites (ID No. Components) of the identified peaks are shown in Table 3. (c), Venn diagrams of the peaks (KOS < YAM, GOM), whose intensity in sake made from KOS (total rice of 70% and 50% polishing ratio) was lower ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (total rice of 70% and 50% polishing ratio). The metabolites (ID No. Components) of the identified peaks are shown in Table 4.

Table 3. The metabolites (ID No. Compound_detected ion) of the identified peaks in Fig. 3(b) (KOS > YAM, GOM).

Category	KOS > YAM, GOM		
	I	II	III
dipeptide			572 Glu-Glu_P
carbohydrate	12 alpha-Ethylglucoside_f10		
		12 alpha-Ethylglucoside_(6; P, f6-9, f11)	
		19 Panose_f3	
		366 D-Maltotriose_f1	
			12 alpha-Ethylglucoside_f4
			21 Raffinose_(2; f5, f10)
organic acid		157 Vanillic acid_P	
		175 Citric acid_(4; f1, f2, f4, f5)	
			172 Adipic acid_f5
			174 Citramalic acid_(4; P, f5-7)
			175 Citric acid_f3
			190 Malic acid_(4; P, f2-4)
			195 n-butyric acid_f4
ester			67 Ethyl 3-hydroxybutyrate_(4; P, f2, f5, f6)
			77 Ethyl lactate_P
phenol compound			149 p-Hydroxybenzoic acid_P
nucleic acid			269 Thymidine_f5
alcohol			53 Tryptophol_f1

The intensity of the peaks in sake made from KOS (total rice of 70% and 50% polishing ratios) was higher ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (total rice of 70% and 50% polishing ratios). Identified compounds (ID No. Compound_detected ion, Table S1) at 70% polishing ratio only (I), at both 70% and 50% polishing ratios (II), and at 50% polishing ratio only (III) are indicated. The detected ions are indicated as Table 1.

Table 4. The metabolites (ID No. Compound_detected ion) of the identified peaks in Fig. 3(c) (KOS < YAM, GOM).

Category	KOS < YAM, GOM		
	I	II	III
nitrogen compound		220 L-Serine_f2	
		223 L-Threonine_f2	
		224 L-Cysteine_f1	
		226 L-Leucine_(2; P, f1)	
		228 L-Aspartic acid_(3; f1-3)	
		229 L-Lysine_(2; P, f1)	
		230 L-Glutamic acid_P	
		231 L-Methionine_(2; f1, f2)	
		232 L-Histidine_(2; P, f1)	
		233 L-Phenylalanine_f1	
		236 L-Tyrosine_(4; P, f1, f3, f4)	
		257 beta-Phenylethylamine_(2; P, f1)	
		258 Agmatine_f3	
			227 L-Ornithine_f2
dipeptide	553 Phe-Pro_P		
	566 Lys-Asn_(2; f1, f4)		
		466 Ala-Trp_P	
		468 Ala-Leu_f2	
		470 Ala-Phe_P	
		474 Leu-Val_P	
		483 Gly-Thr_f2	
		484 Gly-Trp_P	
		490 Gly-Phe_(2; P, f3)	
		491 Gly-Pro_P	
		494 Gly-Tyr_P	
		498 Leu-Tyr_P	
		502 Leu-Leu_P	
		503 Gln-Gln_P	
		513 Val-Val_(2; P, f1)	
		523 Ala-Gln_P	
		525 Val-Gln_P	
		526 Val-Phe_P	
		527 Leu-Phe_P	
		531 Ala-Thr_P	
		532 Leu-Lys_f1	
		533 Asp-Glu_P	
		536 Gln-Leu_P	
		542 Asn-Ile_P	
		558 Arg-Val_(3; P, f2, f3)	
		564 Glu-Ala_P	
		565 Asp-Ser_P	
		566 Lys-Asn_(2; P, f2)	
		574 His-Leu_P	
			526 Val-Phe_f2
			530 Pro-Gln_P
			532 Leu-Lys_f2
			536 Gln-Leu_f1
			566 Lys-Asn_f3
carbohydrate			12 alpha-Ethylglucoside_f1
			367 Melibiose_f16
organic acid		209 Succinic acid_f1	
			179 Gluconolactone_f4
			209 Succinic acid_(3; P, f2, f3)
ester		414 N-(2-phenylethyl)acetamide_(2; P, f3)	
		463 Ethyl-glucopyranoside_f6	
			67 Ethyl 3-hydroxybutyrate_f7
carbohydrate alcohol		2 Erythritol_P	
nucleic acid		271 Uridine_(2; P, f6)	
		273 Inosine_f3	
			266 Hypoxanthine_P
other		456 Phosphoric acid_P	

The intensity of the peaks in sake made from KOS (total rice of 70% and 50% polishing ratios) was lower ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM (total rice of 70% and 50% polishing ratios). Identified compounds(ID No. Compound_detected ion, Table S1) at 70% polishing ratio only (I), at both 70% and 50% polishing ratios (II), and at 50% polishing ratio only (III) are indicated. The detected ions are indicated as Table 1.

