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DOI:
10.1016/j.foodchem.2018.03.022

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Document Version
Peer reviewed version

Citation for published version (Harvard):
Devanthi, PV, Linforth, R, El Kadri, H & Gkatzionis, K 2018, 'Water-in-oil-in-water double emulsion for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce', *Food Chemistry*, vol. 257, pp. 243-251. https://doi.org/10.1016/j.foodchem.2018.03.022

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PII: S0308-8146(18)30436-9
DOI: https://doi.org/10.1016/j.foodchem.2018.03.022
Reference: FOCH 22565

To appear in: *Food Chemistry*

Received Date: 28 November 2017
Revised Date: 25 February 2018
Accepted Date: 6 March 2018

Please cite this article as: Devanthi, P.V.P., Linforth, R., El Kadri, H., Gkatzionis, K., Water-in-oil-in-water double emulsion for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.03.022

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Water-in-oil-in-water double emulsion for the delivery of starter cultures in reduced-salt moromi fermentation of soy sauce

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Abstract

This study investigated the application of water-oil-water ($W_1/O/W_2$) double emulsions (DE) for yeast encapsulation and sequential inoculation of *Zygosaccharomyces rouxii* and *Tetragenococcus halophilus* in moromi stage of soy sauce fermentation with reduced NaCl and/or substitution with KCl. *Z. rouxii* and *T. halophilus* were incorporated in the internal W_1 and external W_2 phase of DE, respectively. NaCl reduction and substitution promoted *T. halophilus* growth to 8.88 log CFU/mL, accompanied with faster sugar depletion and enhanced lactic acid production. Reducing NaCl without substitution increased the final pH (5.49) and decreased alcohols, acids, esters, furan and phenol content. However, the application of DE resulted in moromi with similar microbiological and physicochemical characteristics to that of high-salt. . Principal component analysis of GC-MS data

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demonstrated that the reduced-salt moromi had identical aroma profile to that obtained in the standard one, indicating the feasibility of producing low-salt soy sauce without compromising its quality.

Keywords: Soy sauce; Moromi fermentation; Salt reduction; $W_1/O/W_2$ double emulsion; Yeast encapsulation; Sequential inoculation; Aroma compounds; GC-MS.

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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

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temperature prior to fermentation (Muramatsu, Sano, Uzuka, & Company, 1993). Nevertheless, the absence of salt during fermentation may result in the growth of spoilage microorganisms and the quality of final product can differ from the original. Salt reduction during moromi fermentation could result in lower content of essential acids, alcohols, and esters, and higher acidity content (Song et al., 2015a). Such problems could be counteracted by the addition of mixed cultures of indigenous yeast species (Song et al., 2015a) as well as combining LAB and yeasts (Singracha et al., 2017).

However, a recent study showed that the final aroma profile in moromi fermentation was compromised due to antagonism between co-inoculated *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* while their sequential inoculation could improve the aroma complexity (Devanthi, Linforth, Onyeaka, & Gkatzionis, 2018). The application of sequential inoculation of mixed cultures has been reported to improve flavor quality of fermented foods and beverages. Modulation of the inoculation time was found to be key in achieving the desired quality of apple cider (Ye, Yue, & Yuan, 2014). Furthermore, in whey fermentation, sequential inoculation of *Kluyveromyces lactis* B10 and *Torulaspora delbrueckii* B14 after 48 h improved volatile compounds production (e.g. alcohols and esters) (Andrade, Melo, Genisheva, Schwan, & Duarte, 2017). Higher production of 3-sulfanylhexyl acetate (3SHA) and 3SH (3-sulfanyl-1-hexanol), which are the most important volatiles in Sauvignon blanc aroma, has been achieved with sequential culture of *T. delbrueckii* and *S. cerevisiae*.

A formulation is needed to control the sequential delivery and activity of microbial cultures in soy sauce fermentation. Water-in-oil-in-water ($W_1/O/W_2$) double emulsions (DE) have been studied in recent years for their ability to encapsulate hydrophilic substances, including bacteria for protection and controlled release. Their multi-compartmentalized structure is created by dispersing a water-in-oil (W_1/O) emulsion in another aqueous phase (W_2). Recent studies have focused on probiotic bacteria encapsulation in DE for enhancing survival during

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digestion (Eslami, Davarpanah, & Vahabzadeh, 2016; Shima, Morita, Yamashita, & Adachi, 2006). The instability of DE structure can be used to modulate the release of bacterial cells by utilizing changes in osmotic balance (El Kadri, Gun, Overton, Bakalis, & Gkatzionis, 2016; El Kadri, Overton, Bakalis, & Gkatzionis, 2015) as they would occur during fermentation. A previous study demonstrated that the inherent DE instability acted as a mechanism for gradual release of *Z. rouxii*, which could be linked to changes in glucose concentration in the medium (Devanthi, El Kadri, Bowden, Spyropoulos, & Gkatzionis, 2018).

