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1 # Water-in-oil-in-water double emulsion for the delivery

2 # of starter cultures in reduced-salt moromi fermentation

3 # of soy sauce

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9

10 **Abstract**

11 This study investigated the application of water-oil-water ($W_1/O/W_2$) double emulsions (DE)

12 for yeast encapsulation and sequential inoculation of *Zygosaccharomyces rouxii* and

13 *Tetragenococcus halophilus* in moromi stage of soy sauce fermentation with reduced NaCl

14 and/or substitution with KCl. *Z. rouxii* and *T. halophilus* were incorporated in the internal W_1

15 and external W_2 phase of DE, respectively. NaCl reduction and substitution promoted *T.*

16 *halophilus* growth to 8.88 log CFU/mL, accompanied with faster sugar depletion and

17 enhanced lactic acid production. Reducing NaCl without substitution increased the final pH

18 (5.49) and decreased alcohols, acids, esters, furan and phenol content. However, the

19 application of DE resulted in moromi with similar microbiological and physicochemical

20 characteristics to that of high-salt. . Principal component analysis of GC-MS data

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21   demonstrated that the reduced-salt moromi had identical aroma profile to that obtained in the

22   standard one, indicating the feasibility of producing low-salt soy sauce without compromising

23   its quality.

24   Keywords: Soy sauce; Moromi fermentation; Salt reduction; $W_1/O/W_2$ double emulsion;

25   Yeast encapsulation; Sequential inoculation; Aroma compounds; GC-MS.

1. Introduction

Soy sauce is a traditional fermented seasoning that is popular in Asia and throughout the world, due to its intense umami taste and characteristic flavor. Soy sauce production process involves a 2-step fermentation process, called *koji* and *moromi*. Koji fermentation begins by mixing steam-cooked soybeans and roasted wheat flour with spores of mold, such as *Aspergillus oryzae* or *Aspergillus sojae*, and, after 3 days of incubation, a compact mass is formed due to mycelium growth (Zhu & Tramper, 2013). The resulting koji is then immersed in brine solution, typically containing 18–22% NaCl to initiate the second step of fermentation and produce moromi mash, and left to ferment for approximately 6 months. During this stage, a wide range of microbial species grow spontaneously and produce numerous flavor compounds, which are essential to the organoleptic properties of the final product. *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* have been considered as the most predominant osmophilic lactic acid bacteria (LAB) and yeast, respectively, and play major roles in the aroma formation (Wilfred F.M. Röling, Timotius, Prasetyo, Stouthamer, & Van Verseveld, 1994). The use of brine with high NaCl concentration in moromi fermentation is important to control undesirable microorganisms and improve the flavor profile and texture of the final product (Song, Jeong, & Baik, 2015a). However, high NaCl content contributes to excessive sodium intake, which has been reported to increase risk of hypertension, cardiovascular disease, and renal dysfunction (Kremer, Mojet, & Shimojo, 2009). Furthermore, the World Health Organization (WHO) recommends a limitation of average daily intake of sodium to 2 g, which is equivalent to 5 g of salts (WHO, 2012). As a consequence, producing soy sauce with low NaCl content without compromising its quality and consumer acceptability is a challenge, and low salt soy sauce products are now available. Soy sauce production with reduced NaCl has been investigated by different approaches. Moromi fermentation in the absence of NaCl was possible by autolyzing koji under high

51 temperature prior to fermentation (Muramatsu, Sano, Uzuka, & Company, 1993).

52 Nevertheless, the absence of salt during fermentation may result in the growth of spoilage

53 microorganisms and the quality of final product can differ from the original. Salt reduction

54 during moromi fermentation could result in lower content of essential acids, alcohols, and

55 esters, and higher acidity content (Song et al., 2015a). Such problems could be counteracted

56 by the addition of mixed cultures of indigenous yeast species (Song et al., 2015a) as well as

57 combining LAB and yeasts (Singracha et al., 2017).

58 However, a recent study showed that the final aroma profile in moromi fermentation was

59 compromised due to antagonism between co-inoculated *Tetragenococcus halophilus* and

60 *Zygosaccharomyces rouxii* while their sequential inoculation could improve the aroma

61 complexity (Devanthi, Linforth, Onyeaka, & Gkatzionis, 2018). The application of sequential

62 inoculation of mixed cultures has been reported to improve flavor quality of fermented foods

63 and beverages. Modulation of the inoculation time was found to be key in achieving the

64 desired quality of apple cider (Ye, Yue, & Yuan, 2014). Furthermore, in whey fermentation,

65 sequential inoculation of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 after 48

66 h improved volatile compounds production (e.g. alcohols and esters) (Andrade, Melo,

67 Genisheva, Schwan, & Duarte, 2017). Higher production of 3-sulfanylhexyl acetate (3SHA)

68 and 3SH (3-sulfanyl-1-hexanol), which are the most important volatiles in Sauvignon blanc

69 aroma, has been achieved with sequential culture of *T. delbrueckii* and *S. cerevisiae*.

70 A formulation is needed to control the sequential delivery and activity of microbial cultures

71 in soy sauce fermentation. Water-in-oil-in-water ($W_1/O/W_2$) double emulsions (DE) have

72 been studied in recent years for their ability to encapsulate hydrophilic substances, including

73 bacteria for protection and controlled release. Their multi-compartmentalized structure is

74 created by dispersing a water-in-oil (W_1/O) emulsion in another aqueous phase (W_2). Recent

75 studies have focused on probiotic bacteria encapsulation in DE for enhancing survival during

4

76   digestion (Eslami, Davarpanah, & Vahabzadeh, 2016; Shima, Morita, Yamashita, & Adachi,

77   2006). The instability of DE structure can be used to modulate the release of bacterial cells by

78   utilizing changes in osmotic balance (El Kadri, Gun, Overton, Bakalis, & Gkatzionis, 2016;

79   El Kadri, Overton, Bakalis, & Gkatzionis, 2015) as they would occur during fermentation. A

80   previous study demonstrated that the inherent DE instability acted as a mechanism for

81   gradual release of *Z. rouxii*, which could be linked to changes in glucose concentration in the

82   medium (Devanthi, El Kadri, Bowden, Spyropoulos, & Gkatzionis, 2018).

83   In this study, the application of DE for the encapsulation and sequential delivery of *T.*

84   *halophilus* and *Z. rouxii* cultures was tested in conditions reflecting moromi fermentation

85   with reduced NaCl content and/or substitution with KCl. The stability of DE in moromi was

86   examined by monitoring its microstructure, oil globules size, and distribution. Furthermore,

87   microbial population and physicochemical changes as well as volatile compounds formation

88   were monitored.

89   **2.   Materials and Methods**

90   *2.1   Materials, chemicals, and microorganisms*

91   Soy and wheat flour were purchased from a local retailer (UK). *Aspergillus oryzae* 126842

92   was purchased from Centre for Agriculture and Biosciences International (Egham, UK).

93   *Tetragenococcus halophilus* 9477 and *Zygosaccharomyces rouxii* 1682 were purchased from

94   National Collection of Industrial Food and Marine Bacteria Ltd. (Aberdeen, UK) and

95   National Collection of Yeast Cultures (Norwich, UK), respectively. Sodium chloride (NaCl,

96   extra pure) was purchased from Acros Organics (Fairlawn, NJ). Microbiological growth

97   media used were Czapex Dox Agar (CDA; Oxoid Ltd., Basingstoke, UK), Brain Heart

98   Infusion agar (BHI, Oxoid Ltd., UK), de Man, Rogosa, and Sharpe broth (MRS broth, Oxoid

99   Ltd., UK), Yeast Malt agar (YM agar, Sigma-Aldrich, Gillingham, UK), Yeast Malt broth

100 (YM broth, Sigma-Aldrich, UK). Bacteria and yeast growth were controlled using

101 chloramphenicol (Oxoid Ltd., UK) and natamycin (Sigma-Aldrich, UK), respectively. 1-

102 Octen-3-ol (purity ≥98%) was purchased from Sigma-Aldrich. Soybean oil (Alfa Aesar,

103 Heysham, UK) was used as the oil phase of the DE. Polysorbate 80 (Tween 80, Sigma-

104 Aldrich, United Kingdom) and polyglycerol polyricinoleate (PGPR, Danisco A/S,

105 Copenhagen, Denmark) were used as water and oil soluble emulsifiers, respectively.

106 *2.2   Culture preparation*

107 *Aspergillus oryzae* was maintained on CDA at 25 °C. The spore suspension of *A. oryzae* was

108 prepared according to the method described by Chou and Ling (1998) with slight

109 modification. Briefly, spores were obtained by growing *A. oryzae* on CDA at 25 °C for 7

110 days. NaCl solution (0.85%, *w/v*) solution containing 0.01% of Tween 80 (Sigma-Aldrich,

111 UK) was added into the agar slant bottle followed by vigorous mixing to collect the spores.

112 The number of spores were counted using an improved Neubauer hemocytometer and

113 adjusted to 10^6 spores/mL. *Tetragenococcus halophilus* was maintained on BHI with 10%

114 (*w/v*) NaCl and incubated at 37 °C. *T. halophilus* was grown in MRS broth with 7% NaCl for

115 36 h and the cell concentration was adjusted to a final concentration of 10^6 cells/mL.