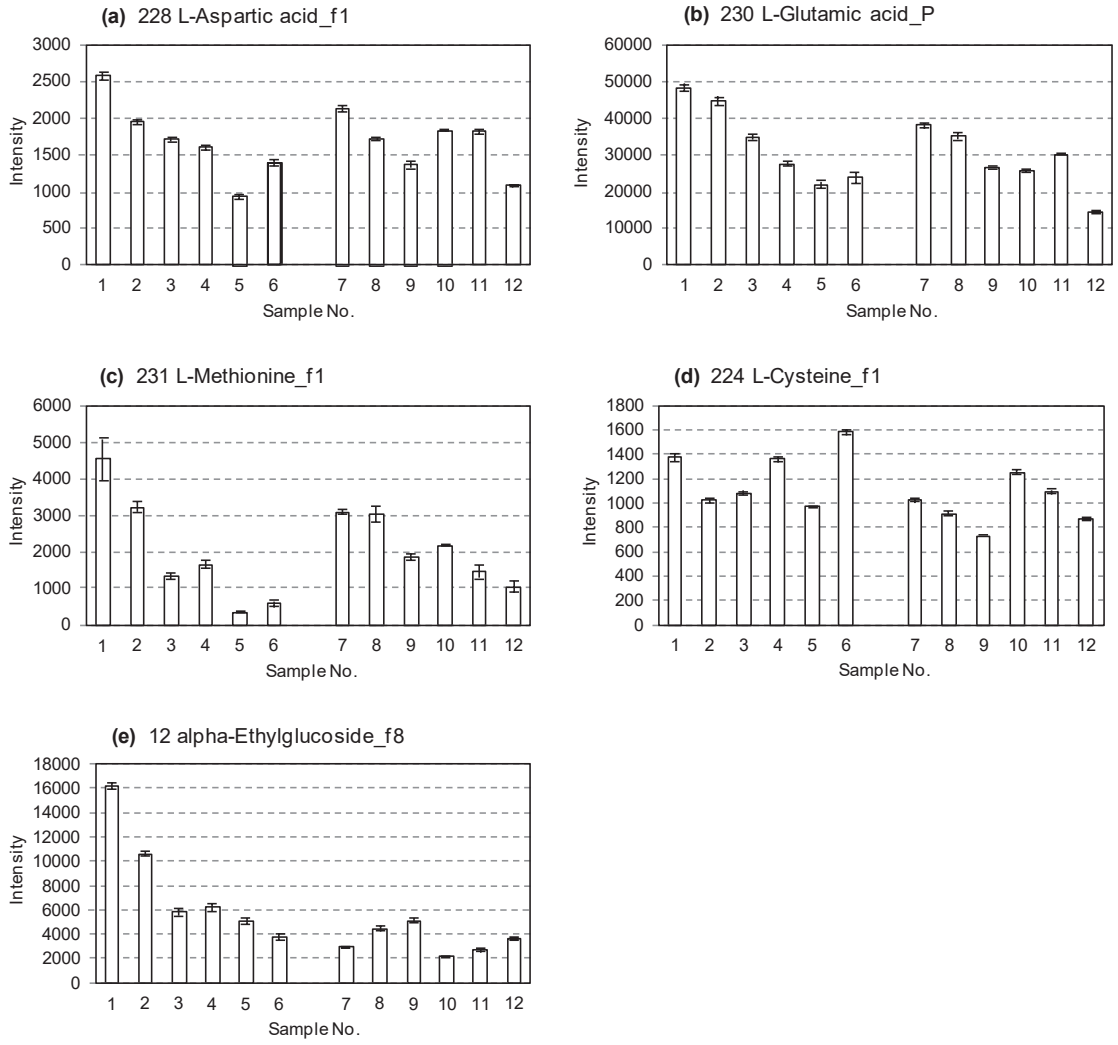


Fig. 4. Typical metabolites (ID No. Compound_detected ion) affected by *Koshitanrei.* Intensity of (a) L-Aspartic acid_f1 (RT, 1.2439; m/z, 116.0346), (b) L-Glutamic acid_P (RT, 1.3348; m/z, 148.0609), (c) L-Methionine_f1 (RT, 2.6324; m/z, 133.0306), (d) L-Cysteine_f1 (RT, 1.2018; m/z, 241.0325), and (e) alpha-Ethylglucoside_f8 (RT, 5.1417; m/z, 145.0499) are indicated.

CHAPTER Ⅱ

Effect of *koji* starter on metabolites in Japanese alcoholic beverage sake made from the sake rice *Koshitanrei*

2.1. Abstract

In sake brewing, the steamed rice is used in 2 ways, added to sake-mash and making *rice-koji*. Rice-*koji* is made from the steamed rice by using *koji* starter, and its quality is an important determinant of the aroma/taste of sake. The sake rice *Koshitanrei* (KOS) was developed in Niigata Prefecture by crossing 2 sake rice varieties, *Gohyakumangoku* and *Yamadanishiki*. Recently, we reported the characteristic components/metabolites in sake made from KOS by conducting metabolome analysis using UPLC-QTOF-MS. In this study, to investigate the effect of *koji* starter and sake rice cultivars on the sake metabolites, I performed small-scale sake-making tests using the above 3 rice cultivars and 3 *koji* starters. Finally, I demonstrated that some of the characteristic components/metabolites of sake from KOS are affected by the *koji* starter. Thus, in addition to rice cultivar, *koji* starter plays an important role for establishment/maintenance of the quality of the final product.

2.2. Introduction

The Japanese alcoholic beverage sake is made from rice and water by using 2 microorganisms, *Aspergillus oryzae* (called *koji*-mold) and the yeast *Saccharomyces cerevisiae* (Yoshizawa, 1999). The main raw material for sake brewing is the rice, and the steamed rice is used in 2 ways, directly added to sake mash (as *kake-rice*) and used to make *koji*. In order to produce the specific taste of the sake, various rice cultivars have been used for sake brewing. Breeding of the sake rice cultivars has been done in many areas of Japan (Maeshige and Kobayashi, 2000). In Niigata Prefecture, the originally developed sake rice *Gohyakumangoku* (GOM) has been used widely. GOM has contributed to high-quality sake having a clear taste, although the hardness of its grains is insufficient for high polishing (especially to less than the 50% polishing ratio for *Daiginjo-shu*), due to the shape and size of its white core (Anzawa *et al.*,. 2013). To improve this polishing property of GOM, the sake rice *Koshitanrei* (KOS) was originally developed in Niigata Prefecture by crossing GOM with *Yamadanishiki* (YAM) (Kobayashi *et al.*, 2006). Anzawa *et al*. (Anzawa *et al.*,. 2013) reported the polishing/brewing properties of KOS determined by using a wide range of polished rice and showed differences in the general properties of the sake between that made from KOS and that from GOM.

In sake brewing, *koji* making is one of the important processes, because *koji* is a determinant of the taste and flavor component in sake. The significance aspects of *koji* are its being the source of diastatic enzyme, the nutrient source for yeast cell growth, and the

source of the component(s) affecting sake quality. The quality of *koji* is determined by the production process, rice cultivar, and *koji* starter. Ito *et al*. (Ito *et al.*,. 2013) reported that amino acid in *koji* is affected by the rice cultivar used to make *koji* and *koji* starters. Also, Takahashi *et al*. (Takahashi *et al.*,. 2008) reported that the activity of each enzyme is affected by the rice cultivar used to make *koji* and *koji* starters. Further, Yanagiuchi *et al*. (Yanagiuchi *et al.*,. 1993) reported that enzyme activity and enzyme balance (glucoamylase and acid carboxypeptidase) are also affected by the rice cultivar used to make *koji* and *koji* starters and that the fatty acid composition of *koji* and amino acid composition of sake are affected by *koji* starters. Therefore, the selection of the *koji* starter for the rice cultivar is important.

The metabolomics technique is an analysis method examining cyclopaedically/co-instantaneously a number of intravital metabolites. Recently, this type of analysis has been applied in various fields with diversification (Putri *et al.*, 2013a; Putri *et al.*, 2013b). Previously, in the analysis of sake done by using the metabolomics technique CE-TOF MS, Sugimoto *et al*. (Sugimoto *et al.*, 2010) reported a correlation between sensory evaluation scores of sake and metabolome profiles, as well as a difference in the charged metabolites between pasteurized and unpasteurized sake during storage (Sugimoto *et al.*, 2012). In the sake analysis using GC/MS, Mimura *et al.* (Mimura *et al.*, 2014) examined the relationship between the features of sake and the component profile; and Tamada *et al*. (Tamada *et al.*, 2017) reported the characteristics of sake according to the sake-making parameters (sake yeast, rice cultivars) assessed by GC/MS and analyzed the correlation between the component profiles of GC/MS and the intensity of "Oshi-aji (Tamada *et al.*, 2018). Further, in the sake analysis using 2-dimensional GC with TOF-MS, Takahashi *et al*. (Takahashi *et al.*, 2016) examined the correlation between sake components and organoleptic properties; and Tokuoka *et al*. (Tokuoka *et al.*, 2017) used HILIC-TOF-MS to analyze the oligosaccharide composition of sake. Recently, Yazawa *et al*. (Yazawa *et al.*, 2019) conducted sake metabolome analysis using UPLC-QTOF-MS (ultra-performance liquid chromatography/time-of-flight mass spectrometry) and examined the relationship between sake-making parameters and sake metabolites. In their study, the influence of the rice cultivar used as *rice-koji* was also analyzed and found to have a significant effect on sake quality. Recently, I reported the characteristic components/metabolites in sake made from KOS by using the method of Yazawa *et al*. (Yazawa *et al.*, 2019) (Ichikawa *et al*. (Ichikawa *et al.*, 2019)). However, the effect of

the *koji* starter on the metabolites in the sake made from KOS was not analyzed at that time.

In this present study, in order to investigate the effect of the *koji* starter on the sake metabolites in the sake made from KOS, I performed small-scale sake fermentation tests using 3 rice cultivars: KOS, YAM, and GOM, and 3 *koji* starters (*Yoikaori/Y, High G/H, and Byakuya/B*). I analyzed the sake metabolome data obtained from small-scale sake fermentation tests by using the recently developed sake metabolome analysis method; (Yazawa *et al.*, 2019) and I also investigated the effect of *koji* starters on the characteristic sake metabolites whose contents in sake made from KOS were higher/lower than those from the other 2 cultivars, YAM and GOM. Finally, I demonstrated that the characteristic components/metabolites of sake made from KOS were affected by the *koji* starter.

