Sphingolipid Properties in Sake Rice Cultivars and Changes During Polishing and Brewing

Shinji Yamashita¹, Chisato Higaki¹, Asuka Kanań¹, Nobuhiro Kikuchi², Daisuke Suzuki³, Mikio Kinoshita¹¼, and Teruo Miyazawa⁴

¹ Department of Life and Food Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, JAPAN
² Fukushima Technology Centre, Aizuakamatsus Technical Support Centre, Aizuakamatsu, Fukushima 965-0006, JAPAN
³ Suzuki Shuzouten Co., Ltd., 10 Higashimukai, Ukedo, Namie-town, Fukushima 979-1522, JAPAN
⁴ Food and Biotechnology Platform Promoting Project, New Industry Creation Hatchery Center (NICHe), Tohoku University, Sendai, Miyagi 980-8579, JAPAN

Abstract: Sphingolipids, including ceramide (Cer) and glucosylceramide (GlcCer), have the characteristic structural units called sphingoid bases, and are constituents of cell and vacuole membranes. Plant sphingolipids have highly diverse base structures and the base composition differs depending on the plant species. It is thought that the composition of sphingolipid classes and sphingoid bases is related to membrane fractions. However, there is little information about differences in sphingolipids among plant cultivars and the changes occurring in sphingolipids during food processing. This study investigated sphingolipids in sake rice (saka-mai) cultivars grown for sake (rice wine), and the changes in sphingolipids during polishing and brewing. In six brown rice samples, there were no large differences of the base composition among Cer or GlcCer of cultivars, whereas there were differences in their sphingolipid contents. When compared to brown rice, highly polished rice contained lower levels of sphingolipids, especially Cer. For three rice brans from different polishing steps, the Cer content was higher in the outer bran than in the inner bran. Sake and sake lees (sake-kasu) were produced by three different starter cultures (shubo preparations: the mixture of koji rice as an enzyme cocktail containing amylases, sake yeast, and adding rice as a carbohydrate source). The Cer/GlcCer ratio in sake and sake lees depended on the starter culture; Cer and GlcCer in sake lees possessed a fungi-specific base, 9-methyl-trans-4,trans-8-sphingadienine. In addition, sake lees had a higher Cer/GlcCer ratio when compared to highly polished rice as a sake source. These results suggest that the sphingolipid content of brown rice differs depending on the rice cultivar; further, the sphingolipids and the sphingolipid composition in sake and sake lees are affected by fungal sphingolipids and self-digestion during brewing.

Key words: bran, cerebroside, lees, rice, sake

1 Introduction

Complex sphingolipids are composed of a sphingoid base with an amide-linked fatty acid (i.e., ceramide, Cer) and a polar head group, such as a phosphocholine or a hexose. Sphingolipids are primarily found in the cell and vacuole membranes of most eukaryotes and some prokaryotes. The compositions of the classes and sphingoid bases are highly diverse in nature. Mammalian sphingolipid classes mainly consist of sphingomyelin, galactosylceramide, Cer, and ganglioside, and also contain glucosylceramide (GlcCer) at a relatively low level. In contrast, plant and fungal sphingolipid classes are mainly composed of GlcCer, Cer, inositol phosphoceramide (IPC), and glycosyl IPC (GIPC). In mammalian sphingolipids, trans-4-sphingenine (sphingosine, d18:1n9) is the most prevalent sphingoid base; whereas sphinganine (d18:0) and 4-hydroxyxypiphanine (phytosphingosine, t18:0) also occur frequently in smaller amounts. Plant sphingolipids have diverse sphingoid base structures such as trans-8-sphingenine (d18:1n9), cis-8-sphingenine (d18:1n9), trans-4, trans-8-sphingadienine (d18:2n9), trans-4, cis-8-sphingadienine (d18:2n9), 4-hydroxy-trans-8-sphingine (t18:1n9), and 4-hydroxy-cis-8-sphingine (t18:1n9). Specifically, 9-methyl-trans-4, trans-8-sphingadienine (9-Me d18:2n9) is a unique base

⁎Correspondence to: Mikio Kinoshita, Department of Life and Food, Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, JAPAN
E-mail: kinosita@obihiro.ac.jp
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found only in fungi.

Sphingolipids play important roles in various biological functions (e.g., apoptosis, autophagy, cellular differentiation, and cell proliferation) in animals, as well as plants. Plant sphingolipids have a highly diverse sphingoid base composition with Δ8-unsaturation as described above. GlcCer possesses different base compositions depending on the plant species, while Cer and GIPC are mainly composed of trihydroxy bases in all plants. In terms of the base composition in edible plant parts, the predominant base of GlcCer is d18:2sn in rice and maize, d18:2sn in soybeans, and d18:1sn in wheat and rye. Sphingolipids show different functions depending on the class, and sphingoid base species are thought to be used as selective items from the ceramide pool when synthesizing GlcCer and GIPC; for example, defects in Δ8-desaturase decrease plant GlcCer levels and their tolerance to cold. GIPC is reported to act as the receptor for cytotoxins produced by plant pathogenic bacteria and is necessary for sensing salt stress via Ca2+ influx. In addition, sphingoid base compositions of sphingolipids also affect membrane fluidity.

GlcCer has also been reported to have various nutritional functions, including alleviation of colon inflammation and improvement of skin moisture. Although monoglycosylceramides with different sphingoid base compositions and saccharides have been reported to show same levels of colon protection in vivo and in vitro, highly polar sphingolipids (i.e., IPC and GIPC) have a stronger beneficial effect in vitro in intestinal cells when compared to Cer and GlcCer. In contrast, sphingolipids are digested and absorbed as sphingoid bases and partly as ceramides, and sphingoid bases with different structures display different absorption ratios and exert different effects on the skin and adipocytes in vitro. Overall, it is important to understand the characteristics of food processing and the utilization of food functionality to clarify the composition of sphingolipid classes and sphingoid bases in plant cultivars and in products and byproducts during food processing.