116 *Zygosaccharomyces rouxii* was maintained on YM agar with 5% (*w/v*) NaCl and incubated at

117 25 °C. The inoculum was prepared by growing *Z. rouxii* in YM broth containing 5% (*w/v*)

118 NaCl in a 30 °C shaker incubator for 24 h and cell concentration was adjusted to 10^6

119 cells/mL.

120 *2.3   DE preparation*

121 The DEs were prepared using a 2-step emulsification method at ambient temperature by

122 using a high shear mixer (Silverson L5M). In the first step, W_1/O primary emulsion was

123 prepared by mixing sterile 6% (*w/v*) NaCl solution into the oil phase (soybean oil with 2% wt

6

124 PGPR) at W_1:oil phase ratio of 20:80 at 1700 rpm for 2 min. For yeast encapsulation, *Z.*

125 *rouxii* suspension in 6% (*w/v*) NaCl solution (10^7 cells/mL) was used as W_1.

126 In the second stage, W_1/O was re-emulsified in the continuous phase (W_2;sterile 6% (*w/v*)

127 NaCl in water with 1% wt Tween 80) at 2000 rpm for 1 min (W_1/O:W_2 ratio of 20:80). The

128 final concentration of encapsulated *Z. rouxii* cells was ~10^5 cells/mL. DEs containing *T.*

129 *halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL)

130 into the W_2 after the mixing process.

131 *2.4   Soy sauce fermentation*

132 **Koji preparation:** Koji was prepared using the modified method of Su et al. (2005). Soy and

133 wheat flour were sterilized at 121 °C for 15 min in an LTE Series 300 autoclave (LTE

134 Scientific Ltd, Oldham, UK). Soy flour moisture was maintained by mixing 100 g of soy

135 flour with 120 mL of sterile distilled water. The cooked soy flour was cooled to room

136 temperature and then mixed thoroughly with the wheat flour (1:1 *w/w*). The mixture was

137 inoculated with *A. oryzae* spore to a final concentration of 10^5 spores/g substrate (Chou &

138 Ling, 1998). The inoculated substrates were transferred into sterile Petri dishes (d:140 mm)

139 and incubated at 30 °C for 3 days.

140 **Moromi preparation:** Different types of brine (18% *w/v* NaCl; 6% *w/v* NaCl and 12% *w/v*

141 KCl; 6% *w/v* NaCl) were added to the koji with ratio of 1:5 (koji:brine) to create moromi

142 $A_{[18\%]}$, $B_{[6:12\%]}$, and $C_{[6\%]}$ respectively, followed by inoculation as shown in Figure 1. Moromi

143 $A_{[18\%]}$ and $B_{[6:12\%]}$ were simultaneously inoculated with *T. halophilus* and *Z. rouxii*. Three

144 different moromi C were prepared according to the inoculation method of *Z. rouxii*. Moromi

145 $C1_{[6\%]}$ was simultaneously inoculated with *T. halophilus and Z. rouxii*, while moromi $C2_{[6\%]}$

146 and $C3_{[6\%]}$ were inoculated with *Z. rouxii* after 1 week and 2 weeks, respectively. Moromi

147 $C4_{[6\%]}$ was inoculated with DE (10% *v/v*) containing *T. halophilus* and *Z. rouxii*, which had

148   been incorporated in its W_2 and W_1 phase, respectively, prior to inoculation. The inoculated

149   moromi mashes were then incubated at 30 °C for 4 weeks and samples were taken at Week 0,

150   1, 2, 3, and 4. *T. halophilus* was grown on BHI agar supplemented with 7% (*w/v*) NaCl and

151   natamycin while the cell count of *Z. rouxii* was done on YM agar with the addition of 5%

152   (*w/v*) NaCl, and 100 mg/L chloramphenicol. In order to study the effect of koji:brine ratio on

153   DE stability, koji was mixed with 18% *w/v* NaCl solution with koji:brine ratio of 1:3, 1:5, and

154   1:7 followed by incubation at 30 °C for 7 days.

155   *2.5   Rheological measurements*

156   Rheological characterization of moromi was done by measuring the viscosity of koji mixed

157   with varying concentrations of brine solution (18% NaCl *w/v*). The viscosity was measured

158   for moromi containing koji:brine ratio of 1:3, 1:5, 1:7 and brine only at 30 °C using AR-G2

159   rheometer (TA instruments, New Castle, DE) on a parallel plate geometry (d: 40 mm). The

160   apparent viscosity was measured over a shear rate range of $0.1–100 \ s^{-1}$. Briefly, 1 mL of

161   sample was placed between the cone and the plate, and measurement was started

162   immediately. In total, 30 data points were recorded at 10-s intervals during the shearing.

163   Shear stress was determined as a function of shear rate. Data were fitted to power-law model

164   (Barnes et al., 1989):

165
$$\eta = K \cdot \gamma^{n-1}$$
(1)

166   where; η refers to viscosity (Pa s), *K* to consistency coefficient (Pa s^n), γ to shear rate (s^{-1}),

167   and *n* to flow behavior index (dimensionless).

168   *2.6   Physicochemical analysis*

169   Soy mash samples were centrifuged at 15000 *g* for 15 min at ambient temperature. The

170   supernatant regarded as raw soy sauce was transferred to microtubes and kept at –20 °C until

171   analysis. Total reducing sugar (D-glucose and D-fructose), total lactic acid (L-lactic acid and

172   D-lactic acid), ethanol, and L-glutamic acid were analyzed using enzymatic assay kit

173   (Megazyme, International Ireland Ltd., Bray, Ireland) according to the manufacturer's

174   instructions. Changes in pH were monitored using a pH meter (SevenCompact S220, Mettler

175   Toledo, Germany).

176   *2.7   Volatile compound analysis (SPME GC-MS)*

177   An automated headspace solid-phase microextraction method (SPME) followed by GC-MS

178   analysis was used for evaluating the *in vitro* production of microbial volatile organic

179   compounds. Soy sauce mash samples (1.5 g) were transferred into 20-mL headspace vials

180   (22.5 mm × 75.5 mm, Grace Alltech, Thermo Fisher UK) and the vials were sealed with

181   magnetic cap (20 mm diameter, 5 mm center, PTFE / Silicone Liner; Grace Alltech). Samples

182   were allowed to equilibrate at 22 °C for 30 min before analysis. Three replicates were

183   prepared for all samples.

184   The volatiles extraction was performed using a 1-cm Stableflex fiber coated with 50/30 μm

185   divinylbenzene-Carboxen on polydimethylsiloxane bonded to a flexible fused silica core

186   (Supelco, Bellefonte, PA). It was conditioned for 90 min at 300 °C in the injection port. The

187   fiber was pushed out of the housing and inserted into the vials through the center of the vial

188   cap. The penetration depth was fixed at 22 mm. The extraction was carried out by exposing

189   the fiber to the headspace for 10 min at 40 °C. For all analyses, desorption time was set to 10

190   min at 230 °C.

191   Chromatography was carried out using a Trace GC Ultra gas chromatography (Thermo

192   Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m

193   × 0.25 mm I.D.; film thickness: 1 μm) from Phenomenex (Torrance, CA). Mass spectrometry

194   (MS) was performed with a DSQ mass spectrometer (Thermo Electron Corporation, Hemel

195   Hempstead, UK)). GC-MS parameters were set according to a previous study (Gkatzionis,

196   Linforth, & Dodd, 2009): The temperature of the injection port was 230 °C. Helium was

197   employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature program

198   was as follows: an initial temperature of 40 °C was maintained for 2 min, increasing at a rate

199   of 8 °C /min to a final temperature of 220 °C. The transfer line from the gas chromatograph

200   to the mass spectrometer was held at 250 °C. The mass spectrometer was operated in positive

201   ionization electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2

202   scans/s) scanning from m/z 20 to 250. Source temperature was 200 °C.

203   Compounds were identified by comparing their retention times and mass spectra with those

204   of standards or their retention indices (RI) with those published in databases and their mass

205   spectra with the National Institute of Standards and Technology (NIST) mass spectral library

206   using XCalibur Software (Thermo Electron Corporation, UK). The signal intensity for each

207   compound was expressed relative to the signal observed when the headspace above a 0.1

208   μg/mL 1-octen-3-ol solution was sampled.