2.3. Materials and Methods

2.3.1. Rice

Rice cultivars used for the small-scale sake fermentation test were the following (Table 5): YAM (grown in Hiroshima Prefecture), GOM (grown in Niigata Prefecture), and KOS (grown in Niigata Prefecture), all harvested in 2016. These rice cultivars were the same as those used in a previous study (Ichikawa *et al.*, 2019). The rice grains were polished to a polishing ratio of 50% by using a milling machine (RP-5 and NF-26FA, Shin-Nakano Industry, Japan).

2.3.2. Small-scale koji making

Small-scale *koji* making was done as previously reported (Ichikawa *et al.*, 2019). The *koji* starters used for the small-scale *koji* making were as follow (Table 5); *Yoikaori*/Y (Bioc, Japan), *High G/H* (Higuchi Matsunosuke Shoten, Japan), and *Byakuya/B* (Hishiroku, Japan).

2.3.3. Small-scale sake-making test

Three rice cultivars (YAM, GOM, and KOS) each with a 50% polishing ratio were used for both *kake*-rice and *koji*-rice (Table 5). In the sake-making test, 500 g of total rice (100 g for *koji*-rice and 400 g for *kake*-rice) was used for three-step addition to the sake mash as follows.

As the first-step, the steamed rice (*kake*-rice) of 56 g was added to 87 mL of water with 1 mL (10^8 cells) of the sake yeast K701, 24 g of *koji*, and 450 μL of 50% lactic acid. As the second-step, the steamed rice (*kake*-rice) of 117g was added to the sake mash with 180 mL of water, and 33 g of *koji.* As the third-step, the steamed rice (*kake*-rice) of 227g

was added to the sake mash with 382 mL of water, and 43 g of *koji.*

The *kake*-rice for the sake-making test was prepared as follows: The rice was washed at 15 °C for 30 sec with tap water, drained for 15 sec, and washed for 30 sec again with more tap water. After the washing step, the rice was drained for 15 sec and then soaked to reach 130% water absorption. Next, the soaked rice was drained at 15 °C for 30 min and subsequently steamed for 60 min. The water absorption of the steamed rice was adjusted to 130%, assuming that the water content of the original white rice was 13.5%.

The sake yeast strain K701 for the sake-making test was cultured as follows: The sake yeast cells were pre-cultured overnight at 30 °C in the rice *koji* extract medium (Baume scale 10). The pre-cultured cells were then inoculated into fresh medium at 100-fold dilution and incubated for 2 days at 30 °C as a standing culture. The cultured cells were washed twice with sterilized water and thereafter added to the sake mash (final concentration, approx. 1 x 10^6 cells/mL).

Sake fermentation was performed at 15 °C (in a water bath) for 20 days, and was monitored by measuring the weight reduction of the sake mash, corresponding to CO_2 evolution. After fermentation, the clear sake sample was obtained by centrifugation (5000 rpm, 10 min, 4 °C).

2.3.4. Analysis of sake

General properties (Sm, sake meter; Alc, sake alcohol; TA, total acidity; AA, amino acidity), and flavor components (EtOAc, ethyl acetate; iAmOH, isoamyl alcohol; iAmOAc, isoamyl acetate; EtCap, ethyl caproate) of the *sake* were determined by the standard method established by the National Tax Agency of Japan (Brewing Society of Japan, 2017).

2.3.5. Enzyme activity analysis of rice koji

Enzyme activity of *koji* was analyzed by the standard method established by the National Tax Agency of Japan (Brewing Society of Japan, 2017). The activities of 3 enzymes, i.e., α-amylase, glucoamylase, and acid carboxypeptidase, were measured by using the respective assay kits (Kikkoman, Japan) (Imai *et al.*, 1996; Shirokane *et al.*, 1996).

2.3.6. Sake metabolome analysis method

A model sake sample was prepared as in the previous report (Ichikawa *et al.*, 2019). Sake samples were filtered through an Amicon Ultra 0.5 3K (Merk Millipore, Germany) and diluted 10-fold with MS-grade water. UPLC-QTOF-MS analysis was performed with a UPLC Xevo Q/TOF-MS system (Waters, Milford, USA). The method for sake metabolome analysis established by Yazawa *et al*. (Yazawa *et al.*, 2019) was followed.

2.3.7. Data processing of sake metabolome

Data processing by Yazawa *et al*. (Yazawa *et al.*, 2019) and Ichikawa *et al*. (Ichikawa *et al.*, 2019) was basically followed. Peak detection, alignment peak data, and calculation of peak area were conducted with Peaklynx XS Rev. 1.0 software (Waters, Milford, USA). Mass range and retention-time range for peak detection were determined by the error range of 14 selected peaks from the model sake samples (Table S4 (Chapter II Table S4.xlsx)). To evaluate the accuracy of the metabolome data, I measured the model sake sample once among 9 sake samples. The model sake sample analyzed total 7 times. The error range of each mass spectrum was calculated by 5-fold standard deviation of each of the 14 selected peaks. The largest value among these values was used as the error range for the peak-picking method of Peaklynx. The error range of the retention time was also calculated as the mass spectrum error. Other settings of the peak picking-method for metabolome data were as follow: initial and final retention times of monitoring, 0 to 15 min; peak width at 5% height, 4 sec; intensity threshold of peak picking, 40; mass window, 0.03 Da; and retention time window, 0.1 min. Other settings followed the manufacturer's instruction. After making a peak table, preliminary data processing was performed according to method by Ichikawa *et al* (Ichikawa *et al.*, 2019).

2.3.8. Multiple comparison technique using metabolome data

The software JMP 13.1 (commercially available) and Microsoft Excel were used for principal component analysis (PCA) and welch's test (two-tailed, Bonferroni corrected), respectively. To examine the difference in sake metabolites generated by the rice cultivars in the small-scale sake-making tests, I performed Welch's test (two-tailed, Bonferroni corrected) by using the intensity of each peak. The *P* value was calculated by using the average of the intensity of each sample, and a value of less than 0.05 was regarded as indicating a significant difference between rice cultivars.

2.4. Results and Discussion

2.4.1. Effect of koji starters on the general properties of sake and the enzyme activities of koji

In a previous report (Ichikawa *et al.*, 2019), to investigate the characteristics of sake metabolites in sake prepared from KOS, I performed the small-scale sake fermentation test using 3 rice cultivars (YAM, GOM, and KOS) and 1 *koji* starter (*Byakuya/B*). Then, in the "*kake*+*koji*-rice (total rice) sake-making test," the contents of the 2 tasterelated amino acids,

glutamic acid and aspartic acid, were lower in the sake from KOS than in those from the other 2 rice cultivars. In the "*kake*-rice sake-making test," alpha-ethylglucoside (α-EG) content in the sake of KOS was lower than that in the sake made from the other rice cultivars; in contrast, in the "*kake*+*koji*-rice sake-making test," the α-EG content in the sake of KOS was higher than that in the other rice cultivars.

In this present study, to investigate whether the characteristic metabolites in sake made from KOS varied according to the *koji* starters, I performed the small-scale sake fermentation test followed by the small-scale *koji* making, using 3 rice cultivars (YAM, GOM, and KOS) and 3 *koji* starters (*Yoikaori/Y, High G/H, and Byakuya/B*) (Table 5). These *koji* starters are commercially available. In this small-scale fermentation test, the 4 enzyme activities (α-amylase, glucoamylase, acid carboxypeptidase, and acid protease) of the *koji*, fermentation rates (CO2 decrement) during the test, and the general properties and flavor components of the obtained sake samples were determined and are shown in Table 5 and Fig. 5(b), Fig. S2 a, b, and c (Chapter II Fig S2.pptx), and Table 6, respectively. The α-amylase activity of *koji* from KOS was lower than that of the *koji* prepared from the other rice cultivars regardless of the type of *koji* starter used, especially, it was significantly lower in *koji* starter-Y and -H (Table 5 and Fig. 5(b)). In either *koji* starters, acid carboxypeptidase activity in *koji* from KOS was intermediate between that from YAM and that from GOM, and the glucoamylase activity in *koji* from KOS was comparable to that in YAM *koji*. In the *koji* made by *koji* starter-B, the 3 enzymatic activities (α-amylase, glucoamylase, and acid protease) were higher than those in the *koji* made from the other 2 *koji* starters (Table 5 and Fig. 5(b)). In the *koji* made by *koji* starter-H, 2 enzymatic activities (α-amylase and acid carboxypeptidase) were lower than those in the *koji* made from the other 2 *koji* starters. In the *koji* made by *koji* starter-Y, 2 enzymatic activities (glucoamylase and acid protease) were lower than those in the *koji* made from the other *koji* starters, -H and -B (Table 5 and Fig. 5(b)). The G/α ratio (glucoamylase activity/α- amylase activity) for the *koji* made from the *koji* starter-B was higher than that for *koji* made from *koji* starter-H or -Y (Table 5and Fig. 5(b)).

