Glycosylceramide modifies the flavor and metabolic characteristics of sake yeast

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In the manufacture of sake, Japanese traditional rice wine, sake yeast is fermented with koji, which is steamed rice fermented with non-pathogenic fungus *Aspergillus oryzae*. During fermentation, sake yeast requires lipids, such as unsaturated fatty acids and sterols, in addition to substances provided by koji enzymes for fermentation. However, the role of sphingolipids on the brewing characteristics of sake yeast has not been studied. In this study, we revealed that glycosylceramide, one of the sphingolipids that is abundant in koji, affects yeast fermentation. The addition of soy, *A. oryzae*, and *G. frondosa* glycosylceramide conferred similar effect on the flavor profiles of sake yeast. In particular, the addition of *A. oryzae* and *G. frondosa* glycosylceramide were very similar in terms of the decreases in ethyl caprylate and ethyl 9-decenoate. Addition of soy glycosylceramide induced metabolic changes to sake yeast such as a decrease in glucose, increases in ethanol and glycerol and changes in several amino acids and organic acids concentrations. TCA cycle, pyruvate metabolism, starch and sucrose metabolism, and glycerolipid metabolism were overrepresented in the cultures incubated with sake yeast and soy glycosylceramide. This is the first study of the effect of glycosylceramide on the flavor and metabolic profile of sake yeast.

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ABSTRACT

In the manufacture of sake, Japanese traditional rice wine, sake yeast is fermented with koji, which is steamed rice fermented with non-pathogenic fungus *Aspergillus oryzae*. During fermentation, sake yeast requires lipids, such as unsaturated fatty acids and sterols, in addition to substances provided by koji enzymes for fermentation. However, the role of sphingolipids on the brewing characteristics of sake yeast has not been studied. In this study, we revealed that glycosylceramide, one of the sphingolipids that is abundant in koji, affects yeast fermentation. The addition of soy, *A. oryzae*, and *G. frondosa* glycosylceramide conferred similar effect on the flavor profiles of sake yeast. In particular, the addition of *A. oryzae* and *G. frondosa* glycosylceramide were very similar in terms of the decreases in ethyl caprylate and ethyl 9-decenoate. Addition of soy glycosylceramide induced metabolic changes to sake yeast such as a decrease in glucose, increases in ethanol and glycerol and changes in several amino acids and organic acids concentrations. TCA cycle, pyruvate metabolism, starch and sucrose metabolism, and glycerolipid metabolism were overrepresented in the cultures incubated with sake yeast and soy glycosylceramide. This is the first study of the effect of glycosylceramide on the flavor and metabolic profile of sake yeast.

INTRODUCTION

Japanese traditional rice wine, sake, uses rice as its raw material. Therefore, rice starch is used as a source of carbohydrates, and saccharification of starch is necessary for subsequent fermentation by sake yeast *Saccharomyces cerevisiae*. As a saccharifier of starch, koji, which is steamed rice fermented with the non-pathogenic fungus *Aspergillus oryzae*, has been used traditionally in Japan, as a Japanese version of the malt used in beer in western countries. Koji is then mixed with steamed rice and sake yeast, and thus simultaneous saccharification and fermentation occurs (*Kitagaki & Kitamoto, 2013*).

Koji contains various enzymes, including glycosidases, proteases, and lipases. The role of these koji enzymes on sake brewing has been studied intensively. Indeed, yeast can synthesize most substances, including saturated fatty acids, from substances provided from rice and degraded by koji enzymes, allowing them to proliferate and ferment. However, in addition to these substances, yeast needs lipids other than saturated fatty acids. Past studies have been limited to unsaturated fatty acids and β-sitosterol. For example, unsaturated fatty acids have been shown to facilitate fermentation and decrease isoamylacetate production (*Ohta & Hayashida, 1983*; *Fujii et al., 1997*; *Mason & Dufour, 2000*). Unsaturated fatty acids, β-sitosterol, and phospholipids synergistically affect the fusel alcohols content of beer and decrease the content of volatile esters and medium chain-length fatty acids (*Taylor, Thurston & Kirsop, 1979*). In addition, grape phytosterol increases the fermentation rate of yeast (*Luparia et al., 2004*). However, there has been no report on the effect of sphingolipids on yeast fermentation.

Sphingolipids are a class of lipids that contain amide bonds of sphingoid bases and fatty acids. Serine and palmitoyl-CoA are conjugated to form sphinganine, which is reduced to sphingoid bases, such as phytosphingosine, sphingosine, and dihydrosphingosine. Fatty acids are amide-linked to the sphingoid bases to form ceramides. In ceramides, either phosphate or carbohydrates are linked to the hydroxyl bond at the 1st position. Phosphate is ester-linked to the 1st hydroxyl bond of ceramides, and further diester-linked to inositol or choline to form acid complex sphingolipids, such as inositol-phosphoceramide (IPC), mannose-inositol-phosphoceramide (MIPC), and mannose-(inositol phosphate)$_2$-ceramide (M(IP)$_2$C) (*Dickson et al., 1997*) or sphingomyelin (*Okazaki, Bell & Hannun, 1989*). Carbohydrates, including glucose, galactose, sialic acid, and/or their conjugates, are acetal-linked to the 1st hydroxyl bond of ceramide to form glycosylceramides. Exceptionally, *Saccharomyces cerevisiae* lacks a glycosylceramide-synthesizing enzyme (*Saito et al., 2006*). Sphingolipids form rafts in the lipid bilayer (*Simons & Ikonen, 1997*) and also function as signaling lipids (*Hannun & Obeid, 2008*).