209   *2.8   DE stability characterization*

210   DE samples were placed onto the microscope slides and the microstructure was observed

211   under a light microscope (Olympus BX50) with a 10× objective lens. Images were taken

212   using a Moticam 10 camera *via* Motic Images Plus video acquisition software at 17fps. The

213   oil droplets size distribution of DE was determined from microscopic images using image

214   analysis software (ImageJ), by measuring the diameter of at least 500 oil droplets from 3

215   different samples of DE.

216 *2.9   Statistical analysis*

217 Microbial cell enumeration, physicochemical tests, and volatile compounds analysis were

218 conducted in triplicate and repeated in two independent experiments. The results were

219 presented as mean ± standard deviation. Significant differences among means were tested by

220 one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software Version 21 at

221 $p < 0.05$ and Tukey's test was applied for means comparison. Principal component analysis

222 (PCA) was performed using XLSTAT^{TM} version 2015.6.01.24027 (Addinsoft, New York,

223 NY) to reduce the dimensionality of the dataset and show the differences in volatile

224 compounds among the soy sauce samples. Observations/variables were chosen as data format

225 and Pearson's correlation matrix was used as PCA type.

226 **3.   Results and Discussion**

227 *3.1   The effect of viscosity on the stability of DE in moromi*

228 DEs were formulated using ingredients relevant to moromi constituents and soybean oil was

229 used as the oil phase. Since the reduced-salt moromi contained 6% NaCl, the internal W_1 and

230 external W_2 phase of DE also contained 6% NaCl. This aimed to balance the osmotic

231 pressure between the two phases, thus reducing instability of DE due to water movement

232 across the oil phase (Mezzenga, Folmer, & Hughes, 2004).

233 In order to describe the relationship between the viscosity of moromi and DE stability,

234 moromi formulations with different viscosities were tested by varying the ratio of koji:brine

235 (1:3, 1:5, and 1:7). The Power-Law model was used to describe the flow curves of the

236 moromi. The rheological parameters of this model are presented in Table S1. All the moromi

237 formulations exhibited non-Newtonian behavior at shear rates ranging between 0.1 and 100 s^{-}

238 ^{1} at 30 °C (Figure S1a). Moreover, the plot of the viscosity against shear rate of the koji and

239 brine mixtures yielded a flow index (n) of less than 1 (shear thinning), indicating that their

240   flow behavior had a non-Newtonian profile. Similar non-Newtonian behavior has been

241   reported for semi-solids of similar composition to koji which could be attributed to the

242   presence of high molecular weight components, such as proteins or dextrin (Manohar,

243   Manohar, & Rao, 1998).

244   DE maintained its microstructure after 4 weeks of fermentation (Figure 2a). However, the oil

245   globule size significantly decreased from 27.88 μm to 11.40 μm (Figure 2b and 2c). This

246   could be attributed to the high viscosity of the moromi system. The viscosity increased when

247   the amount of brine added was decreased (Figure S1a). After incorporation into the moromi

248   system, the DE stability was determined by observing its microstructure (i.e. inner W_1 phase)

249   using microscopy and monitoring the oil globule size. The initial oil globule size (31.84 μm)

250   decreased immediately after incorporation into the moromi slurry and during storage (Figure

251   S1b and S1c). However, the decrease in koji:brine 1:3 was more noticeable compared to

252   those with higher fractions of brine. By the end of storage, the oil globule size of DE in koji :

253   brine 1:3, 1:5, and 1:7 was 6.84 μm, 18.02 μm, and 15.29 μm, respectively. Moreover, all the

254   oil globules in koji:brine 1:3 completely lost their inner phase, while in koji:brine 1:5 and 1:7,

255   the DE structure was maintained (Figure S1d). These data indicate that DEs were destabilized

256   in the moromi system; however, the destabilization was not proportional to the viscosity of

257   the moromi.

258   *3.2   The effect of salt reduction and inoculation sequence on the growth of T. halophilus*

259   *and Z. rouxii*

260   Salt concentration is a significant parameter that determines soy sauce fermentation process

261   by affecting microbial growth. High salt concentration is typically used in soy sauce

262   fermentation, in order to suppress the growth of undesirable microorganism as well as

263   improving the organoleptic properties of the final product. *T. halophilus* growth was

12

264   suppressed during the first 2 weeks of fermentation (from 6.30 log CFU/mL to 4.17 log

265   CFU/mL) when 18% NaCl ($A_{[18\%]}$) was present in moromi (Figure 3a). Meanwhile, its

266   growth was significantly enhanced when part of the NaCl was replaced with KCl ($B_{[6:12\%]}$)

267   and maintained high viability, reaching 7.88 log CFU/mL. Interestingly, the growth of *T.*

268   *halophilus* in $A_{[18\%]}$ recovered after 2 weeks and exceeded $B_{[6:12\%]}$ by the end of incubation.

269   In any case, the growth was higher at the lowest salt concentration ($C1_{[6\%]}$, $C2_{[6\%]}$, $C3_{[6\%]}$)

270   throughout the fermentation, where the cell count sharply increased to 8.49 log CFU/mL

271   within the first week and remained stable throughout the incubation period. Although *T.*

272   *halophilus* is an osmophilic LAB that can tolerate up to 26% NaCl, it grows best at 5 to 10%

273   *w/v* (Taniguchi et al., 1988). Therefore, raising the NaCl concentration can increase the

274   osmotic stress, reducing the ability of *T. halophilus* to grow (Kobayashi et al., 2004). This

275   indicated that *T. halophilus* could not grow immediately after inoculation in the presence of

276   high NaCl concentration, as previously described by Taniguchi et al. (1988).

277   The growth of *T. halophilus* under reduced-salt environment was enhanced when it was

278   simultaneously inoculated with *Z. rouxii* ($C1_{[6\%]}$) compared to sequential inoculation ($C2_{[6\%]}$,

279   $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$). The addition of *Z. rouxii* from the early stage of

280   fermentation might have supplied a variety of metabolites such as pyruvate, amino acids, and

281   vitamins, which are essential for the early stage of bacterial growth (Devanthi et al., 2018;

282   Sudun, Wulijideligen, Arakawa, Miyamoto, & Miyamoto, 2013).

283   *Z. rouxii* was not affected significantly by salt concentration during the first 3 weeks of

284   fermentation. However, low-salt moromi ($C1_{[6\%]}$) suffered a decrease in its population at

285   Week 4, in contrast to the enhanced growth of *T. halophilus*. *Z. rouxii* is typically added to

286   enhance flavor and aroma formation in soy sauce production through alcoholic fermentation

287   (Van Der Sluis, Tramper, & Wijffels, 2001; Wah, Walaisri, Assavanig, Niamsiri, & Lertsiri,

288   2013). In a previous study by Singracha et al. (2017), the addition of *Z. rouxii* in combination

289   with *T. halophilus* and *Pichia guilliermondii* was shown to increase the total population of

290   lactic acid bacteria and yeast in reduced-salt moromi fermentation. Since *Z. rouxii* grows

291   optimally at low pH, *Z. rouxii* would be better added at the later stage of fermentation, once

292   moromi is acidified due to organic acids production by *T. halophilus*. In the present study, *Z.*

293   *rouxii* sequential inoculation (C2$_{[6\%]}$and C3$_{[6\%]}$) and gradual release in DE (C4$_{[6\%]}$) did not

294   have significant effect on growth, as this seemed to depend primarily on the salt formulation

295   and less on inoculation sequence (Figure 3b).

296   *3.3   Physicochemical changes during fermentation*

297   The changes in pH, reducing sugar, lactic acid, ethanol, and glutamic acid were measured to

298   monitor the fermentation progress, as they are associated with the growth of microorganisms

299   (Figure 4). Besides increasing in population during soy sauce fermentation, LAB also utilize

300   and convert carbohydrates into organic acids, which can bring the pH down. Reduction in pH

301   can also occur due to the accumulation of free fatty acids, amino acids, and peptides

302   containing carbonylic side chains, resulting from other microbial activities and raw materials

303   hydrolysis (Hoang et al., 2016; Van Der Sluis et al., 2001; Yanfang & Wenyi, 2009). As

304   shown in Figure 4a, pH of all moromi samples decreased from ~5.3 to final pH of ~4.8,

305   which was similar to values reported in previous studies of traditional Korean (Song, Jeong,

306   & Baik, 2015b) and reduced-salt soy sauce (Singracha et al., 2017). The pH decreased within

307   two weeks and then remained constant throughout the fermentation period, except for C1$_{[6\%]}$,

308   where pH increased to 5.49. The reduction in pH was associated with the increase in the

309   lactic acid amount produced by *T. halophilus* (Figure 4c). Although lactic acid production

310   was greatly suppressed by 18% NaCl, the reduction in pH was unaffected, which could be

311   due to production of other organic acids. Although *T. halophilus* is known as

312   homofermentative, some strains are regarded as heterofermentative and they are able to

313   produce acetic acid (Justé et al., 2012). Moreover, homofermentative strains of *T. halophilus*

14

314   are reported to undergo mixed acid fermentation under certain growth conditions (Wilred F.