Sake (rice wine) is a traditional Japanese alcoholic beverage, produced using highly polished sake rice and parallel multiple fermentation with koji (Aspergillus oryzae) and sake yeast (Saccharomyces cerevisiae) from koji (a koji maker, Kojiya Sanzaemon Co., Ltd., Japan); sake yeast from Iwaki-Kotobuki (Suzuki Shuzouten Co., Ltd., Japan); and 90% lactate for food from Showa Chemical Industry Co., Ltd., Japan. To indicate average grain large of each rice cultivar, 1,000 kernels except for immature green ones weighed. The water absorption velocity of sake rice is reported to be the highest in cultivars D, with velocities decreasing in the order D, E, F, A, C, and B. Assimilation of sake rice by amylases is one of the most important characteristics for sake brewing. When compared to assimilation of cultivar A, cultivar D is very easily assimilated, cultivars E and F are easily assimilated, and cultivars B and C indicate lower assimilation. Shimpaku (white core) is loose structure in the center of rice grain which makes easy for koji to grow in it. The size and incidence rate of shimpaku are decreased in the order D, E, F, A, C, and B.

Brown rice E was polished to 45% and prepared as koji rice (an enzyme cocktail containing amylases) by seeding koji (g/100 kg rice) onto steamed rice and culturing. Brown rice F was polished to 65%; the rice bran was divided into the three types (aka-nuka, naka-nuka, and shiro-nuka from the exterior) using a sake rice-polishing machine; the polished rice was used as koji rice and adding rice (kake-mai, a carbohydrate source). Sake and sake lees were produced by three starter cultures using the same materials (koji rice F, adding rice F, and sake yeast), while these starter cultures differed a lactate source, rice-grinding process, and its fermentation time. The soku-moto style is prepared by adding 90% lactate (700 mL/100 L water of starter culture), the ki-moto style is prepared by natural Lactobacillus, which is obtained from the air of the brewery by in-

2 Experimental Procedures

2.1 Sake rice and procedures for polishing and brewing

Six samples of sake rice (Oryza sativa cv. A–F) were obtained from a rice wholesaler, Japan; koji (Aspergillus oryzae) from a koji maker (Kojiya Sanzaemon Co., Ltd., Japan); sake yeast (Saccharomyces cerevisiae) from Iwaki-Kotobuki (Suzuki Shuzouten Co., Ltd., Japan); and 90% lactate for food from Showa Chemical Industry Co., Ltd., Japan. To indicate average grain large of each rice cultivar, 1,000 kernels except for immature green ones weighed. The water absorption velocity of sake rice is reported to be the highest in cultivars D, with velocities decreasing in the order D, E, F, A, C, and B. Assimilation of sake rice by amylases is one of the most important characteristics for sake brewing. When compared to assimilation of cultivar A, cultivar D is very easily assimilated, cultivars E and F are easily assimilated, and cultivars B and C indicate lower assimilation. Shimpaku (white core) is loose structure in the center of rice grain which makes easy for koji to grow in it. The size and incidence rate of shimpaku are decreased in the order D, E, F, A, C, and B.

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cubating the starter culture in low temperature for long-term, with a rice-grinding process in a wooden tub, and the yamahai-moto style is prepared by natural Lactobacillus without a grinding process. Sokujo-moto styles required brewing for 32 days as fermentation duration with growing of a starter culture for 8 days and conditioning (karashi) for 4 days, the ki-moto style had 25 days’ brewing with 29 days’ growing and 31 days’ conditioning, and the yamahai-moto style had 25 days’ brewing with 27 days’ growing and 26 days’ conditioning. After brewing, each fermentation mash (moromi) was divided to sake and sake lees using a filter press. The other conditions were described as follows. Total rice amount used of sokujo-moto, ki-moto, and yamahai-moto styles was 390 kg, 700 kg, and 700 kg, respectively. The ratio of koji rice to total rice for sokujo-moto, ki-moto, and yamahai-moto styles was 20.5%, 21.9%, and 21.9%, respectively. The ratio of water to rice (kuminizu-buai) for sokujo-moto, ki-moto, and yamahai-moto styles was 129.0%, 135.7%, and 135.7%, respectively. The ratio of sake lees to total rice (kasu-buai) for sokujo-moto, ki-moto, and yamahai-moto styles was 53.5%, 38.1%, and 35.9%, respectively; that for sokujo-moto was higher because of insufficient filtration caused by pump trouble. The water contents of lees in Table 3 contain alcohol due to calculation by heating and drying, and the ratio of dried sake lees to total rice was 14.3%, 14.4%, and 13.1%, respectively. The alcohol concentration of sake was 17.2%, 16.2%, and 16.2% in sokujo-moto, ki-moto, and yamahai-moto, respectively. The final sake volume produced from 100 kg total rice was 164.1 L, 206.1 L, and 229.4 L, respectively.