In the small-scale fermentation test using the *koji* made from either *koji* starter-Y or -H, fermentation rates (CO2 decrement) in the sake mash using KOS showed the same profiles as those using GOM. While, in the test using the *koji* made from *koji* starter-B, the fermentation profile (CO2 decrement) in the sake mash using KOS indicated an intermediate value between that using YAM and using GOM (Fig. S2a, b and c (Chapter II

Fig S2.pptx)). Regardless of the type of *koji* starter, the concentration of the flavor component isoamyl acetate (iAmOAc) in the sake sample from KOS was lower than that in the sake made from the other rice cultivars, GOM and YAM. The concentration of isoamyl alcohol (iAmOH) in the sake samples using *koji* made from *koji* starter-Y or -H from KOS was lower than that in the case of GOM or YAM (Table 6).

2.4.2. Principal component analysis of the metabolome data of the sake fermentation test

To investigate the effect of the *koji* starter on the characteristic sake metabolites from KOS, I constructed a peak table (Table S5 (Chapter II Table S5.xlsx)) containing all metabolome data of 1116 peaks, whose intensity was higher than the threshold of peak-picking, as described in Materials and Methods (*Data processing of sake metabolome*). Among these 1116 peaks, I identified 130 peaks (counting "number of fragment ion(s) from one compound") and categorized 191 peaks. Among the identified 130 peaks, I detected 77 compounds (counting "1" = "more than 1 ion from 1 compound") among 496 compounds (Table S4 (Chapter II Table S4.xlsx)), based on the retention time and m/z of the ion(s) (P, precursor/parent ion; fx, in-source fragment ion; derived from the compounds) in Table S4 (Chapter II Table S4.xlsx). I used this peak table (Table S5 (Chapter II Table S5.xlsx)) for further analysis.

At first, to investigate the character of each sake sample, I performed principal component analysis (PCA) by using this peak table (Fig. 6(a,b)). As the result of PCA score plotting of the metabolome data from the small-scale sake fermentation test, the contribution ratios of PC1 and PC2 were 53.5% and 20.3%, respectively (Fig. 6(a,b)).

In the classification based on the rice cultivars, on the PC2 axis, the sake samples from KOS (sample Nos. 3, 6, and 9) were concentrated in a negative direction; whereas those from YAM and GOM (sample Nos. 1, 2, 4, 5, 7 and 8) were concentrated in a positive one (Fig. 6(a)). The sake samples from YAM and GOM were closely located in almost the same region (Fig. 6(a)). The analysis/result of loading plot of the PCA indicated that unknown peaks contributed mainly to the distribution to a negative direction on the PC2 axis (Fig. S3 (Chapter II Fig S3.pptx)).

Next, in the classification based on *koji* starters, the sake samples from *koji* starter-B (sample Nos. 7–9) and *koji* starter-Y (sample Nos. 1–3) were concentrated on the PC1 axis in a positive and negative direction, respectively; and the sake samples from *koji* starter-H (sample Nos. 4–6) were located intermediate between those from *koji* starter-B and –Y (Fig.

6(b)). These results suggested that the sake components were changed by the *koji* starters (Fig. 6(b)). Indeed, the analysis/result of loading plot of the PCA indicated that many peaks contributed to a distribution on the PC1/2 axis, and that as the peaks contributing to the distribution. α-EG and nitrogen compound/dipeptide were located on the PC1 axis in a negative and positive direction, respectively (Fig. S3 (Chapter II Fig S3.pptx)). Previously, Yazawa et al. (Yazawa *et al.*, 2019) reported that α-EG was negatively correlated with glucose. As the glucoamylase activity of the *koji* made from starter-B was higher than those from other *koji* starters, the amount of glucose in the *koji* made from starter-B would be higher than those from other *koji* starters. Therefore, it is suggested that the *koji* made from starter-B was concentrated in a positive direction on the PC1 axis, the opposite of α-EG.

2.4.3. Effect of koji starters on the sake metabolites obtained with Koshitanrei

Next, to analyze/explore in detail the peaks affecting the distribution on the axis in Fig. 6 and the characteristic peaks for KOS, I performed Welch's test (two-tailed, Bonferroni corrected) using the constructed peak table (Table S5 (Chapter II Table S5.xlsx)). In this analysis, I eliminated 75 peaks (the intensity 0 in some of the samples Nos.1–9). With either *koji* starter, by focusing on the peaks in samples of sake made from KOS, I selected the peaks whose intensities were higher ($p < 0.025$, Bonfferoni corrected; KOS > YAM, GOM) or lower ($p < 0.025$, Bonfferoni corrected; KOS < YAM, GOM) than those for the other rice cultivars. Further, the selected peaks were classified according to *koji* starters. In order to clarify the difference of components/metabolites affected by three *koji* starters, I analyzed the components/metabolites using Venn diagram. Regarding the selected peaks, Venn diagrams of the higher peaks (KOS > YAM, GOM) and lower peaks (KOS < YAM, GOM) are shown in Fig. 7(a,b), respectively.

In the higher peaks with KOS (KOS > YAM, GOM; Fig. 7(a)), the number of the higher peaks for *koji* starter-Y was only 16 ("I" in Fig. 7(a)); that of those for *koji* starter-H, only 20 ("III" in Fig. 7(a)); that of those for *koji* starter-B, only 17 ("V" in Fig. 7(a)); and that of those for either *koji* starter, 41 ("VII" in Fig. 7(a)). Thus, in the characteristic sake metabolites from KOS, there were 2 peak groups, one affected by *koji* starter and the other unaffected by rice cultivar.

On the other hand, in the lower peaks found for KOS (KOS < YAM, GOM; Fig. 7(b)), the number of the lower peaks for *koji* starter-Y was only 53 ("I" in Fig. 7(b)); that of those for *koji* starter-H, only 23 ("III" in Fig. 7(b)); that of those for *koji* starter-B, only 107 ("V" in Fig. 7(b)); and that of those for either *koji* starter, 301 (VII in Fig. 7(b)). Further, the

number of lower peaks for KOS in the sake using the *koji* made from starter-B was larger than those from other *koji* starters (Fig. 7(b) 107/V vs. 53/I and 23/III), indicating that in the sake using the *koji* made from starter-B the character of sake metabolites derived from rice cultivar is likely to emerge. With either *koji* starter, the number of the lower peaks was about 7-fold (301/B-VII vs. 41/A-VII) over the number of the higher peaks, suggesting that some metabolites in the sake from KOS tended to be lower than those from the other rice cultivars. Thus, as in the previous report (Yazawa *et al.*, 2019; Ichikawa *et al.*, 2019), the sake metabolites were affected by the rice cultivar used, and, further, the characteristic sake metabolites of the rice cultivars were changed by the *koji* starter used.