315   M. Röling & van Verseveld, 1997).

316   The production of lactic acid was significantly lower in the presence of high salt

317   concentration, and high sodium content had a greater impact on the suppression (Figure 4c).

318   In low salt concentration, microorganisms are able to perform faster metabolic activity,

319   therefore producing higher amount of acids (Hoang et al., 2016). In the present study, lactic

320   acid production in reduced-salt moromi was enhanced when the inoculation of *Z. rouxii* was

321   modulated, sequentially or gradually by using DE. In co-inoculation, *Z. rouxii* might have

322   changed the physicochemical properties of the substrate, which could suppress the

323   fermentation of lactic acid by *T. halophilus*, as reported in a previous study (Devanthi et al.,

324   2018).

325   Reducing sugar is important during fermentation as it serves as a carbon source for microbial

326   growth as well as flavor and aroma formation. The initial content of total reducing sugar in

327   all moromi samples ranged from 2.68 to 3.49 g/L and it constantly decreased throughout the

328   incubation period (Figure 4b), which was in agreement with the previous study by Zhang,

329   Zhou, Cui, Huang, and Wu (2016). The reduction patterns were comparable regardless of salt

330   concentration and sequence of inoculation. During fermentation, reducing sugar is consumed

331   by microbes or possibly reacts with free amino acids during the Maillard reaction (Kim &

332   Lee, 2008). Since the fungal amylase, which breaks down the polysaccharide into simple

333   sugars, was heat-inactivated prior to the moromi stage, the amount of reducing sugar was

334   expected to decrease over time. The reducing sugar content in moromi decreased faster when

335   low salt concentration ($B_{[6:12\%]}$, $C1_{[6\%]}$, and $C4_{[6\%]}$) was used. This could be attributed to

336   faster metabolic activity of the microbes, which also corresponded to higher *T. halophilus*

337   population and lactic acid production (Hoang et al., 2016). Furthermore, the reducing sugar

338   content decreased at a slower rate when *Z. rouxii* was inoculated sequentially after 1 or 2

15

339   weeks of fermentation, but not when DE was used. This was expected since *Z. rouxii* is the

340   main user of sugar for biomass and ethanol production (Devanthi et al., 2018). The activity of

341   the released *Z. rouxii* cells might have caused faster sugar depletion in DE (C4$_{[6\%]}$).

342   Ethanol production was highly affected by variation in salt concentration and sequence of

343   inoculation (Figure 4d). In low-salt moromi (C1$_{[6\%]}$), the amount of ethanol constantly

344   decreased after 2 weeks of fermentation compared to a high concentration of salt (A$_{[18\%]}$and

345   B$_{[6:12\%]}$). However, the decrease in ethanol production was compensated when *Z. rouxii* was

346   added simultaneously (C2$_{[6\%]}$ and C3$_{[6\%]}$) or using DE (C4$_{[6\%]}$). Interestingly, ethanol

347   production with a similar pattern to A$_{[18\%]}$ and B and at highest concentration was achieved

348   when *Z. rouxii* was encapsulated in DE.

349   *Z. rouxii* is known to produce extracellular glutaminase, which is a proteolytic enzyme that

350   converts L-glutamine derived from soy protein to L-glutamic acid (Iyer & Singhal, 2008;

351   Kashyap, Sabu, Pandey, Szakacs, & Soccol, 2002). Unlike the glutaminase produced by koji

352   mold, *Z. rouxii* glutaminase is more tolerant against high salinity. L-Glutamic acid is essential

353   for improving the flavor of the final product since it contributes to the "umami" taste of the

354   soy sauce. Therefore, high activity of glutaminase is desirable, in order to increase the

355   production of L-glutamic acid. As shown in Figure 4e, the amount of glutamic acid increased

356   after the fermentation process and the final concentration of glutamic acid between samples

357   did not differ significantly ($p > 0.05$).

358   *3.4   Formation of volatile compounds*

359   A total of 38 volatile compounds was detected in the moromi samples using SPME-GC/MS,

360   including 15 alcohols, 5 acids, 8 aldehydes, 4 esters, 1 furan, 1 phenol, 3 ketones, and 1

361   alkene (Table 1). Alcohol was found to be the most abundant compound in all samples,

362 comprising more than 90% of the total volatiles, as previously found in high-salt liquid state

363 fermentation, low-salt solid-state fermentation, and Koikuchi soy sauce (Feng et al., 2015).

364 Salt reduction ($C1_{[6\%]}$) was shown to have a great influence on the volatiles production in

365 moromi, especially alcohols (Table 1). Yeasts contribute to the formation of alcohols through

366 the reduction of related aldehydes (Sun, Jiang, & Zhao, 2010; Van Der Sluis et al., 2001).

367 Lowering salt concentration to 6% *w/v* ($C1_{[6\%]}$) significantly ($p < 0.05$) enhanced the

368 production of 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-methyl-1-butanol, 5-

369 nonanol, and phenylethyl alcohol. On the other hand, the production of ethanol and propanol

370 was reduced in low salt concentration ($C1_{[6\%]}$), which was in agreement with the previously

371 studied reduced-salt Korean soy sauce (Song, Jeong, & Baik, 2015a). Partial salt substitution

372 with KCl ($B_{[6:12\%]}$) did not affect the production of most volatile compounds, except for 2-

373 furanmethanol, 2-methoxy-5-methyphenol, and 2-methyl-1-propanol which were

374 significantly ($p < 0.05$) lower compared to sample $A_{[18\%]}$. In previous studies reported by

375 Sasaki (1996) and Jansen, Veurink, Euverink, & Dijkhuizen (2003), the production of higher

376 alcohols, including phenylethyl alcohol, 3-methyl-1-butanol, 1-propanol, and 2-methyl-1-

377 propanol, was found to decrease with an increase of NaCl concentration. However, the

378 amounts of 1-propanol and 2-methyl-1-propanol decreased under reduced NaCl conditions

379 ($B_{[6:12\%]}$). This might have arisen from decreasing uptake of the related amino acid by yeast,

380 since these compounds are mainly produced by *Z. rouxii* from their corresponding branched-

381 chain amino acids *via* the Ehrlich pathway (Van Der Sluis et al., 2001). The method of

382 inoculation was found to affect the production of most alcohols in the reduced-salt moromi.

383 Moromi with similar flavor pattern to those containing high salt concentrations ($A_{[18\%]}$ and

384 $B_{[6:12\%]}$) was achieved when *Z. rouxii* was added sequentially at Week 1 ($C2_{[6\%]}$) or using DE

385 ($C4_{[6\%]}$). The addition of *Z. rouxii* at Week 2 resulted in significantly ($p < 0.05$) lower

386 amounts of 2-furanmethanol, 3-methyl-1-butanol, ethanol, 1-heptanol, 1-hexanol, and 1-

387 propanol. This result corresponds to the ethanol measurement during fermentation by using

388 enzymatic reaction (Figure 4d).

389 Salt reduction was also found to affect the production of several acids. The amount of 4-

390 methyl-2-oxovaleric acid was enhanced in reduced-salt moromi, only when *Z. rouxii* was

391 added simultaneously. Meanwhile, 2-methylpropanoic acid, which contributes to cheese/fatty

392 odor, was found to be significantly lower in all reduced-salt moromi samples. However,

393 noticeably higher amount of 2-methylpropanoic acid was detected when *Z. rouxii* was added

394 at Week 2. The production of some acids, including 3-methylbutanoic acid (cheese/sweet)

395 and acetic acid (sour/vinegar-like odor), was found to be enhanced when *Z. rouxii* inoculation

396 was delayed for 2 weeks. Acetic acid production was also similar when DE was used. These

397 acids have been reported as the highest odor-active compounds in Chinese soy sauce (Feng et

398 al., 2014). Among these acids, 2-methylpropanoic acid and 3-methylbutanoic acid are formed

399 *via* branched-chain α-keto acid catabolism (Song et al., 2015b)

400 Aldehydes contribute to nutty and malty aroma in soy sauce (Feng et al., 2015). In the present

401 study, most aldehyde compounds were not affected by salt reduction, except for 2-

402 methylpropanal which was significantly enhanced in reduced-salt moromi when mixed

403 cultures were added simultaneously ($C1_{[6\%]}$). This branched-chain aldehyde is considered as

404 an important flavor compound, perceived as malty, chocolate-like, with low taste threshold

405 (Smit, Engels, & Smit, 2009). It is generated from branched-chain amino acid valine *via*

406 Strecker degradation or microbial activity, which then can be converted to its corresponding

407 alcohol (2-methyl-1-propanol) and/or acid (2-methylpropanoic acid) (Ardö, 2006; Song et al.,

408 2015a). The effect of modulating the inoculation time of *Z. rouxii* on aldehydes formation

409 was hardly seen, except for benzaldehyde (burnt sugar/sweet) and furfural (bread/sweet),

410 which were significantly enhanced in $C2_{[6\%]}$ and $C4_{[6\%]}$, respectively.