2.2 Lipid analysis

GlcCer and Cer in samples were separated using thin-layer chromatography (TLC), and their sphingoid bases were analyzed using a slight modification of previous method. Briefly, crude lipids were extracted using a chloroform/methanol/water solvent system and the weights were considered as total lipid amounts. The crude lipids were saponified with 0.4 M KOH in methanol at 38°C for 2 h to obtain the alkali-stable lipids (e.g., sphingolipids and sterols). GlcCer and Cer were separated by TLC. The isolated sphingolipids were hydrolyzed with or without d18:0 (Cayman Chemical, Michigan, USA) as the internal standard (IS) using aq. methanolic 1 M HCl at 70°C for 18 h. The reaction mixture was washed with hexane and adjust-

| Table 1 | Lipid profiles in brown rice of 6 sake rice cultivars. |
|---------|---------------------------------------------------------|
|         | TKW (g) | Water (%) | Total Lipid | GlcCer | Cer | Cer/GlcCer | Total FA | Saturated FA% | n-6/n-3 |
| Brown rice A | 27.1 | 14.2 | 2.3 | 4033 | 1881 | 2.0 | 9517 | 28.9 | 27.1 |
| Brown rice B | 27.7 | 14.6 | 2.9 | 3332 | 2445 | 1.4 | 10520 | 29.0 | 28.0 |
| Brown rice C | 29.7 | 14.6 | 3.0 | 2929 | 2185 | 1.3 | 11942 | 27.1 | 29.6 |
| Brown rice D | 25.5 | 14.3 | 2.7 | 4217 | 1614 | 2.6 | 11083 | 26.9 | 29.3 |
| Brown rice E | 24.9 | 14.1 | 2.6 | 2413 | 1181 | 2.0 | 10042 | 27.9 | 28.7 |
| Brown rice F | 26.6 | 12.6 | 2.5 | 4003 | 1342 | 3.0 | 9362 | 29.0 | 22.8 |

*Because rice E was not enough amount for analysis of the water content, the average of the water contents in rice A, B, C, D, and F was used for rice E.

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|         | t18:0 | t18:1<sup>9</sup> | t18:1<sup>9</sup> | t20:0 | d18:0 | d18:1<sup>9</sup> | d18:1<sup>9</sup> | d18:2<sup>9</sup> | d18:2<sup>9</sup> | 9Me-d18:2<sup>9</sup> |
|---------|-------|-----------------|-----------------|-------|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cer     |       |                 |                 |       |       |                 |                 |                 |                 |                 |
| Brown rice A | 81.3 | 3.7 | 9.0 | n.d. | 2.8 | n.d. | n.d. | n.d. | 1.7 | 1.5 | n.d. |
| Brown rice B | 78.5 | 4.6 | 10.1 | n.d. | 3.0 | n.d. | n.d. | n.d. | 1.3 | 2.4 | n.d. |
| Brown rice C | 83.9 | 3.1 | 6.8 | n.d. | 1.8 | n.d. | n.d. | n.d. | 1.1 | 3.2 | n.d. |
| Brown rice D | 83.7 | 3.4 | 6.3 | n.d. | 2.1 | n.d. | n.d. | n.d. | 1.1 | 3.3 | n.d. |
| Brown rice E | 81.1 | 3.6 | 6.3 | n.d. | 2.5 | n.d. | n.d. | n.d. | 2.6 | 3.8 | n.d. |
| Brown rice F | 79.8 | 4.8 | 10.0 | n.d. | 2.9 | n.d. | n.d. | n.d. | 0.3 | 2.3 | n.d. |

| GlcCer  |       |                 |                 |       |       |                 |                 |                 |                 |                 |
|---------|-------|-----------------|-----------------|-------|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Brown rice A | 6.1 | 2.9 | 24.5 | n.d. | 1.0 | 0.2 | 0.8 | 1.4 | 15.8 | 47.3 | n.d. |
| Brown rice B | 5.4 | 3.1 | 33.7 | n.d. | 1.7 | 0.2 | 0.7 | 0.6 | 12.0 | 42.6 | n.d. |
| Brown rice C | 5.1 | 2.6 | 25.6 | n.d. | 1.7 | 0.2 | 0.7 | 1.3 | 16.4 | 46.4 | n.d. |
| Brown rice D | 4.1 | 1.7 | 19.3 | n.d. | 1.7 | 0.1 | 0.4 | 1.0 | 16.5 | 55.1 | n.d. |
| Brown rice E | 6.2 | 3.0 | 28.2 | n.d. | 2.5 | 0.1 | 0.2 | 1.4 | 14.1 | 44.3 | n.d. |
| Brown rice F | 3.9 | 1.4 | 11.2 | n.d. | 3.5 | n.d. | 0.3 | 5.1 | 18.1 | 56.4 | n.d. |

(a) indicates TKW and contents of water and lipids in samples; (b) indicates sphingoid base composition of Cer and GlcCer in samples. Cer, ceramide; FA, fatty acid; GlcCer, glucosylceramide; n.d., not detected; TKW, thousand kernel weight.
ed to a pH of more than 9 with 6 M KOH. The liberated sphingoid base component was extracted with diethyl ether and converted to fatty aldehydes using NaIO4 oxidation. The resultant fatty aldehydes were analyzed using gas chromatography (GC)–mass spectrometry (MS). The GC-MS system was equipped with GC-2030 and GCMS-QP2020NX instrument (Shimadzu, Kyoto, Japan). The peak area ratio of fatty aldehydes was calculated by adjusting the main peak ratio to 1.0, and the ratio of aldehyde from d18:0 without IS was subtracted from that in the same sample with IS. The sphingolipids were quantified based on the peak area difference as added IS amount. In terms of fatty acid analysis, without extraction, the composition in sake was determined by GC-MS after direct ethylation using heptadecanate as IS and those in other samples were by direct methylation25. Briefly, sample with or without IS was reacted in ethanol or methanol containing acetyl chloride at 100°C. After mixing with hexane and water, the upper layer was washed with 2% KHCO3 and analyzed.

3 Results
3.1 Sphingolipid levels and sphingoid base composition in the six sake rice cultivars
Table 1a shows the amounts of Cer and GlcCer in the six brown rice samples. The Cer content was the highest in rice D, with levels decreasing in the order D, A, F, B, C, and E, whereas the GlcCer content was highest in rice B, decreasing in the order B, C, A, D, F, and E. In addition, the Cer/GlcCer ratio decreased in the order rice F, D, E, A, B, and C. The highest Cer and GlcCer levels were approximately double the lowest. In contrast, the total content, saturated ratio, and n-6/n-3 ratio of fatty acids (FA) were similar among cultivars. The 1,000 kernel weight (TKW) of sake rice was decreased in the order rice C, B, A, F, D, and E.