In previous studies, we revealed that one of the sphingolipids, glycosylceramide, having galactose (19.2%) or glucose (80.8%) as the monohexosyl moiety, 9-methyl-4,8-sphingadienine as the sphingoid base moiety, and 2'-hydroxyoctadecanoic acid as the fatty acid moiety, is contained in

69   shochu and sake koji (*Hamajima et al., 2016*; *Hirata et al., 2012*). Furthermore, we have revealed
70   that the glycosylceramide contained in shochu koji increased the alkali tolerance and modified the
71   flavor profile of shochu yeast (*Sawada et al., 2015*). However, the effect of koji glycosylceramide
72   on the fermentation profile of sake yeast remains unknown. Therefore, in the present study, we
73   investigated the effect of koji glycosylceramide on the brewing characteristics of sake yeast.

74

MATERIALS AND METHODS

Strains, reagents, and materials

77   The *S. cerevisiae* sake yeast (isolated and distributed in 2003 from the Brewing Society of Japan,
78   K7-4) was obtained from the Brewing Society of Japan (Tokyo, Japan). Conidia of *A. oryzae* were
79   obtained from Higuchi Moyashi Co., Ltd. (Osaka, Japan). Analytical grade reagents were used.
80   Glycosylceramides of soy and *Grifola frondosa* were purchased from Funakoshi Co. Ltd. (Tokyo,
81   Japan), and that of *A. oryzae* was purified from the mycelia of *A. oryzae*.

82

Culture of *A. oryzae*

84   Conidia of *A. oryzae* (10 mg) were dissolved in 1 ml of sterile water, and 100 µl of the solution
85   was inoculated onto potato dextrose agar (0.4 % potato starch, 2 % dextrose, 2 % agar) and
86   incubated at 30 °C for 3 days. An aliquot of the culture was inoculated into 500 ml of potato
87   dextrose medium (0.4 % potato starch, 2 % dextrose) and incubated at 30 °C and 200 rpm for 5
88   days. Mycelia of *A. oryzae* were washed with sterile water three times, freeze-dried for 2 days, and
89   ground with a mortar.

90

Lipid extraction

92   Dried *A. oryzae* mycelia (0.12 g) in a glass tube with a screw cap were added with 2 ml of
93   chloroform/methanol (1:1 v/v) and vortexed for 1 min. To the solution, 2 ml of 0.8 M KOH
94   dissolved in methanol was added and incubated at 42 °C and 160 rpm for 30 min. Subsequently, 5
95   ml of chloroform and 2.25 ml of water was vortexed until saponification, and centrifuged at 700 ×
96   *g* for 10 min. The lower organic layer was recovered and evaporated under a vacuum.

97

Separation and detection of lipids using thin layer chromatography (TLC)

99   Separation and detection of lipids were performed basically as previously described (*Takahashi et*
100 *al., 2014*).The extracted lipids (500 µl) were dried, dissolved in 50 µl of chloroform/methanol (2:1
101 v/v), and 20 µl of the solution was spotted onto a TLC plate (Silica gel 60 plate, Merck Millipore
102 Inc.,   Darmstadt,   Germany).   The   dried   plate   was   developed   with   saturated
103 chloroform:methanol:acetic acid:water (20: 3.5: 2.3: 0.7 v/v). Cerebroside (Matreya Inc., Pleasant

104 Gap, PA, USA) was used as an internal standard. To detect glycosylceramide, 2 mg/ml of orcinol

105 in 70 % sulfuric acid was sprayed onto the TLC plate and heated at 100 °C for 40 min.

106

Semi-purification of glycosylceramide using column chromatography

108 Crude extracts of sphingolipids were evaporated under a vacuum, solubilized in 3 ml of

109 chloroform, and applied to silica gel chromatography (diameter 30 mm, column length 300 mm,

110 Silica Gel 60 70–230 mesh, Nacalai Tesque Inc., Kyoto, Japan). First, 600 ml of chloroform was

111 used as an eluent to remove contaminants. Then, 10 ml of ethyl acetate:methanol (v/v, 9:1) was

112 added 24 times. An aliquot (800 µl) of each 10 ml fraction was dried, dissolved in 50 µl of

113 chloroform/methanol (2:1 v/v), and was spotted onto a TLC plate as described above. An Rf value

114 of 0.4 was adopted as the glycosylceramide fraction, as previously reported (*Takakuwa et al.,*

115 *2005*).

116

Purification of glycosylceramide using high performance liquid chromatography (HPLC)

118 Fractions that contained glycosylceramides were evaporated under a vacuum, dissolved in 1.5 ml

119 of chloroform, and injected into a 0.5 ml injection loop. Purification was performed as follows;

120 particle size: 5 µm, diameter: 4.6 mm, length: 250 mm (Inertsil SIL 100A, GL Science Inc., Tokyo,

121 Japan), column temperature; 30 °C, buffer A: chloroform, buffer B: 95 % methanol, gradient: 0.01

122 min A 100 %, 60 min A 85 %, 80 min A 10 %, 90 min A 10 %, 90.01 min A 100 %, 100 min A

123 100 %, 100.01 min STOP; rate: 0.7 ml/min. Eluents at 40–50 min were sampled at 1 min intervals,

124 and aliquots of the fractions were dried and applied to TLC analysis. The fractions that were

125 confirmed to contain purified glycosylceramide were dried under a vacuum (Supplementary Fig.