18

411 Replacing NaCl with KCl decreased the amount of 2-phenylethyl acetate, which contributes

412 to honey, rosy odor. However, this could be compensated for by adding *Z. rouxii* at Week 1

413 of the fermentation process. In our previous study, it was found that the production of 2-

414 phenylethyl acetate could be enhanced by adding *Z. rouxii* sequentially rather than

415 simultaneously (Devanthi et al., 2018). *Z. rouxii* enhances the production of esters (Van Der

416 Sluis et al., 2001), although the production of isoamyl acetate (banana aroma) was

417 significantly enhanced in reduced-salt moromi. This was only observed in $C1_{[6\%]}$, while the

418 amount of isoamyl acetate in $C2_{[6\%]}$, $C3_{[6\%]}$, and $C4_{[6\%]}$ was similar to that at high salt

419 concentration ($A_{[18\%]}$ and $B_{[6:12\%]}$).

420 The only furan and phenol compounds detected in all moromi samples were 3-acetyl-2,5-

421 dimethylfuran and 2-methoxy-5-methylphenol, respectively. These were produced in

422 negligible amount when either salt or NaCl were reduced, except when *Z. rouxii* was added at

423 Week 1. Several ketones, such as 3-methyl-2-pentanone and acetoin, were produced at

424 significantly higher concentrations in reduced-salt moromi. The amounts of these compounds

425 were similar to moromi containing high salt ($A_{[18\%]}$ and $B_{[6:12\%]}$) when *Z. rouxii* was added

426 sequentially, with or without DE.

427 *3.5 Principal component analysis*

428 PCA analysis was conducted, in order to gain more understanding on the relationship

429 between the fermentation conditions and profiles of volatile compounds. The first (PC1) and

430 second principal component (PC2) accounted for 30.60% and 21.43% of the total variance,

431 respectively (Figure 5a-b). The PCA score plot demonstrates distinct separation of some

432 moromi samples (Figure 5a). In the case of co-inoculated samples, low salt moromi sample

433 ($C1_{[6\%]}$) was differentiated from high salt moromi sample ($A_{[18\%]}$) while reduced NaCl sample

434 (substituted with 12% KCl; B) was positioned in the middle of PC1. This indicates that salt

435   reduction affected the aroma profile of moromi. Replacing part of NaCl with KCl (B$_{[6:12\%]}$)

436   was associated with lower content of 2-furanmethanol, 2-methyl-1-propanol, 2-phenylethyl

437   acetate, 3-acetyl-2,5-dimethylfuran, and 2-methoxy-5-methylphenol. C1$_{[6\%]}$ was associated

438   with high amounts of 3-methyl-1-butanol, phenylethyl alcohol, 2,4-dimethyl-3-pentanol, 2,6-

439   dimethyl-4-heptanol, isoamyl acetate, 2-methylpropanal, 4-methyl-2-oxovaleric acid, 3-

440   methyl-2-pentanone, and acetoin (Figure 5b).

441   The method of inoculation was found to affect the aroma profiles, and adding *Z. rouxii*

442   encapsulated in DE or sequentially after 1 week matched the aroma profile obtained with

443   high salt concentration. This was not the case when *Z. rouxii* was added sequentially after 2

444   weeks of fermentation. Clustering of samples A$_{[18\%]}$, C2$_{[6\%]}$, and C4$_{[6\%]}$was influenced by

445   compounds such as 2-furanmethanol, 2,4-dimethyl-3-pentanol, 2,6-dimethyl-4-heptanol, 3-

446   methyl-1-butanol, 5-nonanol, ethanol, 1-hexanol, methanol, phenylethyl alcohol, 4-methyl-2-

447   oxovaleric acid, 3-methylbutanoic acid, acetic acid, propionic acid, 2-methylbutanal, 2-

448   methylpropanal, 3-methylbutanal, furfural, hexanal, pentanal, propanal, ethyl acetate, ethyl

449   propionate, isoamyl acetate, 3-methyl-2-pentanone, acetoin, acetone, and D-limonene.

450   **4.   Conclusion**

451   Salt reduction could affect microbial growth and physicochemical changes during moromi

452   fermentation. Low salt concentrations could promote *T. halophilus* growth and enhance lactic

453   acid production. However, the final overall aroma balance differed from the original soy

454   sauce fermented with high salt concentration, indicated by lower content of some alcohols,

455   acids, esters, furan, and phenol. The use of DE for delivering the mixed cultures of *T.*

456   *halophilus* and *Z. rouxii* in reduced-salt moromi could compensate for such changes by

457   promoting the formation of some essential volatile compounds, including alcohols (e.g., 2-

458   furanmethanol and ethanol) and esters (e.g., 2-phenylethyl acetate). This indicates the

459 possibility of producing soy sauce in a low salt environment with a volatile profile pattern

460 identical to the original high-salt soy sauce. The results obtained in this study provide the soy

461 sauce industry with a new technique for standardizing the microbial activity and aroma

462 development, which also offers health benefits to the consumers, due to low salt content in

463 the final product. However, since modulating the release has a great impact on the aroma

464 formation, further study is needed in order to tailor the physicochemical properties of DE,

465 therefore enabling the cell release in a more controlled manner.

466 **5.   Acknowledgement**

469 **6.   References**

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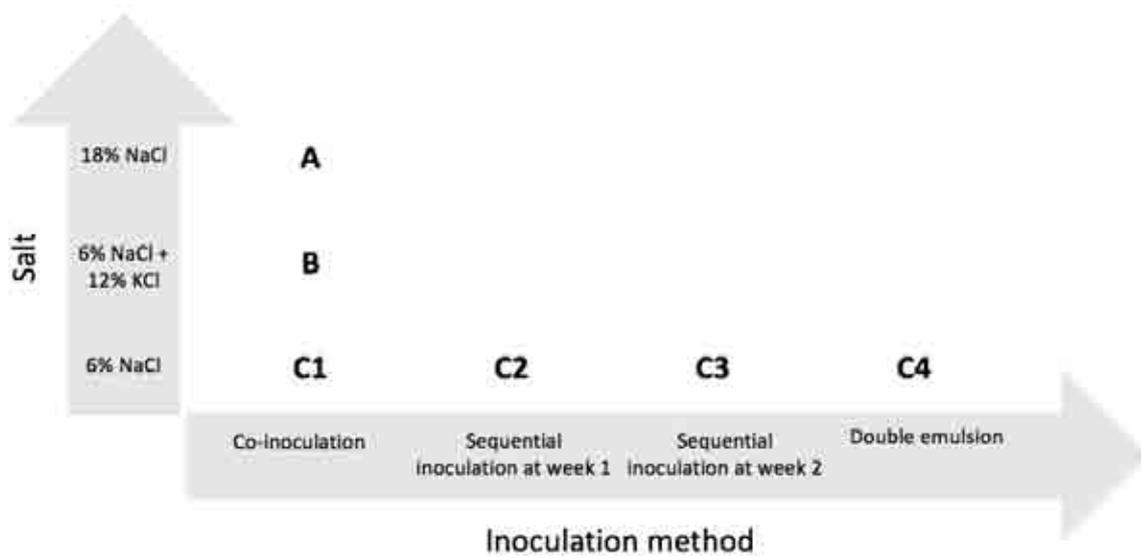
593   Figure 1. Set of moromi samples varying in salt composition and inoculation method
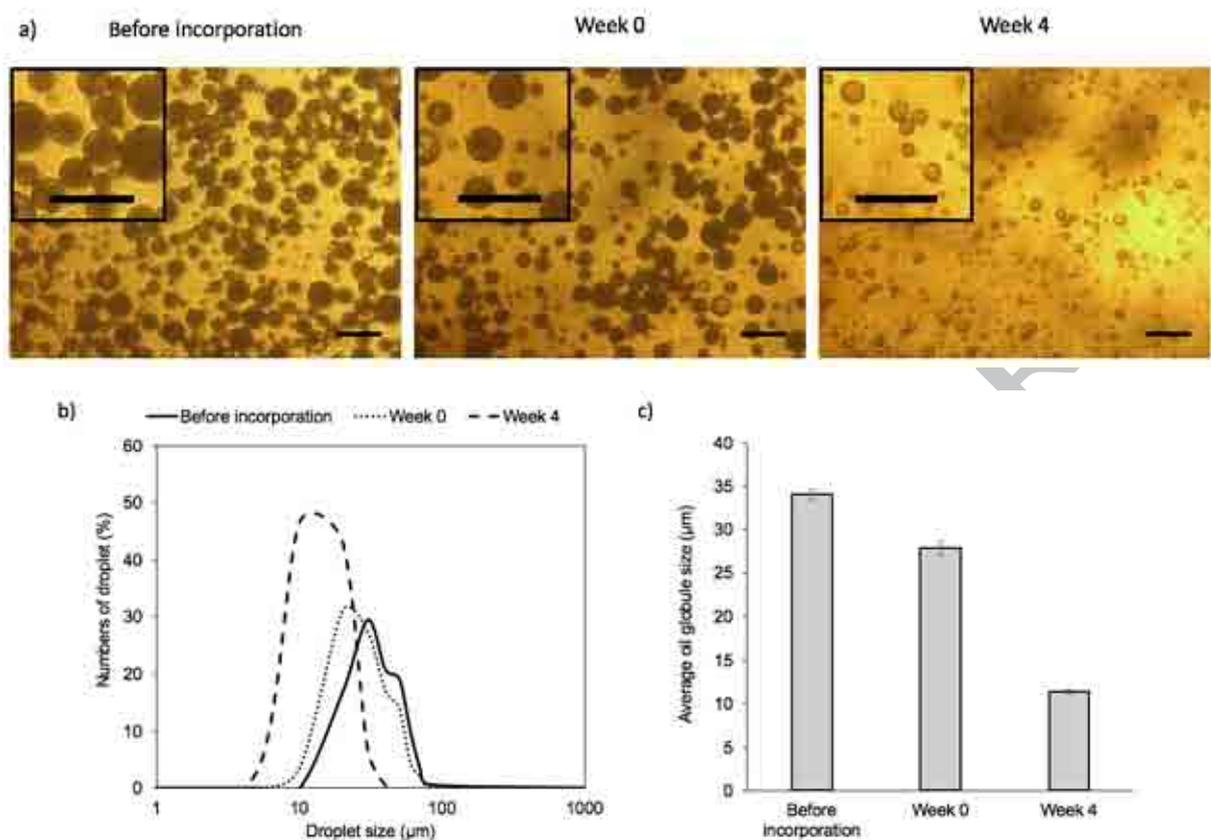
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606 Figure 2. (a) Optical micrograph of $W_1/O/W_2$ DE before and after incorporation into moromi,