2.4.4. Identification of the sake metabolites affected by Koshitanrei and koji starters

Further, I categorized or identified the metabolites of the affected peaks by using the metabolite list (Table S4 (Chapter II Table S4.xlsx)). The results (Fig. 7(a) and Table 7) showed that the numbers of the identified/categorized higher peaks for KOS were 2/2 among 16 for *koji* starter-Y (I), that no peaks were identified/categorized among the higher peaks for *koji* starter-H (III) and -B (V), and that the numbers of identified/categorized peaks were 9/9 among 41 for either *koji* starter (VII). On the other hand, the identified/categorized lower peaks (Fig. 7 (b) and Table 8) for KOS were 2/5 among 53 for *koji* starter-Y (I), 6/9 among 23 for *koji* starter-H (III), 10/18 among 107 for *koji* starter-B (V), and 41/61 among 301 for either *koji* starter (VII). The metabolites (ID No. Compound_detected ion, in Table S4 (Chapter II Table S4.xlsx)) of the identified peaks (Fig. 7(a,b)) are indicated in Tables 7 and 8. With either *koji* starter, the contents of alphaethylglucoside (α-EG) in the samples of sake made from KOS were higher than those in the samples from the other 2 rice cultivars (Table 7, VII, 8 peaks). On the other hand, the lower peaks in the KOS sake samples were amino acids and dipeptides (Table 8, VII).

2.4.5. Effect of koji starter on the taste- and aroma-related sake metabolites affected by KOS

Various components including carbohydrates, organic acids, amino acids, and inorganic ones are related to the taste and aroma of sake (Brewing Society of Japan, 1999). A previous report showed 5 components (aspartic acid, glutamic acid, methionine, cysteine, and alpha-ethylglucoside) related to the taste and aroma of sake among the identified metabolites/components found in sake made from KOS (Ichikawa *et al.*, 2019). Therefore, I investigated whether these components were affected by the *koji* starter used.

Previously, it was shown that the concentrations of the 2 taste-related amino acids,

glutamic acid and aspartic acid (Iwano *et al.*, 2004), in the sake made from KOS were lower than those in the sake from other rice cultivars. At first, I examined these 2 amino acids. As the result (Fig. 8(a), Nos. 3, 6, 9), with any of the *koji* starters, the aspartic acid content in the sake from KOS was lower than that in the sake prepared from the other rice cultivars. In regard to aspartic acid, I make standard curve using four different concentration of standard material (Fig. S4a (Chapter II Fig S4.pptx)), and I confirmed that standard curve showed good linearity in the scope of intensity of aspartic acid (Fig. 8(a)). As the low content of aspartic acid in sake from KOS was found regardless of the *koji* starter used, this finding suggests that the content of aspartic acid would be affected by the rice cultivar rather than by the *koji* starter.

Previously, it has been reported that the major component of "*hineka*," one of the unpalatable flavors in sake, is dimethyl tri-sulfide (DMTS) (Isogai *et al.*, 2006) and that the production of 1,2-dihydroxy-5-(methylsulfinyl) prentan-3-one (DMTS-P1), the precursor of DMTS, is related to the methionine salvage pathway genes in *Saccharomyces cerevisiae* (Wakabayashi *et al.*, 2013). In our previous study, I found that the content of methionine in the sake from KOS was lower than that in the sake from the other rice cultivars.

Next, therefore, I examined the methionine content. In regard to methionine, as with aspartic acid, I confirmed that standard curve showed good linearity in the scope of intensity of methionine (Fig. 8(b) and Fig. S4b (Chapter II Fig S4.pptx)). As the result (Fig. 8(b), Nos. 3, 6, 9), regardless of the *koji* starter used, the content of methionine in the sake from KOS was lower than that in the sake from the other rice cultivars, suggesting that the low contents of sulfur-containing amino acids in the sake from KOS would be unaffected by the *koji* starter used.

Finally, I examined the carbohydrate alphaethylglucoside (α-EG), which is responsible for the bitterness following the sweet taste of sake (Oka and Sato, 1976). Furthermore, in regard to α-EG, as with aspartic acid, I confirmed that standard curve showed good linearity in the scope of intensity of α-EG (Fig. 8(c) and Fig. S4c (Chapter II Fig S4.pptx)). Previously, it was shown that the α-EG content was affected by rice cultivar and that the α-EG content in sake using *koji*-rice made from KOS was higher than that in the sake using *koji*-rice made from other rice cultivars. As the result (Fig. 8(c), Nos. 3, 6, 9), with any of the *koji* starters, the α-EG content of sake made from KOS was higher than that from the other rice cultivars, suggesting that the α-EG content of sake would be affected by both rice cultivar and the *koji* starter.

Previously, I showed that sake metabolites affected by the sake rice cultivars, especially KOS. In this study, I further examined the effect of the *koji* starter used on the metabolites of sake made from KOS. For future clarification of the effect of *koji* starter and KOS on the sake metabolites, it will be important to identify the unknown peaks affected by KOS and *koji* starter.

Table 5. Combination of sake-making parameters and properties of koji-rice

Sample No.	Rice cultivar		Koji starter	Yeast strain	Enzyme activity (U/g *koji*)				
	kake-rice	*koji*-rice			Aaase	Gaase	G/α ratio	ACPase	Apase
1	YAM	YAM	*Yoikaori*	K701	765	140	0.18	2,659	1,863
2	GOM	GOM			935	169	0.18	3,804	2,489
3	KOS	KOS			612	153	0.25	3,380	2,186
4	YAM	YAM	*High G*		797	204	0.26	2,532	1,804
5	GOM	GOM			903	237	0.26	4,014	2,354
6	KOS	KOS			553	196	0.35	3,096	2,337
7	YAM	YAM	*Byakuya*		804	368	0.46	2,729	2,261
8	GOM	GOM			948	474	0.50	4,418	2,520
9	KOS	KOS			708	373	0.53	3,388	2,514

Sake-making parameters, such as rice cultivar (YAM, Yamadanishiki; GOM, Gohyakumangoku; and KOS, Koshitanrei), *koji* starter, yeast strain, and enzyme activities (AAase, a-amylase; GAase, glucoamylase; G/a ratio, GAase/AAase; ACPase, acid carboxypeptidase; and APase, acid protease) of *koji*-rice are indicated.