Table 1b shows the sphingolipid base composition of Cer and GlcCer in the six brown rice samples. The base composition of Cer and GlcCer in brown rice is almost the same in all the cultivars; the predominant base in Cer is t18:0, and the primary base in GlcCer was d18:2ω6c.

3.2 Distribution of sphingolipids during the polishing process
We compared sphingolipids in brown rice, highly polished rice, and three types of rice bran (aka-nuka, naka-nuka, and shiro-nuka from the exterior) of rice F (Table 2). The Cer content was highest in aka-nuka, decreasing in the order aka-nuka, naka-nuka, shiro-nuka, brown rice, and highly polished rice, whereas the GlcCer content decreased in the order naka-nuka, aka-nuka, shiro-nuka, brown rice, and highly polished rice.

Table 2 Comparison among lipid profiles of sake rice F during polishing process.

|          | Water (%) | Total lipid1 | Cer2 | GlcCer2 | Cer/GlcCer | Total FA3 | Saturated FA% | n-6/n-3 |
|----------|-----------|--------------|------|---------|------------|-----------|---------------|---------|
| Brown rice | 12.6      | 2.5          | 4003 | 1342    | 3.0        | 9362      | 29.0          | 22.8    |
| Highly polished rice | 11.2 | 0.2 | 1218 | 1135    | 1.1 | 2597 | 48.6 | 51.1 |
| Aka-nuka | 11.6      | 18.3         | 29050 | 3283    | 8.8        | 51669     | 23.5          | 27.3    |
| Naka-nuka | 12.3     | 9.7          | 9755 | 7123    | 1.4        | 27797     | 23.7          | 31.3    |
| Shiro-nuka | 11.8      | 3.0          | 7269 | 1903    | 3.8        | 11423     | 27.4          | 38.7    |

![Image](image.png)

(a) indicates contents of water and lipids in samples; (b) indicates sphingoid base composition of Cer and GlcCer in samples. Cer, ceramide; FA, fatty acid; GlcCer, glucosylceramide; n.d., not detected.

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and highly polished rice. The Cer/GlcCer ratio was highest in aka-nuka, decreasing in the order aka-nuka, shiro-nuka, brown rice, naka-nuka, and highly polished rice. The ratio of Cer and GlcCer to total lipids was highest in the highly polished rice among the rice and bran samples. Figure 1 also shows that the GlcCer to total lipids ratio was the highest in highly polished rice, but the Cer to total lipids ratio does not reflect GC-MS data because the Cer fractions contained other components. In terms of sphingoid base composition, Cer in rice and bran were predominantly composed of t18:0, whereas GlcCer in those were d18:2\(^{a,b,0}\).

The polishing process increased the proportion of d18:2\(^{a,b,0}\) in Cer and that of t18:1\(^{8,0}\) in GlcCer.

### 3.3 Changes in sphingolipids during brewing

We investigated how rice sphingolipids were affected by fermentation using three kinds of starter cultures (Table 3). When compared to highly polished rice F, sake lees contained markedly higher levels of Cer and GlcCer. Among sake lees, Cer and GlcCer contents were highest in ki-moto, decreasing in yamahai-moto, and lower still in sokujo-moto. In particular, the Cer content was significantly higher in ki-moto and yamahai-moto than in sokujo-moto. The ratio of GlcCer to total lipids was about 2 times lower in sake lees compared to highly polished rice, and the ratio of Cer to total lipids was about 2 times higher in sake lees compared to that in highly polished rice. Figure 1 also reflects the ratio of GlcCer to total lipids, but the ratio of Cer to total lipids does not reflect GC-MS data because the Cer fractions contained other components. In addition, we found Cer and GlcCer in sake at low levels; notably, the Cer/GlcCer ratio in sake was highest in yamahai-moto, ki-moto, and sokujo-moto.

In terms of sphingoid base composition, when compared to highly polished rice, all the three sake lees and the three types of sake had much higher ratio of d18:0 in Cer and GlcCer. Notably, sake lees contained GlcCer bearing the fungi-specific base 9-Me d18:2\(^{a,b,0}\), but sake did not. GlcCer in sake lees had higher ratio of 9-Me d18:2\(^{a,b,0}\) in ki-moto and yamahai-moto than sokujo-moto and Cer in sake lees had a small ratio. As rice F was limited, we compared the highly polished rice and koji rice prepared from rice E (Table 4). GlcCer in koji rice had markedly ratio of the

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**Fig. 1** Profiles of alkali-stable lipids in brown rice, highly polished rice, rice bran, and sake lees from sake rice E on silica TLC.

To remove lipid components except for alkali-stable lipids (sphingolipids and sterols), extracted lipids were treated with an alkali. Alkali-treated samples were adjusted to the same lipid levels before treatment and subjected to TLC: 1, GlcCer standard; 2, brown rice; 3, highly polished rice; 4, aka-nuka; 5, naka-nuka; 6, shiro-nuka; 7, sake lees using sokujo-moto; 8, sake lees using ki-moto; 9, sake lees using yamahai-moto. Mobile phase, chloroform:methanol (95:12). Detection, 50% sulfate.

ASG, acylsterylglucoside; Cer, ceramide; FAME, fatty acid methylester; GlcCer, glucosylceramide; SG, sterylglucoside; TLC, thin-layer chromatography.
fungi-specific base 9-Me d18:2 \( t^4 \) and Cer in koji rice had a small ratio, whereas it could not be detected in Cer and GlcCer in highly polished rice. In addition, Cer and GlcCer in koji rice contained t20:0, which was not observed in highly polished rice.