607 and after 4 weeks of fermentation. Scale bar: 100 μm. (b) Oil globule size distribution before

608 and after fermentation. (c) Average oil globule size before and after fermentation.

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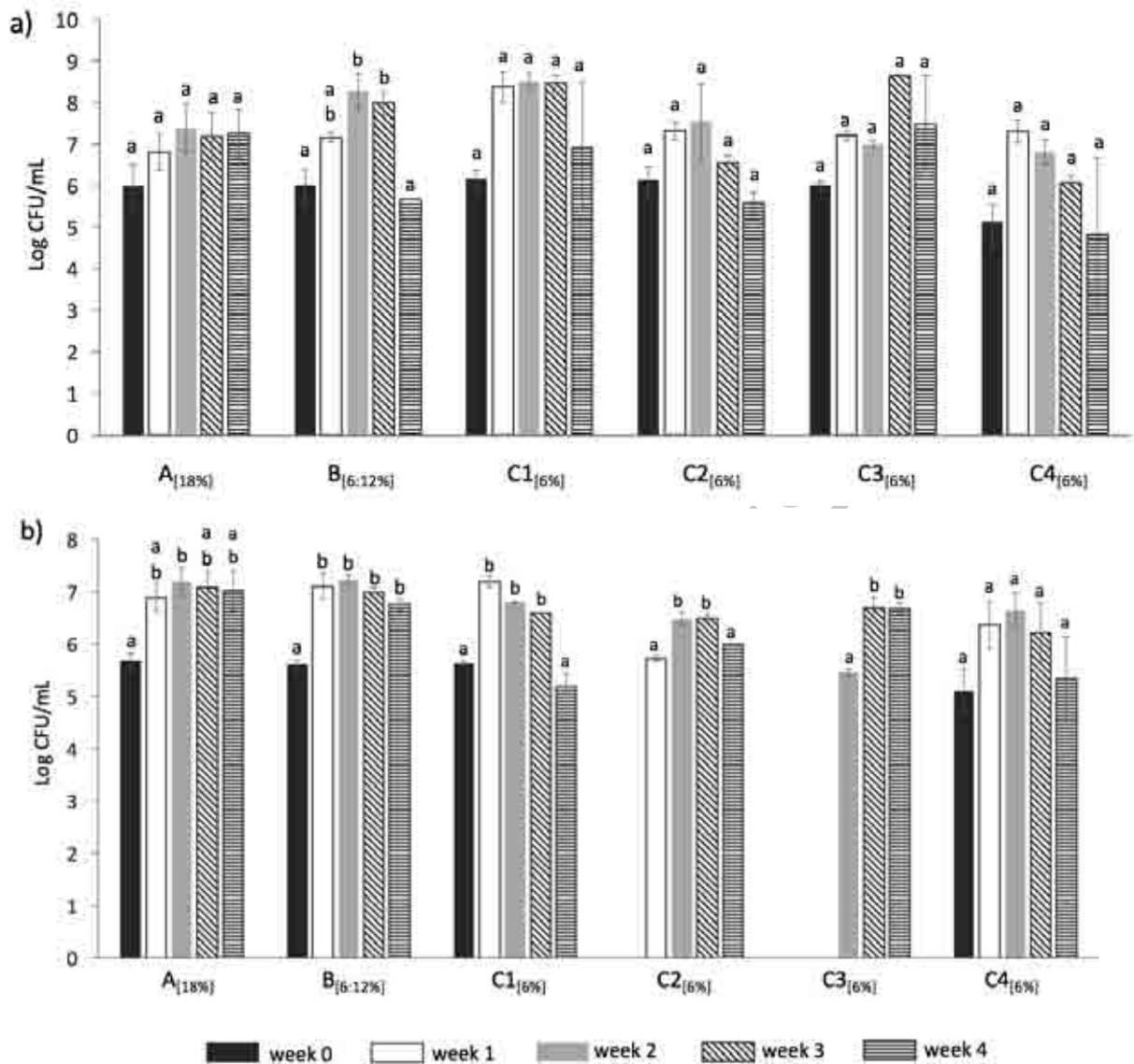
Figure 3. Changes in population of (a) *T. halophilus* and (b) *Z. rouxii* during fermentation of low and high salt moromi at 30 °C. The samples contained co-inoculated *T. halophilus* and *Z. rouxii* in 18% NaCl (A$_{[18\%]}$), 6% NaCl and 12% KCl (B$_{[6:12\%]}$), 6% NaCl (C1$_{[6\%]}$), and sequentially inoculated *T. halophilus* and *Z. rouxii* at week 1 (C2$_{[6\%]}$), week 2 (C3$_{[6\%]}$), or with DE (C4$_{[6\%]}$). The addition time of *Z. rouxii* cells for sequential inoculation is indicated by the asterisk mark (*). Means within the same group with different letters (a, b, c) are significantly different (p<0.05).