126 S1) and dissolved in ethanol (4 µg/µl).

127

Co-culture of sake yeast and glycosylceramide

129 Sake yeast was incubated in 3 ml of YPD medium (1 % yeast extract, 2 % bactopeptone, 2 %

130 glucose) at 30 °C for 24 h. Cells were recovered and washed with sterile water. Koji extract

131 solution (5 ml, pregelatinized koji weighing 5 g was mixed with water and incubated at 55 °C

132 overnight, and the liquid fraction was recovered) was inoculated with nonidet P-40 (final 0.0015%

133 v/v), with or without 50 µl of 4 µg/µl glycosylceramide (either from *A. oryzae*, soy, or *G. frondosa*)

134 dissolved in ethanol and 10^6 cells/ml sake yeast (the final glycosylceramide concentration was 40

135 µg/ml). Sake yeast is exposed to 40 µg/ml koji glycosylceramide during fermentation; therefore,

136 this concentration was adopted. Sterile liquid paraffin (1 ml) was overlaid onto the medium, and

137 the samples were incubated at 15 °C for 1 week. The volatile and non-volatile compounds of the

138 culture were analyzed. Flow chart of the experimental procedures is shown in Fig. 1.

139

Sample collection for metabolites analysis

After 1 week of fermentation with or without glycosylceramide, the fermented cultures were centrifuged at −9 °C and 3200 × g for 3 min. The supernatant (5 ml) was divided into two plastic tubes (2.5 ml each) and stored at 4 °C for volatile compound analysis or at −27 °C for non-volatile compounds analysis. The pellet was resuspended in 3 ml of MilliQ water (Millipore Inc., Darmstadt, Germany) and centrifuged again under the same conditions. The pellet was soaked in liquid nitrogen for 3.5 min, freeze-dried for 12 h, and stored at −80 °C.

Sample preparation for the analysis of volatile compounds

Fermented cultures were centrifuged at 4 °C and 3200 × g for 3 min, and the supernatant was collected. For target products analysis, 900 µl of fermentation broth and 100 µl of internal standard mixture (methyl hexanoate at 5 mg/L and *n*-amyl alcohol at 200 mg/L) were placed into a 10-ml glass vial on ice. The vial containing the fermentation broth and an internal standard mixture was sealed with a magnetic cap and subjected to GC/MS analysis, as previously described (*Yoshizaki et al., 2010*).

GC/MS analysis of volatile compounds

Aroma components of the fermented cultures were analyzed using headspace gas chromatography mass spectrometry (GC/MS) (GC-2010, GCMS-QP2010; Shimadzu, Kyoto, Japan) equipped with a DB-WAX column (60 m; internal diameter, 0.25 mm; 0.5 m; Agilent Technologies, Palo Alto, CA, USA) as previously described (*Yoshizaki et al., 2010*). Experiments were performed in triplicate from respective independent cultures.

Sample preparation for extracellular non-volatile compounds

Sample preparation for extracellular non-volatile compounds was performed as described previously (*Kadowaki et al., 2017*) with minor modifications. The fermented cultures (100 µl) or freeze-dried cells (10 mg) were mixed with 1 ml of chloroform:methanol:water (2:5:2) for extraction. Ribitol solution (0.2 mg/ml, 60 µl) was added to the solution and incubated for 30 min at 30 °C with shaking at 1500 rpm. The supernatant (900 µl) was collected by centrifugation (4 °C 16000 × g for 3 min) and 400 µl of MilliQ water (Millipore Inc., Darmstadt, Germany) was added, mixed, and centrifuged again under the same conditions. The supernatant (800 µl) was evaporated for 3 h and freeze-dried for 12 h. Methoxyamine (20 mg/ml dissolved in pyridine, 100 µl) was mixed with the freeze-dried extract and incubated at 30 °C for 90 min with shaking at 1500 rpm. *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (50 µl) was added and again incubated at 37 °C for 30 min with shaking at 1500 rpm. The solution (70 µl) was transferred to a vial and subjected to gas chromatography flame ionization detector (GC/FID) analysis.

176

177 **GC/FID analysis of non-volatile components.** Non-volatile metabolic components, which are

178 produced during fermentation with or without glycosylceramide, were analyzed using a GC/FID

179 (GC-2014, Gas Chromatograph, Shimadzu, Kyoto, Japan) with a CP Sil8CB column (30 m × 0.25

180 mm × 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). The carrier gas was nitrogen, with a

181 column headspace pressure of 73.9 kPa and a flow rate of 0.97 ml/min. The gas chromatography

182 temperature program was as follows: 60 °C for 2 min, increased to 320 °C at 13 °C /min, and held

183 for 17 min. The split ratios for extracellular metabolites were 10 and 2, respectively. The data were

184 analyzed using GC/FID solution software (Labsolution, Shimadzu, Kyoto, Japan). All metabolite

185 concentrations were normalized using ribitol as an internal control. Experiments were performed

186 from respective independent 7 (control) or 8 (soy glycosylceramide-added) cultures.

187

188 **Measurement of ethanol concentration**

189 The ethanol concentrations of the fermented cultures were analyzed using a contact combustion

190 system with an alcohol densitometer (Alcohol Checker YSA-200; Yazaki Meter Co. Ltd., Tokyo,

191 Japan) according to the manufacturer's instructions, and as described previously (*Katou et al.,*

192 *2008*).