Level of Cer and GlcCer is respectively 1,082 nmol (740 \( \mu \)g at t18:0/24h:0 species) and 1,008 nmol (776 \( \mu \)g as d18:2/20h:0 species) in total rice when total rice is converted to sphingolipid contents of highly polished rice without koji (Aspergillus oryzae) (Table 3). On the other hand, GlcCer level is respectively 1,318 nmol, 1,339 nmol, and 1,339 nmol in 100 g of total rice of sokujo-moto, ki-moto, and yamahai-moto styles when the sphingoid base of koji GlcCer is hypothesized to be mostly 9-Me d18:2 \( t^4 \) (Table 4). In terms of sake lees generated from 100 g of total rice, Cer level is 6,084 nmol, 11,316 nmol, and 9,789 nmol in sake lees of sokujo-moto, ki-moto, and yamahai-moto styles, respectively. GlcCer level in sake lees is 1,825 nmol, 2,535 nmol, and 2,024 nmol, respectively. In terms of sake generated from 100 g of total rice, Cer level is 1.6 nmol, 2.4 nmol, and 3.0 nmol in sake of sokujo-moto, ki-moto, and yamahai-moto styles, respectively. GlcCer level is 3.3 nmol, 1.6 nmol, and 0.9 nmol in sake of sokujo-moto, ki-moto, and yamahai-moto styles, respectively. When compared to highly polished rice used, Cer level in sake lees of sokujo-moto, ki-moto, and yama-

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**Table 3** Comparison among lipid profiles of sake rice F during brewing process.

|                      | Water (%) | Total lipid \(^1\) | Cer \(^2\) | GlcCer \(^2\) | Cer/GlcCer | Total FA \(^3\) | Saturated FA% | n-6/n-3 |
|---------------------|-----------|--------------------|-----------|--------------|------------|----------------|----------------|---------|
| Highly polished rice| 11.2      | 0.25               | 1218.3    | 1135.5       | 1.1        | 2596.7         | 48.6           | 51.1    |
| Sokujo-moto sake lees| 73.3      | 4.48               | 42548.3   | 12765.3      | 3.3        | 15345.5        | 51.1           | 39.8    |
| Ki-moto sake lees   | 62.2      | 5.95               | 78583.0   | 17607.1      | 4.5        | 24825.1        | 49.4           | 31.5    |
| Yamahai-moto sake lees | 63.6   | 6.30               | 74727.8   | 15447.3      | 4.8        | 32224.2        | 48.2           | 30.5    |
| Sokujo-moto sake*   | -         | 0.05               | 1.0       | 2.0          | 0.5        | 2.8            | 54.3           | n.d.    |
| Ki-moto sake*       | -         | 0.05               | 1.2       | 0.8          | 1.5        | 1.7            | 55.0           | n.d.    |
| Yamahai-moto sake*  | -         | 0.05               | 1.3       | 0.4          | 3.4        | 1.5            | 58.8           | n.d.    |

\(^1\)g/100 g dry wt., \(^2\)nmol/100 g dry wt., \(^3\)umol/100 g dry wt.

*As \(^{1,2,3}\) units of sake, 100 mL is used instead of 100 g dry wt.

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\( t^{18:0} \), \( t^{18:1t^4} \), \( t^{18:1t^8} \), \( t^{20:0} \), \( d^{18:0} \), \( d^{18:1t^4} \), \( d^{18:1t^8} \), \( d^{18:2t^{20}} \), \( d^{18:2t^{26}} \), \( d^{18:2t^{32}} \), \( 9Me-d^{18:2t^{26}} \), \( 9Me-d^{18:2t^{32}} \).
hai-moto styles is 5.6 times, 10.5 times, and 9.0 times, respectively, whereas GlcCer level in those is 1.8 times, 2.5 times, and 2.0 times, respectively.

### 4 Discussion

Sphingolipids display diverse classes and sphingoid base composition, and they are currently receiving a lot of attention owing to their potential as biomarkers and drug targets because of changes in their levels and composition in diseases and mutant strains of animals and plants. Although it is well known that edible parts of plants possess different sphingoid base compositions, dependent on the plant species, it is not well established whether the diversity of sphingolipids may affect the characteristics of food processing in plant cultivars. In this study, to obtain the basic data, we investigated differences in sphingolipids in six cultivars of sake rice, and changes in sphingolipids in sake rice during polishing and brewing. The levels of Cer and GlcCer differed among the six brown rice cultivars, whereas the sphingoid base composition was nearly the same (Table 1). Comparing among sake rice before and after polishing and rice bran as the byproduct, brown rice had higher levels of Cer and GlcCer than highly polished rice because sphingolipids were abundant in rice bran, whereas highly polished rice had the highest sphingolipid levels per total lipids (Table 2 and Fig. 1). Sake had low sphingolipid contents; sake lees contained markedly larger amounts, and these levels were dependent on the starter culture (Table 3).

Sphingolipid levels varied markedly more depending on the sake rice cultivars than levels of total lipids and total FA, and were not related to TKW. It is important for brewing that sake rice possesses highly starch utilization, and it is reported that the utilization is concerned with the branch chain length of amylopectin. Sake rice cultivars D, E, and F, which contained lower GlcCer levels, were known to be easily assimilated by amylases during brewing, whereas sake rice A, B, and C, which contained higher GlcCer levels, were not.

Because GlcCer in the membrane indicates stress resistance in plants, GlcCer levels may also be related to reactivity with extrinsic enzymes by membrane strength and may affect cultivar-specific characteristics of food processing. In terms of sphingoid base composition among sake rice and bran, Cer and GlcCer was predominantly composed of t18:0 and d18:2(4,8)t,8,c, respectively (Table 2b). It has been reported that Cer and GlcCer in eating rice bran, not sake rice bran, have the same predominant bases; therefore, we think that there are not significant differences in sphingoid base composition among rice cultivars; in other words, sphingoid base composition is not related to the characteristics of food processing. In this study, the samples analyzed were harvested in a single year and comparison with samples harvested in different years is necessary to confirm this hypothesis.