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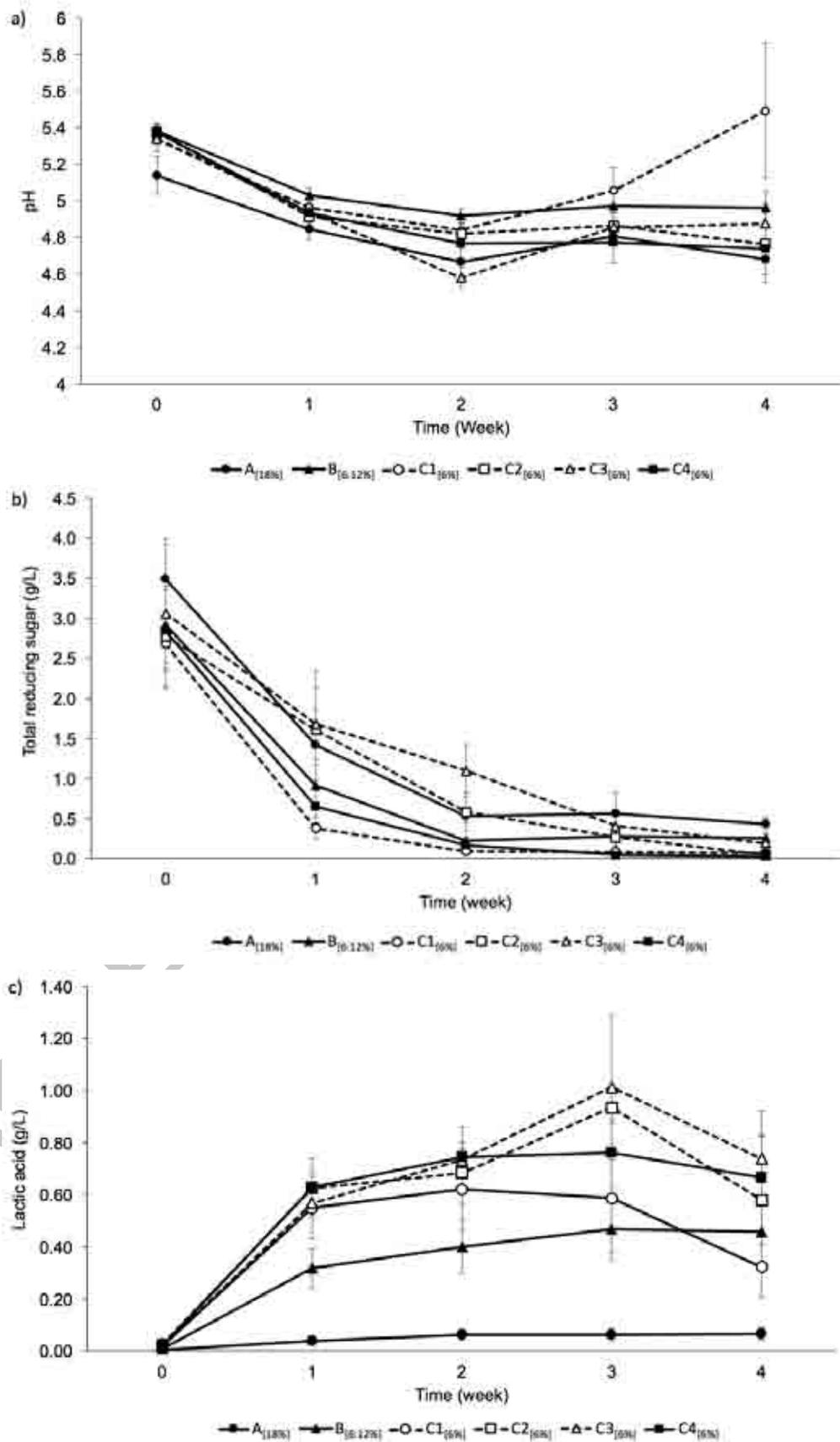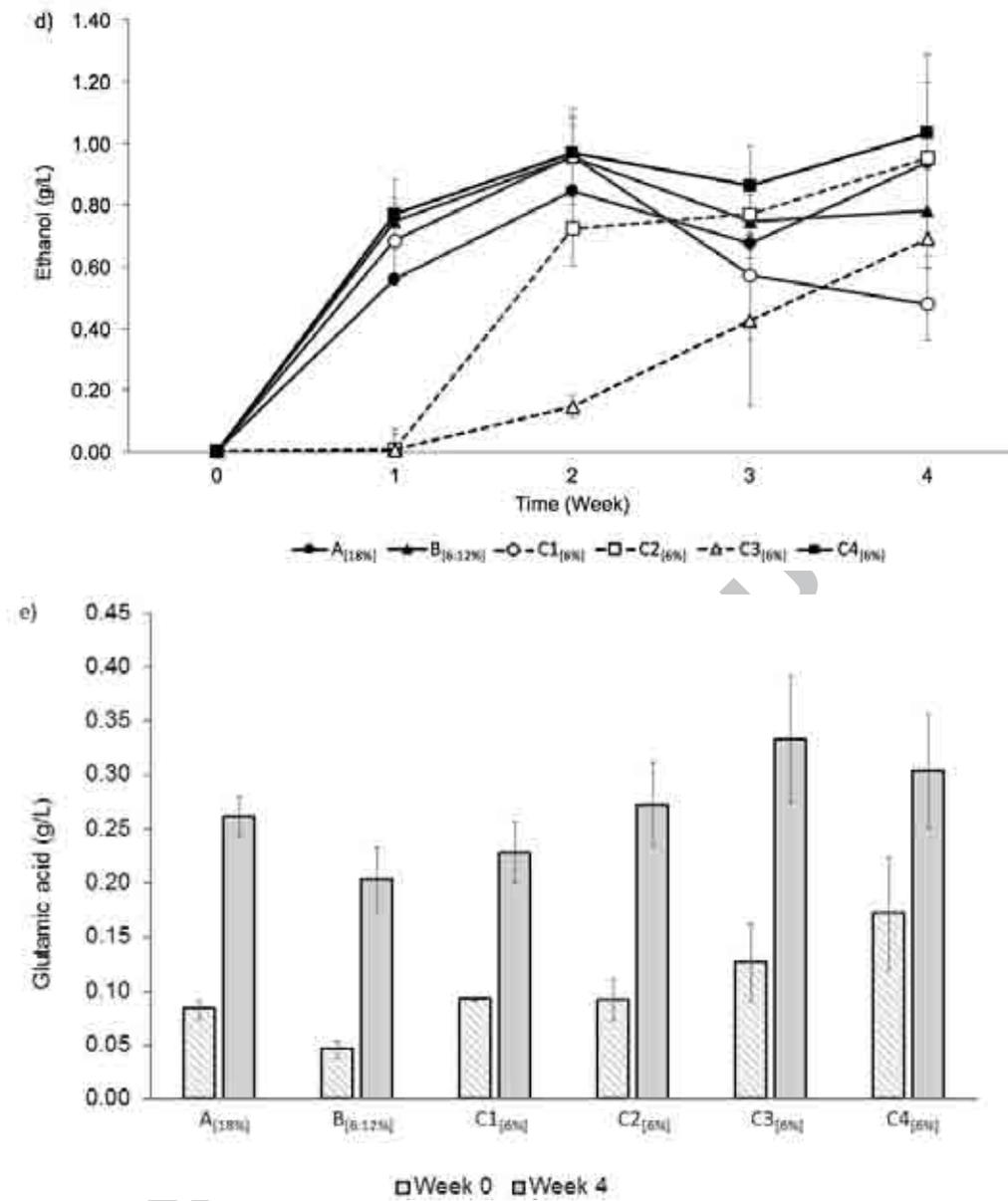
Figure 4. Changes in (a) pH, (b) total reducing sugar, (c) lactic acid, (d) ethanol, and (e) glutamic acid during fermentation of low and high salt moromi at 30 °C.

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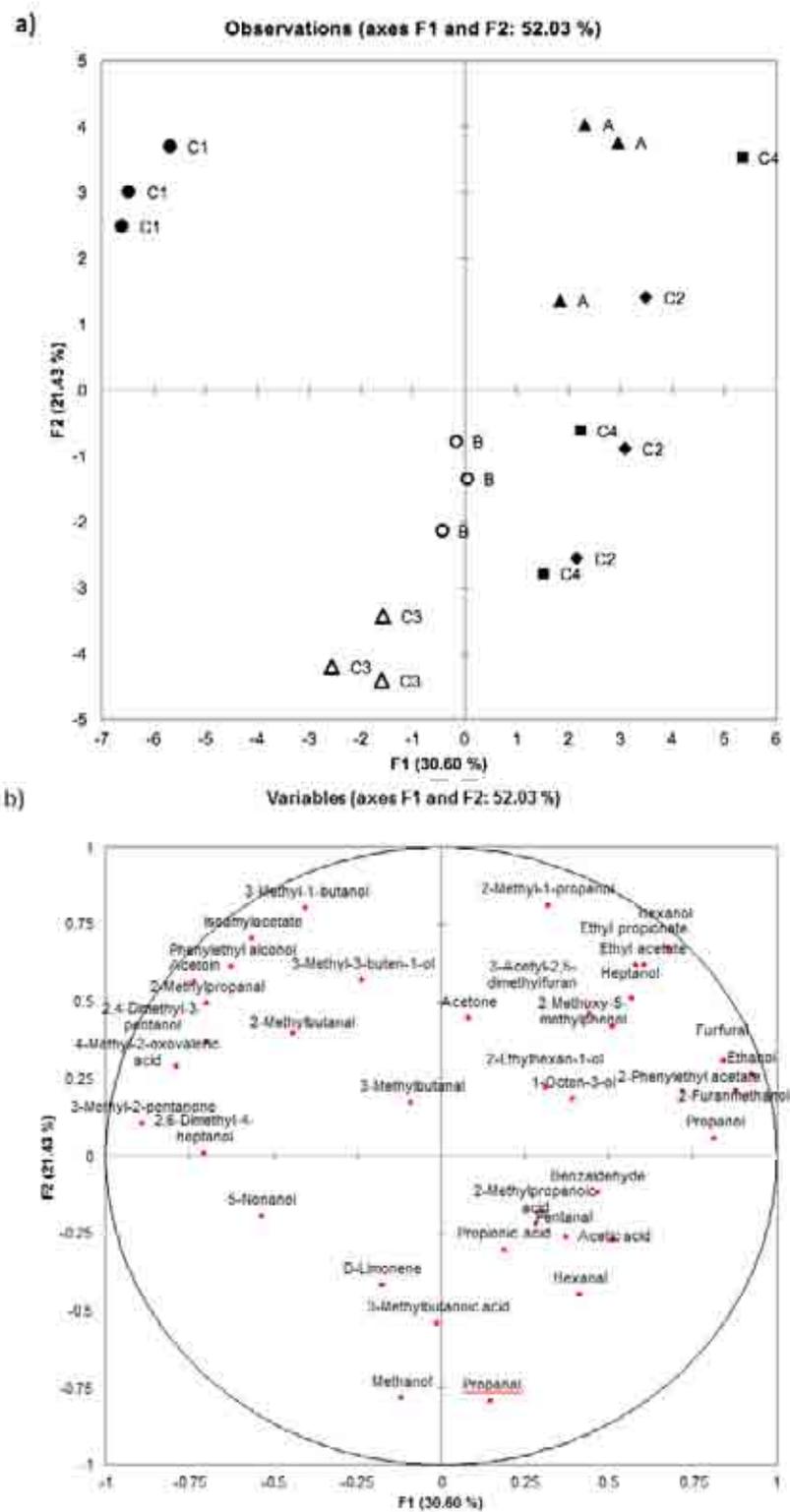
Figure 5. (a) PCA score plot of six moromi samples after 4-week fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements. (b) PCA loading plot of the aroma compounds detected in moromi after 4-week fermentation.