193

194 **Statistical analysis**

195 The statistical significances of differences among the averages of volatile flavors were judged

196 using Student's *t*-test and the false discovery rate. The experimental results were expressed as

197 means ± standard error of the means. For heatmap analysis and integrated pathway analysis,

198 MetaboAnalyst (*Xia et al., 2015*) was used. Heatmaps were based on interquantile range data

199 filtering, normalization by sum, clustering with Ward's method, distance measure with Euclidean,

200 standardization by autoscale features and without data scaling.

201

202 **RESULTS**

203   First, the flavor compounds of the cultures fermented with sake yeast added with or without

204 glycosylceramides were analyzed (Supplementary Table S1–S6). To obtain information on the

205 specificity of the effect of the chemical structures of glycosylceramide, glycosylceramides from

206 *Aspergillus oryzae*, soy, and *G. frondosa* were used. A concentration of 40 μg/ml was adopted,

207 because koji contains approximately 240 μg/g of glycosylceramide (*Sawada et al., 2015*) and koji

208 is contained at a ratio of 1 to 6 of water and rice in the first step of sake brewing. As a result,

209 addition of *A. oryzae*, soy and *G. frondosa* glycosylceramides showed patterns distinct from

210 vehicle control (ethanol-added culture) (Fig. 2). In particular, *A. oryzae* and *G. frondosa*

211 glycosylceramides were clustered in proximity, showing similar decreases in ethyl caprylate and

212   ethyl 9-decenoate and increases in 2-phenylethyl acetate and phenylethyl alcohol (Table 1). Soy
213   glycosylceramide had a similar effect to *A. oryzae* in terms of the increase in 2-phenylethyl acetate
214   and decreases in ethyl 9-decenoate, isoamylalcohol and acetoin (Table 2).

215

216   Next, since all glycosylceramides showed overall similar trends, the metabolites in the cultures
217   fermented with sake yeast added with soy glycosylceramide were analyzed (Fig. 3 and Table 3).
218   As a result, independent cultures incubated with or without soy glycosylceramide were divided
219   into respective distinct clusters (control culture and soy glycosylceramide-added culture), except
220   CONT_6_007, SOY_1_004 and SOY_3_003 (Fig. 3). Many organic acids and amino acids, such
221   as succinate/glycine, malate, glutamate, valine, methionine, pyruvate and threonine, and glycerol
222   were increased and leucine was decreased in sake yeasts added with soy glycosylceramide. (Table
223   3). The decrease in glucose (Table 3) and increase in ethanol (Fig. 4) indicated an increased
224   fermentation ability of sake yeast incubated with glycosylceramide, which was consistent with a
225   previous study of shochu yeast (*Sawada et al., 2015*).

226

227   To extract information about the metabolism occurring in the yeast cells incubated with
228   glycosylceramide, the metabolome data of the medium incubated with or without
229   glycosylceramide were analyzed using MetaboAnalyst (*Xia et al., 2015*). Metabolites (glycerol,
230   succinate/glycine, malic acid, glucose, leucine, valine, glutamate, methionine, pyruvate and
231   threonine), which were significantly different ($p < 0.05$) between medium incubated with or
232   without soy glycosylceramide, were selected and further analyzed. As a result, several
233   metabolisms, such as pyruvate metabolism, the TCA cycle, starch and sucrose metabolism, and
234   glycerolipid metabolism were significantly overrepresented in the medium incubated with
235   glycosylceramide (Fig. 5). This result indicated that extracellularly added glycosylceramide has
236   certain effects on the metabolic pathways described above, although the mechanism remains to be
237   determined.

238

239   **DISCUSSION**
240   In this study, we first revealed the effect of glycosylceramides on the metabolic and flavor
241   profiles of sake yeast. Soy, *A. oryzae* and *G. frondosa* glycosylceramides conferred similar effects
242   on the flavor profiles of sake yeast; addition of *A. oryzae* and *G. frondosa* glycosylceramides
243   showed especially high similarity, providing a specificity of the 9-methyl base of the sphingoid
244   base. Addition of soy glycosylceramide induced significant changes to the concentrations of
245   glucose, ethanol, glycerol and several amino acids and organic acids. Several metabolic pathways
246   were altered in sake yeast incubated with soy glycosylceramide. Considering that the content of
247   glycosylceramide differs among koji samples (*Sawada et al., 2015*), these results suggested that

248   koji exerts its effects on yeast fermentation characteristics through glycosylceramide, and that the
249   effect of koji on sake brewing should be evaluated in terms of the effect of the quantity of
250   glycosylceramide contained in koji.

251   Alcohol fermentation is performed without oxygen, which is required for synthesis of specific
252   lipids; therefore, it has been documented that unsaturated fatty acids (*Fujii et al., 1997*; *Fujiwara*
253   *et al., 1998*) and sterols (*Belviso et al., 2007*), which involve molecular oxygen, are required for
254   progression of yeast fermentation. In addition, yeast incubated with or without oxygen (*Ishtar*
255   *Snoek & Yde Steensma, 2007*) or that incubated after diauxic shift (*Kitagaki et al., 2009*) have
256   different gene expression profiles or yeast incubated without oxygen change their cell wall proteins
257   (*Kitagaki, Shimoi & Itoh, 1997*). However, the role of sphingolipids in yeast during fermentation
258   has not been described until our recent study (*Sawada et al., 2015*).