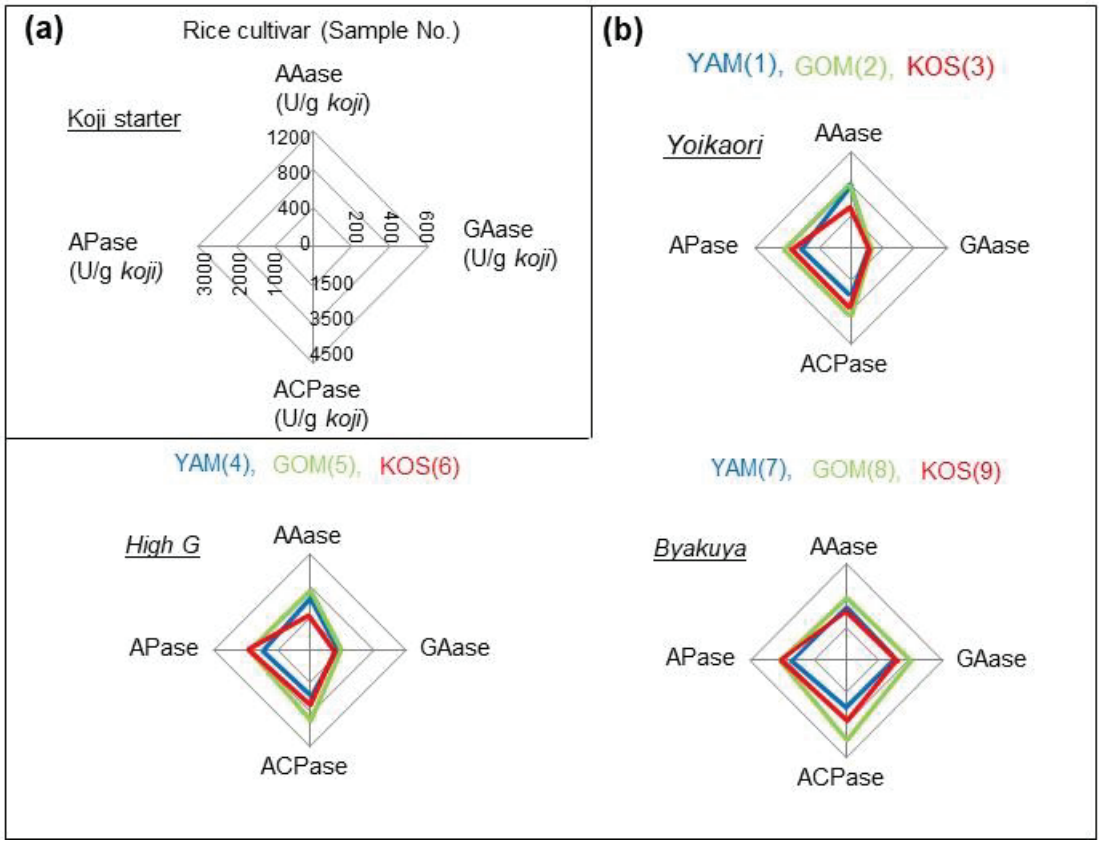


Fig. 5. Combination of sake-making parameters and properties of *koji*-rice and sake samples analyzed in the small-scale sake fermentation tests.

(a), Conceptual diagram of cobweb chart showing 4 enzyme activities of *koji*-rice.

(b), Three cobweb charts of 4 enzyme activities of *koji*-rice prepared by using 3 *koji* starters (Yoikaori, High G, and Byakuya) made from 3 rice cultivars (YAM/blue, GOM/green, and KOS/red) are indicated.

Table 6. sake samples analyzed in the small-scale sake fermentation tests

Sample No.	Rice cultivar	Koji starter	Gneral properties				Flavor components (ppm)			
			Sm	Alc (%)	TA (ml)	AA (ml)	EtOAc	iAmOAc	iAmOH	EtOCap
1	YAM	*Yoikaori*	+2	17.3	2.0	1.3	106	8.7	209	3.0
2	GOM		+10	18.6	2.2	1.8	113	9.7	222	2.3
3	KOS		+11	18.5	2.1	1.5	119	7.4	176	2.7
4	YAM	*High G*	+5	18.6	2.4	1.7	117	8.1	189	3.2
5	GOM		+12	18.7	2.6	1.9	116	8.6	205	2.4
6	KOS		+15	19.3	2.4	1.9	115	5.9	166	2.6
7	YAM	*Byakuya*	+9	19.0	1.9	1.3	124	5.2	129	3.6
8	GOM		+17	19.8	2.2	1.5	128	6.1	141	2.7
9	KOS		+19	19.6	2.1	1.5	117	4.6	133	2.9

General properties and flavor components of sake samples designated in “a.” All data are indicated as the averages for 2 independent tests.

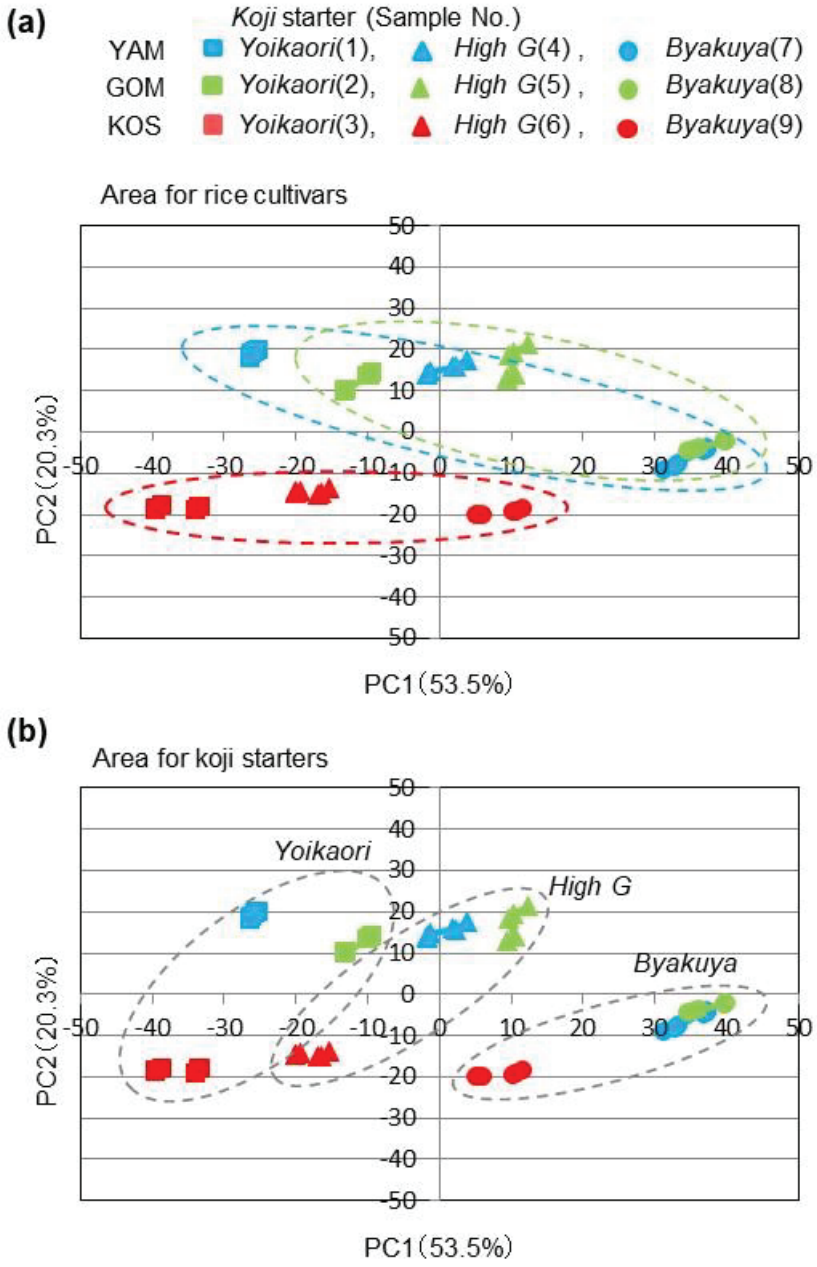
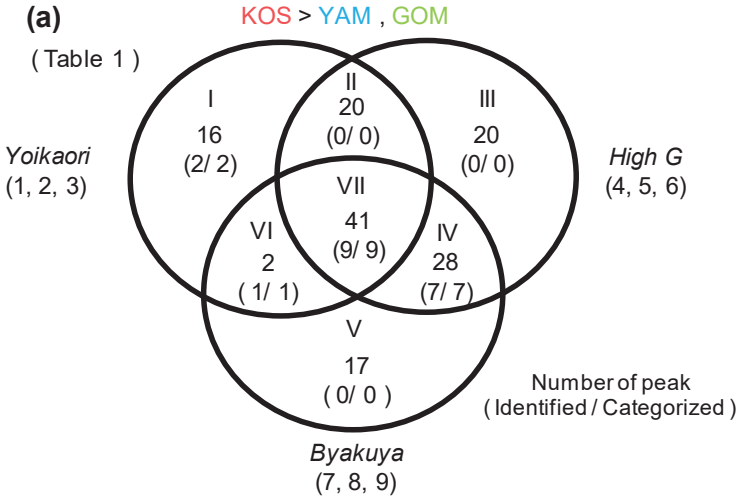


Fig. 6. PCA analysis of small-scale sake making tests.