Rice bran was found to be rich in sphingolipids, and the Cer level was higher in the exterior bran, whereas non-viable organs such as dead leaves are known to have high levels of Cer. In this study, GIPC, one of the main plant sphingolipids, was not analyzed, but sake rice, in particular aka-nuka, was found to contain a markedly high ratio of Cer to GlcCer, which is another main plant sphingolipid. It was thought that rice grains as a plant dormant period have a high Cer content, similar to wheat grains, and aka-nuka, which is mainly composed of pericarp and tegmen, has a much higher Cer content. Naka-nuka is composed of a bran layer and starchy endosperm; therefore, naka-nuka contains higher levels of aleurone and sub-aleurone layers, which show

### Table 4 Comparison between sphingoid base composition of highly polished rice and koji rice from sake rice E.

|        | t18:0 | t18:1t | t18:1c | t20:0 | d18:0 | d18:1t | d18:1c | d18:2t | d18:2c | d18:2t,t | 9Me-d18:2t,t,c |
|--------|-------|--------|--------|-------|-------|--------|--------|--------|--------|----------|-----------------|
| Cer    |       |        |        |       |       |        |        |        |        |         |                 |
| Highly polished rice | 69.2  | 2.4    | 4.4    | n.d.  | 7.8   | n.d.   | n.d.   | 2.9    | 3.9    | 9.4      | n.d.           |
| Koji rice | 71.5  | 1.6    | 2.5    | 6.7   | 9.4   | n.d.   | n.d.   | 0.7    | 3.0    | 3.8      | 0.6            |
| GlcCer |       |        |        |       |       |        |        |        |        |         |                 |
| Highly polished rice | 7.0   | 1.6    | 8.1    | n.d.  | 11.6  | 0.3    | 0.7    | 13.6   | 18.8   | 38.4     | n.d.           |
| Koji rice | 6.1   | 0.3    | 2.6    | 0.7   | 1.3   | 0.2    | 0.3    | 6.5    | 7.8    | 12.3     | 61.9           |

Cer, ceramide; FA, fatty acid; GlcCer, glucosylceramide; n.d., not detected.

### Table 2a Comparison between sphingoid base composition of highly polished rice and koji rice from sake rice E.
strong vacuolar precipitates, than aka-nuka. This may be why naka-nuka had the highest GlcCer content among the three rice brans.

When compared to highly polished rice as a sake source, sake lees had higher levels of total lipids and sphingolipids and a higher ratio of Cer/GlcCer depending on the starter culture; sphingolipids in sake lees had the fungi-specific base 9-Me d18:2\(^{t,8}\) and an increased d18:0 ratio (Table 3). The predominant base of GlcCer in koji rice was 9-Me d18:2\(^{t,8}\) and Cer in koji rice had the same ratio of 9-Me d18:2\(^{t,8}\) as Cer in sake lees prepared by yamahai-moto (Table 4). It is known that Lactobacillus does not have sphingolipids, sake yeast does not have GlcCer, and the predominant base of GlcCer in koji is a fungi-specific base\(^{20}\). The ratio of koji rice to total rice was about 20%. According to the calculation, the 9-Me d18:2\(^{t,8}\) ratio in GlcCer of total rice is about 13 mol% because of about 60 mol% in koji rice and nearly the same as the ratio of that in sake lees. Overall, during brewing, microbes consumed the carbohydrate in highly polished rice and concentrated the lipids. The increased Cer/GlcCer ratio was due to accumulation of Cer from sake yeast and self-digestion of GlcCer to Cer. Cer levels and Cer/GlcCer ratios were higher in ki-moto and yamahai-moto sake lees, which are fermented by Lactobacillus, than in sokujo-moto sake lees. The process of starter culture, especially ki-moto and yamahai-moto, is known to increase the survival ratio of sake yeast during conditioning of a starter culture. Sake yeast is reported to enhance its tolerance to alkaline conditions due to translation from GlcCer in koji to sake yeast during starter culture\(^{20}\); therefore, Lactobacillus without sphingolipids may also be related to the formation of networks among microbes and may increase the amount of sake yeast and Cer from sake yeast in ki-moto and yamahai-moto compared to sokujo-moto.

In terms of the increase in the d18:0 content of sphingolipids in sake lees, the saturated FA ratio of sake lees was almost the same as that of highly polished rice. The mechanism can be thought as follows: 1) the microbe networks or the stress of ethanol and press during brewing affect to change biosynthesis of sphingoid bases; 2) they affect to modify already existing bases (tri- and dihydroxy bases and unsaturated bases); and the possibility of later is higher. Because sphingoid bases are generated from d18:0\(^{29}\), the base synthesis may stop on d18:0 stage. However, only sake yeast without GlcCer biosynthesis performed the biological activity during brewing because rice was steamed and brewing is anaerobic fermentation in which koji cannot act. The rate of d18:0 in Cer and GlcCer of sake lees was nearly among the starter cultures, whereas the increase rate of Cer and GlcCer in sake lees markedly varied depending on the starter culture. Overall, highly polished rice may undergo sphingolipid-specific reaction (e.g., dehydration and hydrogenation) during brewing.

Sphingolipid levels in sake were low and did not contain 9-Me d18:2\(^{t,8}\); the Cer/GlcCer ratio depended on the starter culture (Table 3). The alcohol concentration of sake before addition of water (warimizu), which is performed with the aim of adjusting to favorite flavor and taste after filtration, was 17.2%, 18.0%, and 17.8% in sokujo-moto, ki-moto, and yamahai-moto, respectively. Sphingolipids, especially Cer, are not easily extracted by low alcohol concentrations of about 18%, and it is thought that GlcCer was extracted in small amounts and digested to Cer; and GlcCer bearing 9-Me d18:2\(^{t,8}\) was not extracted due to its low polarity. Different ratios of Cer/GlcCer in sake may be affected by enzyme activity, length of conditioning, and whether or not the rice is ground, depending on the starter culture. Sphingolipids in plant cells cannot easily exert nutritional functions when consumed, although extracted sphingolipids show nutritional functions at low intake levels\(^{31}\). Thus, although sphingolipids in sake are present at low levels, an appropriate dose of sake may contribute to health maintenance because of the extracted sphingolipids.