In this study, the application of DE for the encapsulation and sequential delivery of *T. halophilus* and *Z. rouxii* cultures was tested in conditions reflecting moromi fermentation with reduced NaCl content and/or substitution with KCl. The stability of DE in moromi was examined by monitoring its microstructure, oil globules size, and distribution. Furthermore, microbial population and physicochemical changes as well as volatile compounds formation were monitored.

2. Materials and Methods

2.1 Materials, chemicals, and microorganisms

Soy and wheat flour were purchased from a local retailer (UK). *Aspergillus oryzae* 126842 was purchased from Centre for Agriculture and Biosciences International (Egham, UK). *Tetragenococcus halophilus* 9477 and *Zygosaccharomyces rouxii* 1682 were purchased from National Collection of Industrial Food and Marine Bacteria Ltd. (Aberdeen, UK) and National Collection of Yeast Cultures (Norwich, UK), respectively. Sodium chloride (NaCl, extra pure) was purchased from Acros Organics (Fairlawn, NJ). Microbiological growth media used were Czapex Dox Agar (CDA; Oxoid Ltd., Basingstoke, UK), Brain Heart Infusion agar (BHI, Oxoid Ltd., UK), de Man, Rogosa, and Sharpe broth (MRS broth, Oxoid Ltd., UK), Yeast Malt agar (YM agar, Sigma-Aldrich, Gillingham, UK), Yeast Malt broth

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(YM broth, Sigma-Aldrich, UK). Bacteria and yeast growth were controlled using chloramphenicol (Oxoid Ltd., UK) and natamycin (Sigma-Aldrich, UK), respectively. 1-Octen-3-ol (purity ≥98%) was purchased from Sigma-Aldrich. Soybean oil (Alfa Aesar, Heysham, UK) was used as the oil phase of the DE. Polysorbate 80 (Tween 80, Sigma-Aldrich, United Kingdom) and polyglycerol polyricinoleate (PGPR, Danisco A/S, Copenhagen, Denmark) were used as water and oil soluble emulsifiers, respectively.

2.2 Culture preparation

Aspergillus oryzae was maintained on CDA at 25 °C. The spore suspension of *A. oryzae* was prepared according to the method described by Chou and Ling (1998) with slight modification. Briefly, spores were obtained by growing *A. oryzae* on CDA at 25 °C for 7 days. NaCl solution (0.85%, *w/v*) solution containing 0.01% of Tween 80 (Sigma-Aldrich, UK) was added into the agar slant bottle followed by vigorous mixing to collect the spores. The number of spores were counted using an improved Neubauer hemocytometer and adjusted to 10^6 spores/mL. *Tetragenococcus halophilus* was maintained on BHI with 10% (*w/v*) NaCl and incubated at 37 °C. *T. halophilus* was grown in MRS broth with 7% NaCl for 36 h and the cell concentration was adjusted to a final concentration of 10^6 cells/mL. *Zygosaccharomyces rouxii* was maintained on YM agar with 5% (*w/v*) NaCl and incubated at 25 °C. The inoculum was prepared by growing *Z. rouxii* in YM broth containing 5% (*w/v*) NaCl in a 30 °C shaker incubator for 24 h and cell concentration was adjusted to 10^6 cells/mL.

2.3 DE preparation

The DEs were prepared using a 2-step emulsification method at ambient temperature by using a high shear mixer (Silverson L5M). In the first step, W_1/O primary emulsion was prepared by mixing sterile 6% (*w/v*) NaCl solution into the oil phase (soybean oil with 2% wt

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PGPR) at W_1:oil phase ratio of 20:80 at 1700 rpm for 2 min. For yeast encapsulation, *Z. rouxii* suspension in 6% (*w/v*) NaCl solution (10^7 cells/mL) was used as W_1.

In the second stage, W_1/O was re-emulsified in the continuous phase (W_2;sterile 6% (*w/v*) NaCl in water with 1% wt Tween 80) at 2000 rpm for 1 min (W_1/O:W_2 ratio of 20:80). The final concentration of encapsulated *Z. rouxii* cells was ~10^5 cells/mL. DEs containing *T. halophilus* in the W_2 were prepared by directly adding 2 mL of *T. halophilus* (10^6 cells/mL) into the W_2 after the mixing process.

2.4 Soy sauce fermentation

Koji preparation: Koji was prepared using the modified method of Su et al. (2005). Soy and wheat flour were sterilized at 121 °C for 15 min in an LTE Series 300 autoclave (LTE Scientific Ltd, Oldham, UK). Soy flour moisture was maintained by mixing 100 g of soy flour with 120 mL of sterile distilled water. The cooked soy flour was cooled to room temperature and then mixed thoroughly with the wheat flour (1:1 *w/w*). The mixture was inoculated with *A. oryzae* spore to a final concentration of 10^5 spores/g substrate (Chou & Ling, 1998). The inoculated substrates were transferred into sterile Petri dishes (d:140 mm) and incubated at 30 °C for 3 days.