259   Sphingolipids have several characteristics that are different from other lipids. For example, they
260   are amide-linked and have long fatty acid lengths (C20–26) (*Kitagaki et al., 2007*). The mechanism
261   of glycosylceramide's effect on yeast fermentation characteristics seems to be through its
262   increasing effect on the yeast membrane fluidity. Indeed, we have shown in a previous report that
263   glycosylceramide shortens the average fluorescence lifetime of τ of shochu yeast, as measured by
264   trimethylamine-diphenylhexatriene (*Sawada et al., 2015*). Consistent with this hypothesis, we
265   found that volatile esters were decreased in sake yeast incubated with glycosylceramide (Fig. 2,
266   Table 1 and Table 2).

267   The effect of glycosylceramide on the physiology of yeast elucidated in this study suggests its
268   impact on the membrane characteristics. It was reported that the addition of unsaturated fatty acid,
269   which also increases membrane fluidity, decreases ethyl ester flavors (*Moonjai et al., 2003*;
270   *Saerens et al., 2008*). It was also reported that a 9-methyl base is essential for membrane fluidity
271   (*Singh et al., 2012; Takakuwa et al., 2002*). However, the specificity of 9-methyl base-containing
272   sphingolipids was not known. Glycosylceramide containing the 9-methyl-4, 8-sphingadienine
273   base, such as *A. oryzae* and *G. frondosa* glycosylceramide, had different effects in decreasing
274   volatile esters, including ethyl caprylate and ethyl 9-decenoate, compared with those produced by
275   soy glycosylceramide containing a 4, 8-sphingadienine base (Table 1 and Table 2), suggesting that
276   9-methyl base in the sphingoid base has a strong inhibitory effects on these volatile flavors.
277   However, there are several phenomena that cannot be explained only by the increase in membrane
278   fluidity, such as the increase in glycerol and decrease in leucine. Sphingolipids are signal
279   molecules that stimulate protein phosphatase 2A (*Dobrowsky et al., 1993*) and form rafts in the
280   lipid bilayer (*Pralle et al., 2000*); therefore, glycosylceramide might act via these mechanisms. It
281   will be intriguing to investigate the effect of *A. oryzae* glycosylceramide on yeasts, which will
282   form the target of our next study.

283   The behavior, fate, and effects of koji glycosylceramide during fermentation are now starting to

284   be elucidated. Koji contains abundant (0.15–0.25 mg/ml) glycosylceramides (*Sawada et al., 2015*;
285   *Sakamoto et al., 2016*). Therefore, sake yeast is exposed to a high concentration of
286   glycosylceramide (40 µg/ml) during fermentation. Koji glycosylceramide is bound to the surface
287   of brewery yeasts, and remains there until the end of fermentation (*Sawada et al., 2015*). In
288   addition, koji glycosylceramide alters the brewing profiles of sake yeast. These results shed new
289   light on the interaction of koji glycosylceramide and brewery yeasts.

290   The amount of koji, or the extent of propagation of *A. oryzae* on the surface of koji (*haze*), has
291   been empirically considered as an important criterion to govern yeast fermentation by brewing
292   technicians in the sake industry. It has been proposed that mycelia of *A. oryzae* increase the
293   complex taste of sake, but the precise mechanism has remained unknown. However, together with
294   the data obtained in this study, it can be hypothesized that the amount of lipids, such as
295   glycosylceramide, unsaturated fatty acids, and sterols, contained in koji, is the key to control yeast
296   fermentation.

297   Sake yeast cells incubated with glycosylceramide showed effects on pyruvate metabolism, the
298   TCA cycle, starch and sucrose metabolism, and glycerolipid metabolism. Lactosylceramide and
299   ceramide cause dysfunction of the mitochondria (*Novgorodov et al., 2016*; *Law et al., 2017*);
300   therefore, it might have stimulated the mitochondrial function of sake yeast. Mitochondrial activity
301   has a significant effect on the fermentation profile of sake yeast (*Shiroma et al., 2014*; *Kitagaki &*
302   *Takagi, 2014*; *Motomura, Horie & Kitagaki, 2012*; *Sawada & Kitagaki, 2016*); therefore, the
303   upregulation of mitochondrial function in sake yeast might be responsible for the altered
304   fermentation profile. The mechanism of the effect of glycosylceramide on yeast mitochondria
305   requires further research.

306

307   **CONCLUSIONS**

308   In conclusion, we have determined the altered fermentation characteristics of sake yeast in
309   response to glycosylceramide, which will enable interpretation of the effect of koji on the
310   fermentation characteristics of yeast.

311

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315

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Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and *G. frondosa.*

Sake yeasts were incubated in synthetic medium with or without 40 µg/ml glycosylceramide of *A. oryzae* and *G. frondosa* and nonidet P-40 (final 0.0015% v/v) at 15 °C for 1 week. Volatile compounds were analysed using headspace gas chromatography mass spectrometry (GC/MS). GlcCer indicates glycosylceramide. The results are the mean values with standard errors of triplicate independent experiments. The relative percentage as compared to the mean value of the control culture is shown. Volatile compounds which were significantly different between control and GlcCer-added culture as judged by false discovery rate ($p <$ 0.05, indicated by *) are described. P values were calculated using unpaired one-tailed Student's t-test. Further details are described in Materials and Methods.

1   **Table 1. Volatile compounds in the culture of sake yeast added with or without**

2   **glycosylceramide of *A. oryzae* and *G. frondosa***

Volatile Compounds	Control	GlcCer of *A. oryzae*		GlcCer of *G. frondosa*	
	Relative percentage (%)	Relative percentage (%)	*p* value	Relative percentage (%)	*p* value
Ethyl Caprylate	100.0±13.1	56.4±1.78	0.0272*	50.4±1.07	0.0185*
Ethyl 9-Decenoate	100.0±20.9	38.4±5.49	0.0401*	26.2±2.35	0.0227*
2-Phenylethyl Acetate	100.0±19.6	351±34.6	0.00338*	431±27.7	0.000671*
Phenylethyl Alcohol	100±3.59	159±19.3	0.0342*	216±9.56	0.000376*

Volatile compounds in the culture of sake yeast added with or without glycosylceramide of *A. oryzae* and soy.