In this study, it was clarified that byproducts during polishing and brewing have various sphingolipid contents, sphingoid base compositions, Cer/GlcCer ratios, and sphingolipid purities in total lipids. GlcCer is digested to Cer and sphingoid base and absorbed as sphingoid base and partly Cer into lymph\(^{31}\); therefore, Cer is expected to have a higher level of absorption than GlcCer. In contrast, it has been reported that GlcCer bearing 9-Me d18:2\(^{t,8}\) is difficult to digest when compared to that bearing other bases, and most of the intake of GlcCer can reach the colon to exert nutritional functions such as improvement of intestinal bacterial flora\(^3\). These data may allow the selection of the appropriate sphingolipid sources for specific applications.

In conclusion, sphingolipid levels in sake rice may be related to the characteristics of food processing. In addition, we can obtain basic data for changing the composition of sphingolipids and the sphingoid bases during polishing and brewing.

**Conflict of Interest**

The authors have no conflicts of interest to declare.

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References

1) Yamashita, S.; Kikuchi, N.; Kinoshita, M.; Miyazawa, T. Chemical properties and nutritional value of plant-origin glucosylceramide. J. Nutr. Sci. Vitaminol. (Tokyo) 65, S153-S157 (2019).
2) Michaelson, L.V.; Napier, J.A.; Molino, D.; Faure, J.D. Plant sphingolipids: Their importance in cellular organization and adaption. BBA-Mol. Cell Biol. Lipids 1861, 1329-1335 (2016).
3) Ogretmen, B. Sphingolipid metabolism in cancer signalling and therapy. Nat. Rev. Cancer 18, 33-50 (2018).
4) Miyazawa, T.; Ito, S.; Fujino, Y. Isolation of cerebrosides from pea seeds. Agr. Biol. Chem. 38, 1387-1391 (1974).
5) Aida, K.; Takakuwa, N.; Kinoshita, M.; Sugawara, T.; Imai, H.; Ono, J.; Ohnishi, M. Properties and physiological effects of plant cerebrosides species as functional lipids. in Advanced Research on Plant Lipids (Murata, N. ed.), Kluwer Academic Publishers, Netherlands. pp. 233-236 (2003).
6) Chen, M.; Han, G.; Dietrich, C.R.; Dunn, T.M.; Cahoo, E.B. The essential nature of sphingolipids in plants as revealed by the functional identification and characterization of the Arabidopsis LCB1 subunit of serine palmitoyltransferase. Plant Cell 18, 3576-3593 (2006).
7) Lenarčič, T.; Albert, I.; Bohm, H.; Hodnik, V.; Pirc, K.; Zavec, A.B.; Podobnik, M.; Pahovnik, D.; Žagar, E.; Pruitt, R.; Greimel, P.; Yamaji-Hasegawa, A.; Kobayashi, T.; Zienkiewicz, A.; Gömann, J.; Mortimer, J.C.; Fang, L.; Mamode-Cassim, A.; Deleu, M.; Lins, L.; Oecking, C.; Feussner, I.; Mongrand, S.; Anderluh, G.; Nürnberger, T. Eudicot plant-specific sphingolipid metabolism determined host selectivity of microbial NLP cytolysins. Science 358, 1431-1434 (2017).
8) Jiang, Z.; Zhou, X.; Tao, M.; Yuan, F.; Liu, L.; Wu, F.; Wu, X.; Xiang, Y.; Niu, Y.; Liu, F.; Li, C.; Ye, R.; Byeon, B.; Xue, Y.; Zhao, H.; Wang, H.N.; Crawford, B.M.; Johnson, D.M.; Hu, C.; Pei, C.; Zhou, W.; Swift, G.B.; Zhang, H.; Vo-Dinh, T.; Hu, Z.; Siedow, J.N.; Pei, Z.M. Plant cell-surface GPIC sphingolipids sense salt to trigger Ca2+ influx. Nature 572, 341-346 (2019).
9) Takakuwa, N.; Tanji, M.; Oda, Y.; Ohnishi, M. Distribution of 9-methyl sphingoid base in mushrooms and its effects on the fluidity of phospholipid liposomes. J. Oleo Sci. 51, 741-747 (2002).
10) Yamashita S.; Sakurai R.; Hishiki K.; Aida K.; Kinoshita M. Effects of dietary plant-origin glucosylceramide on colon cytokine contents in DMH-treated mice. J. Oleo Sci. 66, 157-160 (2017).
11) Uchiyama, T.; Nakano, Y.; Ueda, O. Mori, H.; Nakashima, M.; Noda, A.; Ishizaki, C.; Mizoguchi, M. Oral intake of glucosylceramide improves relatively higher level of transepidermal water loss in mice and healthy human subjects. J. Health Sci. 54, 559-566 (2008).
12) Aida, K.; Kinoshita, M.; Tanji, M.; Sugawara, T.; Tamura, M.; Ono, J.; Ueno, N.; Ohnishi, M. Prevention of aberrant crypt foci formation by dietary maize and yeast cerebrosides in 1,2-dimethylhydrazine-treated mice. J. Oleo Sci. 54, 45-49 (2005).
13) Yamashita, S.; Seino, T.; Aida, K.; Kinoshita, M. Effects of plant sphingolipids on inflammatory stress in differentiated Caco-2 cells. J. Oleo Sci. 66, 1337-1342 (2017).