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634

635   **Table 1.** Aroma compounds found in moromi after 4 weeks of fermentation in low and high

636   salt concentration. The values are relative to the peak area observed when the headspace

637   above a 0.1 μg/mL 1-octen-3-ol solution was analyzed. Each value is based on three

638   replicates.

Compound	LRI[1]	Day 30							
		A[18%]		B[6:12%]		C1[6%]		C2[6%]	
		mean	SD	mean	SD	mean	SD	mean	SD
Alcohols									
1-octen-3-ol	1466	0.039 a	0.009	0.020 a	0.002	0.037 a	0.006	0.086 b	0.035
2-ethyl-1-hexanol	1508	0.029 a	0.003	0.028 ab	0.008	0.014 abc	0.007	0.025 abc	0.011
2-furanmethanol	1690	0.084 a	0.006	0.046 b	0.003	0.039 b	0.002	0.089 a	0.011
2-methyl-1-propanol	1115	0.385 a	0.055	0.186 bc	0.019	0.204 b	0.070	0.159 bc	0.030
2,4-dimethyl-3-pentanol	1395	0.013 a	0.001	0.019 a	0.001	0.298 b	0.203	0.021 a	0.004
2,6-dimethyl-4-heptanol	1506	0.031 ab	0.010	0.005 a	0.001	0.090 b	0.025	0.032 ab	0.006

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3-methyl-3-buten-1-ol	1271	0.026	a	0.004	0.021	a	0.002	0.019	a	0.004	0.004	b	0.001
3-methyl-1-butanol	1225	25.061	a	3.033	18.089	ac	1.609	40.297	b	8.876	19.532	ac	3.675
5-nonanol	1473	0.002	a	0.000	0.001	a	0.000	0.006	b	0.003	0.005	ab	0.002
ethanol	950	33.709	a	2.159	27.538	ab	1.129	14.398	b	1.106	39.284	a	3.380
1-heptanol	1473	0.042	ab	0.006	0.016	a	0.002	0.016	a	0.002	0.015	a	0.006
1-hexanol	1371	0.071	a	0.012	0.033	ab	0.002	0.032	ab	0.010	0.046	ab	0.018
methanol	915	0.449	a	0.025	0.511	a	0.065	0.488	a	0.088	0.604	a	0.082
phenylethyl alcohol	1957	2.425	a	0.371	2.059	ac	0.121	3.801	b	0.352	2.010	ac	0.193
1-propanol	1057	0.376	ac	0.030	0.281	ab	0.023	0.180	b	0.030	0.468	cd	0.057
acids												
4-methyl-2-oxovaleric acid	1478	0.016	ac	0.005	0.006	a	0.000	0.040	b	0.006	0.012	ac	0.004
2-methylpropanoic acid	1596	0.113	a	0.046	0.057	ab	0.025	0.000	b	0.000	0.025	bc	0.007
3-methylbutanoic	1699	0.400	ab	0.153	0.205	a	0.081	0.008	a	0.012	0.116	a	0.103

acid

	RI												
acetic acid	1481	0.161	ab	0.098	0.041	ab	0.040	0.000	a	0.000	0.212	ab	0.055
propionic acid	1565	0.010	a	0.010	0.018	a	0.023	0.015	a	0.025	0.061	a	0.090
aldehydes													
2-methylbutanal	929	0.009	a	0.002	0.001	a	0.000	0.026	a	0.027	0.009	a	0.009
2-methylpropanal	824	0.026	a	0.009	0.009	a	0.002	0.127	b	0.057	0.027	a	0.022
3-methylbutanal	934	0.083	a	0.013	0.012	a	0.002	0.091	a	0.063	0.109	a	0.114
benzaldehyde	1568	0.021	a	0.004	0.034	ab	0.001	0.013	a	0.001	0.060	b	0.030
furfural	1500	0.014	ab	0.002	0.010	ab	0.001	0.009	a	0.000	0.015	b	0.003
hexanal	1104	0.001	a	0.001	0.002	a	0.001	0.001	a	0.000	0.003	a	0.001
pentanal	1001	0.014	a	0.007	0.015	a	0.007	0.015	a	0.004	0.029	a	0.010
propanal	807	0.004	a	0.000	0.005	a	0.001	0.003	a	0.001	0.004	a	0.002
esters													
2-phenylethyl acetate	1860	0.316	ac	0.054	0.136	b	0.006	0.094	b	0.024	0.378	c	0.071
ethyl acetate	906	0.196	a	0.046	0.079	a	0.026	0.073	a	0.023	0.100	a	0.077

ethyl propionate	975	0.032	ab	0.008	0.006	a	0.000	0.010	ab	0.005	0.011	ab	0.009
isoamyl acetate	1141	0.256	a	0.074	0.029	a	0.006	0.878	b	0.280	0.115	a	0.119
furan													
3-acetyl-2,5-dimethylfuran	1450	0.186	a	0.061	0.001	bc	0.000	0.001	b	0.000	0.069	c	0.014
phenol													
2-methoxy-5-methylphenol	1614	0.258	a	0.081	0.001	b	0.000	0.001	b	0.000	0.169	a	0.035
ketone													
3-methyl-2-pentanone	1037	0.004	a	0.001	0.009	ac	0.001	0.032	b	0.011	0.000	a	0.000
acetoin	1318	0.656	a	0.039	0.451	a	0.043	1.624	b	0.572	0.066	a	0.013
acetone	829	0.671	a	0.088	0.334	a	0.082	0.507	a	0.084	0.405	a	0.107

Others

D-limonene	1217	0.006	a	0.003	0.001	a	0.001	0.004	a	0.004	0.002	a	0.001

639   [1]LRI: linear retention indices of the compounds relative to an alkane series.

640   Means within the same row with different letters (a, b, c) are significantly different ($p < 0.05$)

641     $A_{[18\%]}$: Co-inoculation; 18% NaCl

642     $B_{[6:12\%]}$: Co-inoculation; 6% NaCl and 12% KCl

643     $C1_{[6\%]}$: Co-inoculation; 6% NaCl

644     $C2_{[6\%]}$: Sequential inoculation starting at Week 1; 6% NaCl

645     $C3_{[6\%]}$: Sequential inoculation starting at Week 2; 6% NaCl

646     $C4_{[6\%]}$: Inoculation with DE; 6% NaCl

647

648

649   Highlights

650   • First study to utilize $W_1/O/W_2$ double emulsion (DE) in low-salt moromi

651   fermentation

652   • DE stability was dependent on but not proportional to moromi viscosity

653   • DE was utilized to control the inoculation of soy sauce starter cultures

654   • Volatile profile of low-salt moromi fermented with DE resembled that of high-salt

655   sample

656   • Sequential inoculation affected fermentation and volatile compounds formation

657