Sake yeasts were incubated in synthetic medium with or without 40 µg/ml glycosylceramide of *A. oryzae* and soy and nonidet P-40 (final 0.0015% v/v) at 15 °C for 1 week. Volatile compounds were analysed using headspace gas chromatography mass spectrometry (GC/MS). GlcCer indicates glycosylceramide. The results are the mean values with standard errors of triplicate independent experiments. The relative percentage as compared to the mean value of the control culture is shown. Volatile compounds which were significantly different between control and GlcCer-added culture as judged by false discovery rate ($p <$ 0.05, indicated by *) are described. P values were calculated using unpaired one-tailed Student's *t*-test. Further details are described in Materials and Methods.

2   **Table 2. Volatile compounds in the culture of sake yeast added with or without**

3   **glycosylceramide of *A. oryzae* and soy**

Volatile Compounds	Control	GlcCer of *A. oryzae*		GlcCer of soy	
	Relative percentage (%)	Relative percentage (%)	*p* value	Relative percentage (%)	*p* value
Isoamyl Alcohol	100±4.16	75.1±5.18	0.0189*	97.6±1.93	0.347
Acetoin	100±7.51	44.7±8.22	0.00772*	84.7±8.58	0.168
Ethyl 9-Decenoate	100±13.4	35.2±2.12	0.00886*	61.5±3.23	0.0427*
2-Phenylethyl Acetate	100±12.9	125±24.3	0.249	192±18.9	0.0150*
Phenylethyl Alcohol	100±5.00	90.2±14.0	0.310	143±10.6	0.0200*

Metabolite concentrations in the culture of sake yeast added with or without soy glycosylceramide.

Sake yeasts were incubated in synthetic medium with or without 40 µg/ml soy glycosylceramide and nonidet P-40 (final 0.0015% v/v) at 15 °C for 1 week. Metabolites derivatized with methoxyamine and MSTFA, analysed using gas chromatography flame ionization detector (GC/FID) and normalized using ribitol. GlcCer indicates glycosylceramide. The results are the mean values with standard errors of 7 (control) or 8 (GlcCer of soy) independent experiments. The relative percentage as compared to the mean value of the control culture is shown. _P_ values were calculated using unpaired one-tailed Student's _t_-test. Further details are described in Materials and Methods.

2 **Table 3. Metabolite concentrations in the culture of sake yeast added with or without soy**

3 **glycosylceramide**

Metabolic compounds	Control	GlcCer of soy	
	Relative percentage (%)	Relative percentage (%)	p value
Glycerol	100±2.55	110±1.14	0.00140
Succinate/Glycine	100±4.05	116±2.68	0.00376
Malate	100±2.54	116±3.55	0.00191
Glucose	100±5.17	71.7±3.05	0.000513
Glutamate	100±0.501	101±0.315	0.0392
Leucine	100±2.73	89.8±1.86	0.00770
Valine	100±5.12	128±4.62	0.00225
Methionine	100±7.23	127±6.12	0.0130
Pyruvate	100±15.5	222±28.3	0.00235
Threonine	100±13.8	146±9.62	0.0125

Figure 1

Flow chart of the experimental procedures.
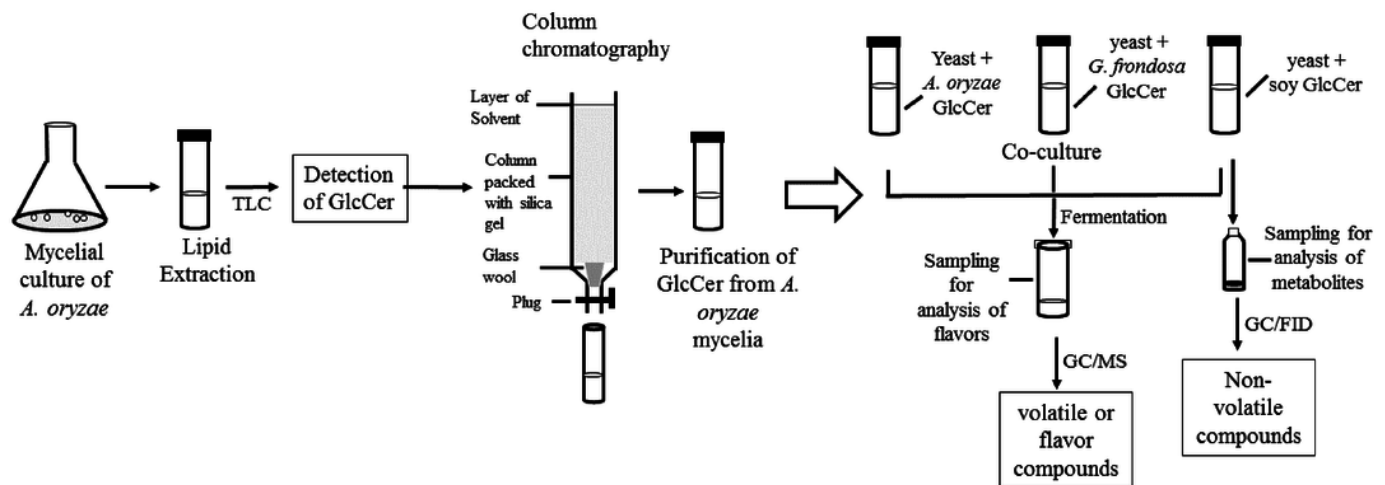
Figure 2

Heatmap of volatile compounds in the culture of sake yeast incubated with or without glycosylceramide.