(a), PCA score plots of metabolome data (3 analyses of each sample) from 2 independent sake-making tests (sample Nos. 1-9 in Table 1). Blue, green, and red symbols indicate the 3 rice cultivars, YAM, GOM, and KOS, respectively. Squares, triangles, and circles indicate the 3 *koji* starters, Yoikaori, High G, and Byakuya, respectively. Blue-, green-, and red-dotted ovals indicate the located areas for the samples from the 3 rice cultivars, YAM, GOM, and KOS.

(b), PCA score plots of metabolome data. All symbols are the same as in “a.” The 3 dotted ovals indicate the located area for the samples from 3 *koji* starters, Yoikaori, High G, and Byakuya.



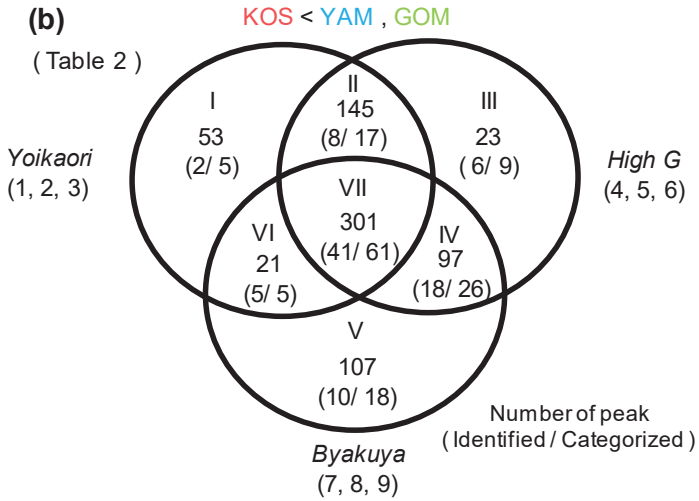


Fig. 7. Venn diagrams of peaks affected by KOS in sake using *koji* from each *koji* starter. (a), For the intensity of peaks in sake made from the 3 rice cultivars (YAM, GOM, and KOS), welch's test (two-tailed, Bonferroni corrected) were performed to compare the intensities between 2 evaluations (KOS vs. YAM, KOS vs. GOM). Venn diagrams of the peaks (KOS > YAM, GOM), whose intensity in sake made from KOS was higher ($p <$ 0.025, Bonfferoni corrected, KOS > YAM, GOM) than that in sake made from the other 2 rice cultivars, YAM and GOM. The numbers in Venn diagrams indicate the numbers of peaks (number of the identified/categorized peaks). The metabolites (ID No. Components) of the identified peaks are shown in Table 3.

(b), Venn diagrams of the peaks (KOS < YAM, GOM), whose intensity in sake made from

KOS was lower ($p < 0.025$, Bonfferoni corrected, KOS < YAM, GOM) than that in sake made from the other 2 rice cultivars, YAM and GOM. The metabolites (ID No. Components) of the identified peaks are shown in Table 4.

Table 7. The metabolites (ID No. Compound_detected ion) of the identified peaks in Fig. 3(a) (KOS > YAM, GOM).

Category	I	III	V	VII
dipeptide				517 Gln-Glu_P
carbohydrate				12 alpha-Ethylglucoside_(8; P, f2, f4, f6-10)
ester	67 Ethyl 3-hydroxybutyrate_f7			
other	456 Phosphoric acid_P			

The intensity of the peaks in sake made from KOS was higher ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM. Identified compounds (ID No. Compound_detected ion, Table S1) at *yoikaori* only (I), at *high G* only (III), at *byakuya* only (V), and at common 3 koji starters (VII) are indicated. The detected ions are indicated as P, precursor/parent ion; fx, in-source fragment ion; more than one (No. of detected ions; kind of ions).

Table 8. The metabolites (ID No. Compound_detected ion) of the identified peaks in Figure. 3(b) (KOS < YAM, GOM).

Category	I	III	V	VII
nitrogen compound			224 L-Cysteine_P	
			232 L-Histidine_f1	
			237 L-Tryptophan_f2	
				228 L-Aspartic acid_(2; f1, f3)
				229 L-Lysine_P
				231 L-Methionine_(2; f1-2)
				236 L-Tyrosine_(5; P, f1-4)
				257 beta-Phenylethylamine_P
				258 Agmatine_P
dipeptide		533 Asp-Glu_P		
			484 Gly-Trp_P	
			496 Leu-Ala_P	
			513 Val-Val_f1	
			526 Val-Phe_f2	
			531 Ala-Thr_P	
			532 Leu-Lys_(2; f1, f3)	
				468 Ala-Leu_(3; P, f2-3)
				474 Leu-Val_P
				476 Gly-Ala_P
				483 Gly-Thr_f2
				490 Gly-Phe_f3
				502 Leu-Leu_P
				510 Pro-Arg_P
				511 Gly-Val_(2; P, f3)
				519 Gln-Tyr_P
				522 Ser-Val_P
				530 Pro-Gln_P
				536 Gln-Leu_f1
				552 Arg-Ala_P
				554 Arg-Gln_P
				566 Lys-Asn_(2; P, f2)
				567 Phe-Asp_P
carbohydrate		387 D-Ribose_f9		
organic acid	206 Pyroglutamic acid_P			
		190 Malic acid_(2; f3-4)		
		209 Succinic acid_(2; P, f3)		
				174 Citramalic acid_(4; P, f5-7)
phenol compound	54 Tyrosol_f1			
ester				67 Ethyl 3-hydroxybutyrate_(2; f5-6)
				414 N-(2-phenylethyl)acetamide_(2; P, f3)
vitamine				318 Inositol_f5

The intensity of the peaks in sake made from KOS was lower ($p < 0.05$) than that in sake made from the other 2 rice cultivars, YAM and GOM. Identified compounds (ID No. Compound_detected ion, Table S1) at *yoikaori* only (I), at *high G* only (III), at *byakuya* only (V) , and at common 3 koji starters (VII) are indicated. The detected ions are indicated as Table 1.

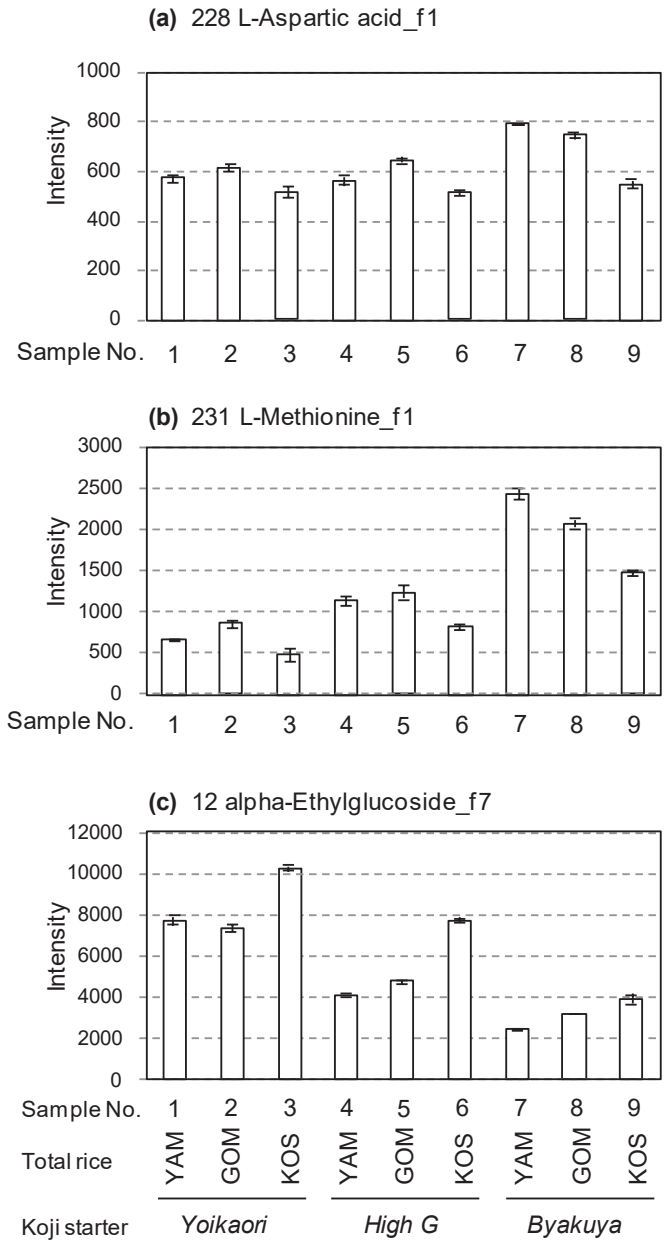


Fig. 8. Typical metabolites (ID No. Compound_detected ion) affected by *Koshitanrei.* Intensity of (a) L-Aspartic acid_f1 (RT, 1.2516; m/z, 116.0348), (b) L-Methionine_f1 (RT, 2.5127; m/z, 133.0299), and (c) alpha-Ethylglucoside_f7 (RT, 4.6532; m/z, 163.0589) are indicated.