14) Yamashita, S.; Seino, T.; Inobe, M.; Jutanom, M.; Matsumoto, S.; Kinoshita, M. Polar lipid fraction from golden oyster mushrooms (Pleurotus citrinopileatus) suppresses colon injuries from inflammatory stresses in vivo and in vitro. J. Oleo Sci. 69, 751-757 (2020).
15) Jutanom, M.; Higaki, C.; Yamashita, S.; Nakagawa, K.; Matsumoto, S.; Kinoshita, M. Effects of sphingolipid fractions from golden oyster mushroom (Pleurotus citrinopileatus) on apoptosis induced by inflammatory stress in an intestinal tract in vitro model. J. Oleo Sci. 69, 1087-1093 (2020).
16) Fujii, A.; Manabe, Y.; Aida, K.; Tsuduki, T.; Hirata, T.; Sugawara, T. Selective absorption of dietary sphingoid bases from the intestine via efflux by P-glycoprotein in rats. J. Nutr. Sci. Vitaminol. (Tokyo) 63, 44-50 (2017).
17) Shirakura, Y.; Kikuchi, K.; Matsumura, K.; Mukai, K.; Mitsutake, S.; Igarashi, Y. 4,8-Sphingadieninedine and 4-hydroxy-8-sphingenine activate ceramide production in the skin. Lipids Health Dis. 11, 108 (2012).
18) Esaki, S.; Nagasawa, T.; Tanaka, H.; Tominaga, A.; Mikami, D.; Usuki, S.; Hamajima, H.; Hanamatsu, H.; Sakai, S.; Hama, Y.; Igarashi, Y.; Kitagaki, H.; Mitsutake, S. The fungal 9-methyl-sphingadiene is a novel ligand for both PPARy and GPR120. J. Food Biochem. 42, e12624 (2018).
19) The story of sake (National Research Institute of Brewing ed.) [Internet]. Hiroshima, Japan: National Research Institute of Brewing. https://www.nrib.go.jp/English/sake/pdf/SakeNo01_en.pdf.
20) A comprehensive guide to Japanese sake/Japan Sake and Shochu Markers Association and National Research Institute of Brewing ed.) [Internet]. Tokyo, Japan: Japan Sake and Shochu Markers Association. https://www.nrib.go.jp/English/sake/pdf/guidesse01.pdf.
21) Takahashi, K.; Izumi, K.; Nakahata, E.; Hirata, M.; Sawada, K.; Tsuge, K.; Nagao, K.; Kitagaki, H. Quantitation and structural determination of glucosylceramides contained in sake lees. J. Oleo Sci. 63, 15-23 (2014).
22) Yamashita, S.; Hata, M.; Kikuchi, N.; Kinoshita, M.; Miyazawa, T. Effects of dietary ethanol extracts from Sphingolipids of Sake Rice, Sake Lees, and Sake J. Oleo Sci. 70, (2) 203-212 (2021)
sake rice and sake lees on intestinal impairment in mice. J. Oleo Sci. 69, 929-939 (2020).
23) Shuzo-yo genryo-mai zenkokutoitsu bunseki kekka (Saka-mai kenkyukai ed.) [Internet]. Saka-mai kenkyukai. http://www.sakamai.jp/. (in Japanese)
24) Imai, H.; Ohnishi, M.; Hotsubo, K.; Kojima, M.; Ito, S. Sphingoid base composition of cerebrosides from plant leaves. Biosci. Biotechnol. Biochem. 61, 351-353 (1996).
25) Yamashita, S.; Shimada, K.; Sakurai, R.; Yasuda, N.; Oikawa, N.; Kamiyoshihara, R.; Otoki, Y.; Nakagawa, K.; Miyazawa, T.; Kinoshita, M. Decrease in intramuscular levels of phosphatidylethanolamine bearing arachidonic acid during postmortem aging depends on meat cuts and breed. Eur. J. Lipid Sci. Technol. 121, 1800370 (2019).
26) Okuda, M. Structural and retrogradation properties of rice endosperm starch and sake making properties of rice grain used in sake production. Bull. Appl. Glycosci. 4, 193-201 (2014). (in Japanese)
27) Fujino, Y.; Ohnishi, M. Constituents of ceramide and ceramide monohexoside in rice bran. Chem. Phys. Lipids 17, 275-289 (1976).
28) Fujino, Y.; Ohnishi, M. Sphingolipids in wheat grain. J. Cereal Sci. 1, 159-168 (1983).
29) Fujino, Y.; Ohnishi, M. Structure of cerebroside in Aspergillus oryzae. Biochim. Biophys. Acta 486, 161-171 (1976).
30) Sawada, K.; Sato, T.; Hanajima, H.; Jayakody, L.N.; Hirata, M.; Yamashiro, M.; Tajima, M.; Mitsutake, S.; Nagao, K.; Tsuge, K.; Abe, F.; Hanada, K.; Kitagaki, H. Glucosylceramide contained in koji mold-cultured cereal confers membrane and flavor modification and stress tolerance to Saccharomyces cerevisiae during coculture fermentation. Appl. Environ. Microbiol. 81, 3688-3698 (2015).
31) Yamashita, S.; Yamamoto, M.; Hiraoka, K.; Kikuchi, N.; Kinoshita, M.; Miyazawa, T. Extraction of lipophilic fraction from polished rice improves its ameliorative effect on intestinal impairment. J. Oleo Sci. 68, 463-470 (2019).
32) Hanajima, H.; Matsunaga, H.; Fujikawa, A.; Sato, T.; Mitsutake, S.; Yanagita, T.; Nagao, K.; Nakayama, J.; Kitagaki, H. Japanese traditional dietary fungus koji Aspergillus oryzae functions as a prebiotic for Blautia coccooides through glucosylceramide: Japanese dietary fungus koji is a new prebiotic. Springerplus 5, 1321 (2016).