Sake yeasts were incubated in synthetic medium containing 40 µg/ml *A. oryzae* (A1 and A2), soy (S), *G. frondosa* (G) glycosylceramide or their vehicle control ethanol (E1 and E2) and nonidet P-40 (final concentration 0.0015% v/v) at 15 °C for 1 week. Volatile compounds were analyzed using headspace gas chromatography mass spectrometry (GC/MS). A heatmap of volatile compounds was created with Metaboanalyst.
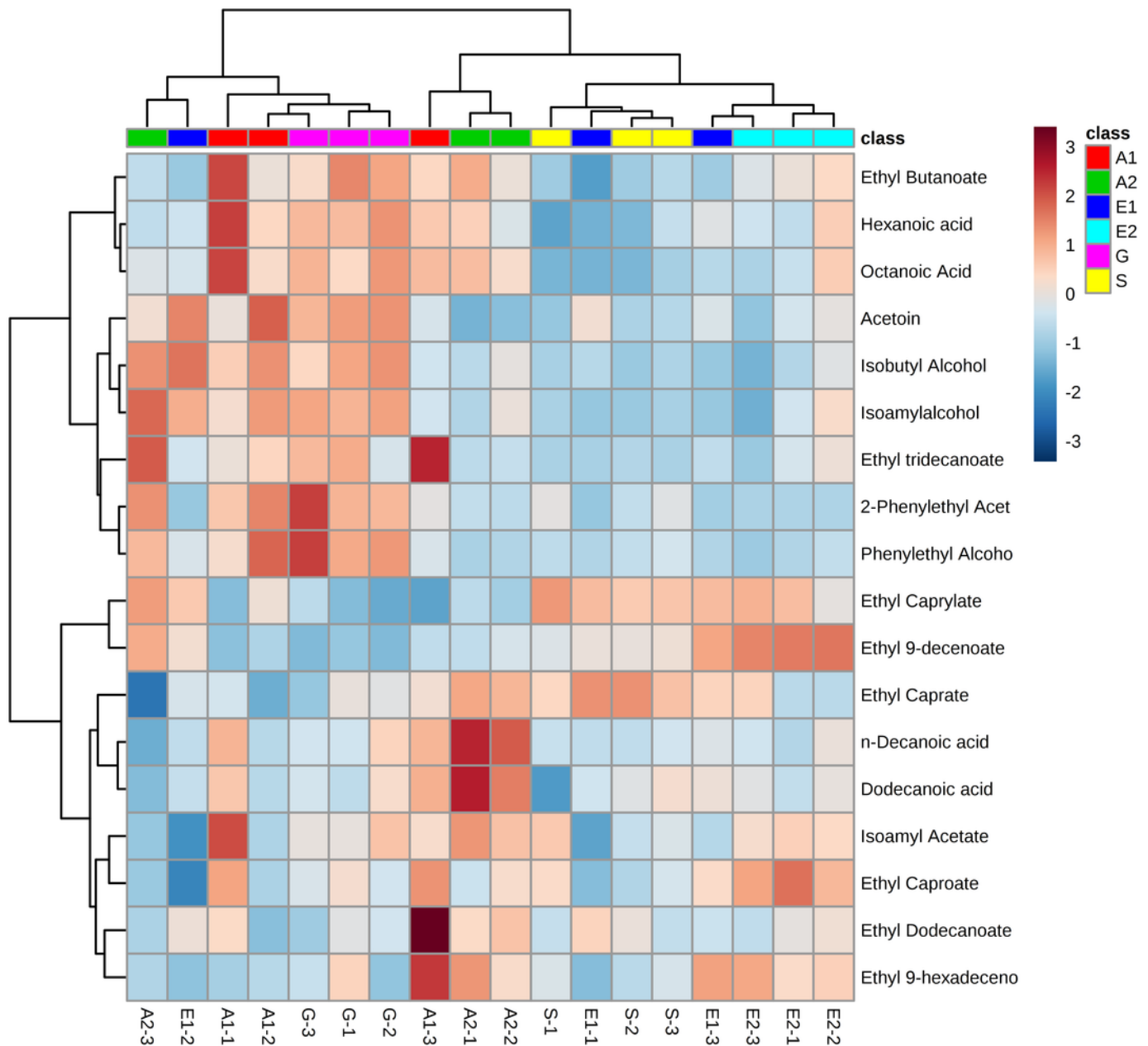
Figure 3

Heatmap of extracellular metabolite concentrations in the culture of yeast incubated with or without soy glycosylceramide.