Concluding remarks

The Japanese alcoholic beverage sake is made from rice and water by using 2 kinds of microorganisms, *A. oryzae* (called *koji*-fungi) and the yeast *S. cerevisiae*. The main raw material for sake brewing is the rice, and the characteristic features of rice are one of the most important factors determining the quality of the final product, sake. Therefore, the selection of the rice cultivar is one of the important concerns for brewing high-quality sake. Moreover, in sake brewing, rice-*koji* making is the other important processes, because rice-*koji* is a determinant of the aroma/ taste component of sake. Therefore, it is necessary to choose the proper *koji* starter for a rice cultivar, with taste designing of the sake in mind. In this study, I examined the suitability of sake rice *Koshitanrei* (KOS), developed in Niigata Prefecture, for the brewing of high-quality sake.

In Chapter I, to investigate the characteristic metabolites in sake from KOS, I performed 2 types of small-scale sake-fermentation tests for comparing KOS with its parental cultivars, YAM and GOM. These rice cultivars were examined as *kake*-rice (rice for *kake*-mai) by using the same YAM koji or total rice (both *kake-mai* and *koji*) at 2 polishing ratios (70% and 50%). Subsequent fermentation was carried out by using the same sake yeast strain and parameters, and then sake components were measured by the sake metabolome analysis method. As a result, in the *kake*-rice sake-making tests, the sake metabolomes of YAM and GOM were significantly affected by the difference in polishing ratio (70% or 50%), whereas that analysis of KOS was less affected, regardless of the progeny of these cultivars. The crude protein content of KOS was lower than that of the other rice cultivars. For that reason, especially the KOS sake made with the 70% polishing ratio had a lower amino acid content than that found for the YAM and GOM sakes. As a result of principal component analysis (PCA), many nitrogen compounds/dipeptides impacted the plot of the 70% YAM and GOM sakes. On the other hand, some other nitrogen compounds affected the 70% KOS sake plot. Thus KOS was the sake with the lowest amino acid content even at the low polishing ratio of 70%.

In the total rice sake-making tests, the sake metabolome for KOS, unlike that for the other 2 rice cultivars, was affected by the difference in the polishing ratio (70% or 50%). A comparison of “kake+koji-rice sake-making test” and “kake-rice sake-making test” suggested that for KOS rice, the effect of the polishing ratio was greater for the total rice KOS sake than that for the *kake*-rice. And, even in the kake+koji-rice sake-making test, the sake components of KOS sake showed characteristics different from those of YAM and

GOM sakes. The amino acid content of the KOS sake was also lower than that of the other rice cultivars at either polishing ratio. At the 70% polishing ratio, the *koji* from KOS indicated low proteolytic enzyme activity. But, at the 50% polishing ratio, the acid carboxypeptidase (ACP) activity of KOS *koji* was higher than that of the YAM *koji,* and the acid protease (AP) activity was similar to that of the YAM *koji.* Thus, KOS sake may be considered to have a lower amino acid content due to the lower crude protein content of the rice. In the "kake-rice sake-making test," the level of α-EG in the sake from 70% or 50% polished KOS was lower than that in the sake made from the other rice cultivars. In contrast, in the "kake+koji-rice sake-making test" at 70% and 50% polishing ratios, the α-EG content in the sake from KOS was higher than that from the other rice cultivars. Yazawa et al. reported that α-EG was negatively correlated with glucose. In the "*kake*-rice sake-making test," fermentation rates (CO_2 decrement) of KOS sake mash, and sake meter (Sm) and alcohol (Alc) of the KOS sake tended to be lower than those of the other 2 cultivars. On the other hand, in the "*kake*-+*koji* rice sake-making test," compared with that from the other rice cultivars, koji from KOS showed lower activity of 4 enzymes at the 70% polishing ratio and slightly lower glucoamylase activity at the 50% polishing ratio. And then, KOS sake mashes indicated fermentation rates (CO_2 decrement) intermediate between those of GOM and YAM sake mash. It may be considered that the enzyme activity of koji and the difference in fermentation process influenced the behavior of α-EG. In this study, the sake components from KOS were different from those of the parental cultivars. In KOS, *kake*-rice had little effect on the polishing ratio, and changing the total rice affected the polishing ratio. The data revealed that KOS had components that were higher or lower than those of the parent cultivars depending on whether it is used for the *kake*-rice only or for the total rice.

In Chapter II, to investigate the effect of the *koji* starter on the rice-*koji* characteristics and sake metabolites, I performed small-scale sake-making tests using the above 3 rice cultivars (KOS, YAM, and GOM) and 3 *koji* starters (*Yoikaori*, *High G*, and *Byakuya*). Based on the PCA analysis of the sake metabolites, KOS sake plotted at an area different from that of the other 2 rice cultivars in the case of all 3 *koji* starters. In addition, *koji* starters also affected the sake components of all 3 rice cultivars, but the effect was more significant for the parental cultivars. To reveal the difference in KOS sake metabolites in the entire *koji* starter, I focused on higher/lower peaks for the KOS sake sample compared with those peaks for sake samples from the other rice cultivars. The KOS-specific peaks could be

classified into 2 groups, one affected by the *koji* starter and the other, is unaffected by it. With either *koji* starter, the number of the lower peaks was about 7-fold over the number of the higher peaks, suggesting that some metabolites in the sake from KOS tended to be lower than those from the other rice cultivars.

In the classification based on the difference in *koji* starters, the number of lower peaks of KOS sake prepared with starter-B was larger than that for the other *koji* starters, indicating that the appearance or disappearance of sake metabolites depended on the kind of *koji* starter used conjugating with difference of rice cultivar. The α-EG content of KOS sake was higher than that for the other rice cultivars independent of the *koji* starter used, suggesting that the α-EG content of sake would be more affected by the rice cultivar. As in previous experiments, the levels of nitrogen compounds and dipeptides in sake from KOS were lower than those in the sake from the other rice cultivars. The ACP activity of KOS *koj*i was intermediate between the YAM and GOM *koji* activities, and the AP activity was almost the same as that of the GOM koji with all *koji* starters. Thus, the lower amino acid content of KOS sake was also due to the low crude protein content of KOS.

In this study, I investigated the suitability of sake rice KOS for high-quality sake brewing by evaluating the difference in sake metabolites between it and YAM and GOM and the effect of *koji* starters. The data clarified that the sake made from KOS possessed a quality and characteristics different from those of the parental cultivars, YAM and GOM, in terms of usage as *kake*-rice or *koji*-rice. In addition, by changing the *koji* starter, it became clear that characteristics of the KOS sake were still observed, but the *koji* starter was also observed to affect the sake components in a qualitative manner. Thus, it is necessary to select the *koji* starter according to the desired sake quality.

As the number of identified metabolites was low, it will thus be important to make an effort to identify the unknown peaks affected by KOS and *koji* starter.

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Related publications

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公表論文

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