Sake yeasts were incubated in synthetic medium containing 40 μg/ml soy glycosylceramide and nonidet P-40 (final concentration 0.0015% v/v) at 15 °C for 1 week. Metabolites of the cultures were derivatized with methoxyamine and MSTFA, analysed using GC-FID and normalized using ribitol. A heatmap of metabolites was created with Metaboanalyst.
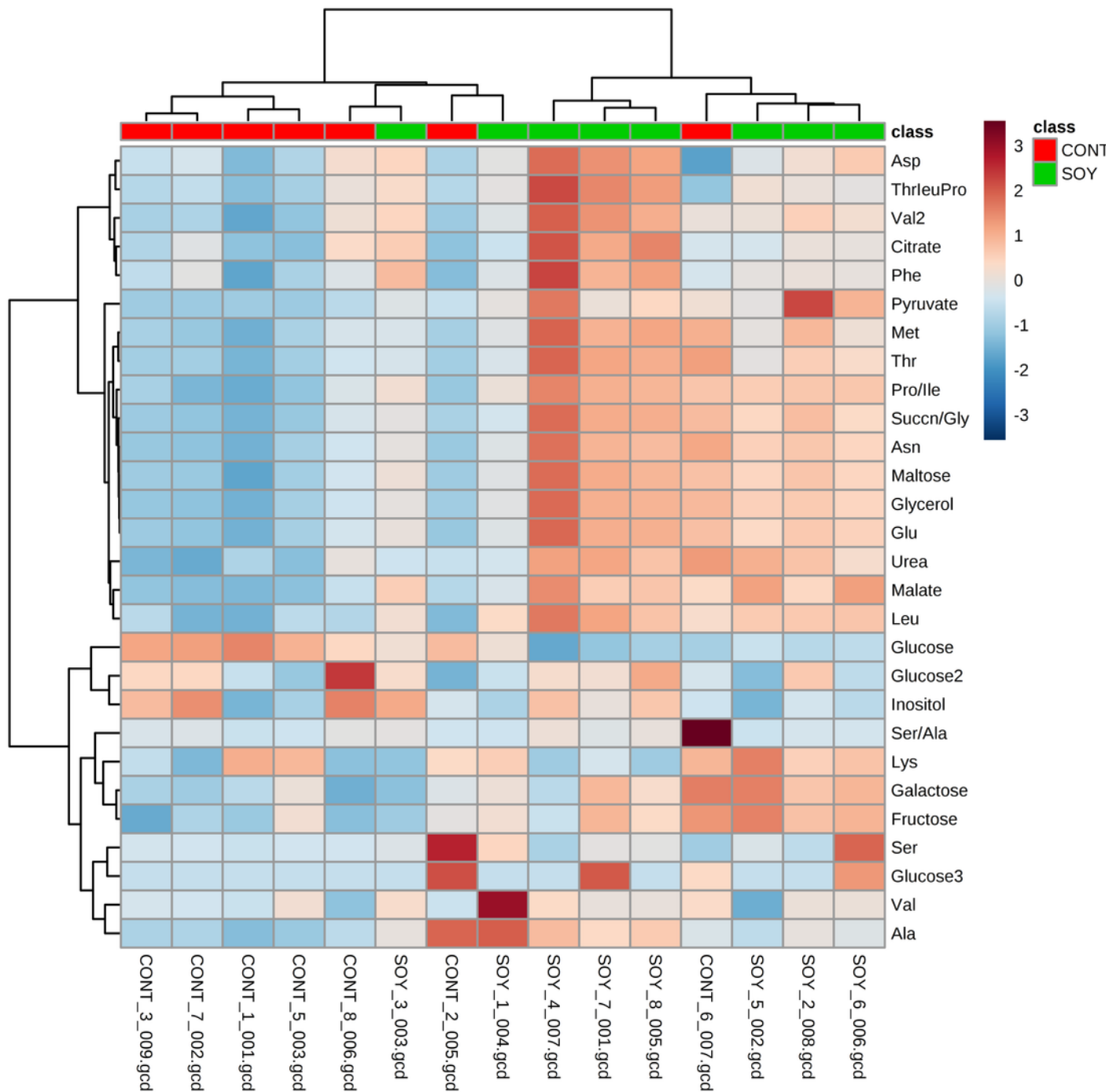
Figure 4

Ethanol concentrations (%(vol/vol)) of the culture of sake yeast added with or without soy glycosylceramide.

Sake yeasts were incubated in synthetic medium with or without 40 µg/ml soy glycosylceramide and nonidet P-40 (final 0.0015% v/v) at 15 °C for 1 week. The ethanol concentration of fermented culture was analyzed using a contact combustion system with an alcohol densitometer. The results are the mean values with standard errors of triplicate independent experiments. The statistical significance of the difference between the averages was assessed using the unpaired one-tailed Student's t-test (***, $p < 0.001$).
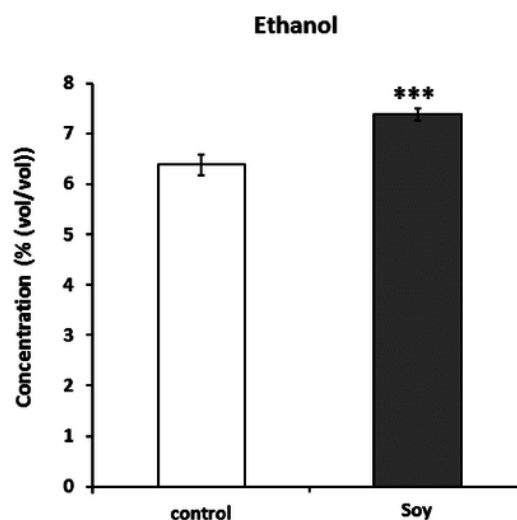
Figure 5

Pathway analysis of extracellular metabolites of sake yeast incubated with or without soy glycosylceramide.

The normalized values of metabolites (glycerol, succinate/glycine, malic acid, glucose, leucine, glutamate, valine, methionine, pyruvate, and threonine), which were significantly different ($p < 0.05$) between medium incubated with and without soy glycosylceramide, were used as independent variables for Metaboanalyst.