Moromi preparation: Different types of brine (18% *w/v* NaCl; 6% *w/v* NaCl and 12% *w/v* KCl; 6% *w/v* NaCl) were added to the koji with ratio of 1:5 (koji:brine) to create moromi $A_{[18\%]}$, $B_{[6:12\%]}$, and $C_{[6\%]}$ respectively, followed by inoculation as shown in Figure 1. Moromi $A_{[18\%]}$ and $B_{[6:12\%]}$ were simultaneously inoculated with *T. halophilus* and *Z. rouxii*. Three different moromi C were prepared according to the inoculation method of *Z. rouxii*. Moromi $C1_{[6\%]}$ was simultaneously inoculated with *T. halophilus and Z. rouxii*, while moromi $C2_{[6\%]}$ and $C3_{[6\%]}$ were inoculated with *Z. rouxii* after 1 week and 2 weeks, respectively. Moromi $C4_{[6\%]}$ was inoculated with DE (10% *v/v*) containing *T. halophilus* and *Z. rouxii*, which had

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been incorporated in its W_2 and W_1 phase, respectively, prior to inoculation. The inoculated moromi mashes were then incubated at 30 °C for 4 weeks and samples were taken at Week 0, 1, 2, 3, and 4. *T. halophilus* was grown on BHI agar supplemented with 7% (*w/v*) NaCl and natamycin while the cell count of *Z. rouxii* was done on YM agar with the addition of 5% (*w/v*) NaCl, and 100 mg/L chloramphenicol. In order to study the effect of koji:brine ratio on DE stability, koji was mixed with 18% *w/v* NaCl solution with koji:brine ratio of 1:3, 1:5, and 1:7 followed by incubation at 30 °C for 7 days.

2.5 Rheological measurements

Rheological characterization of moromi was done by measuring the viscosity of koji mixed with varying concentrations of brine solution (18% NaCl *w/v*). The viscosity was measured for moromi containing koji:brine ratio of 1:3, 1:5, 1:7 and brine only at 30 °C using AR-G2 rheometer (TA instruments, New Castle, DE) on a parallel plate geometry (d: 40 mm). The apparent viscosity was measured over a shear rate range of 0.1–100 s^{-1}. Briefly, 1 mL of sample was placed between the cone and the plate, and measurement was started immediately. In total, 30 data points were recorded at 10-s intervals during the shearing. Shear stress was determined as a function of shear rate. Data were fitted to power-law model (Barnes et al., 1989):

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

where; η refers to viscosity (Pa s), K to consistency coefficient (Pa s^n), γ to shear rate (s^{-1}), and n to flow behavior index (dimensionless).

2.6 Physicochemical analysis

Soy mash samples were centrifuged at 15000 *g* for 15 min at ambient temperature. The supernatant regarded as raw soy sauce was transferred to microtubes and kept at –20 °C until

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analysis. Total reducing sugar (D-glucose and D-fructose), total lactic acid (L-lactic acid and D-lactic acid), ethanol, and L-glutamic acid were analyzed using enzymatic assay kit (Megazyme, International Ireland Ltd., Bray, Ireland) according to the manufacturer's instructions. Changes in pH were monitored using a pH meter (SevenCompact S220, Mettler Toledo, Germany).

2.7 Volatile compound analysis (SPME GC-MS)

An automated headspace solid-phase microextraction method (SPME) followed by GC-MS analysis was used for evaluating the *in vitro* production of microbial volatile organic compounds. Soy sauce mash samples (1.5 g) were transferred into 20-mL headspace vials (22.5 mm × 75.5 mm, Grace Alltech, Thermo Fisher UK) and the vials were sealed with magnetic cap (20 mm diameter, 5 mm center, PTFE / Silicone Liner; Grace Alltech). Samples were allowed to equilibrate at 22 °C for 30 min before analysis. Three replicates were prepared for all samples.

The volatiles extraction was performed using a 1-cm Stableflex fiber coated with 50/30 μm divinylbenzene-Carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA). It was conditioned for 90 min at 300 °C in the injection port. The fiber was pushed out of the housing and inserted into the vials through the center of the vial cap. The penetration depth was fixed at 22 mm. The extraction was carried out by exposing the fiber to the headspace for 10 min at 40 °C. For all analyses, desorption time was set to 10 min at 230 °C.

Chromatography was carried out using a Trace GC Ultra gas chromatography (Thermo Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m × 0.25 mm I.D.; film thickness: 1 μm) from Phenomenex (Torrance, CA). Mass spectrometry

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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.

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2.9 *Statistical analysis*

Microbial cell enumeration, physicochemical tests, and volatile compounds analysis were conducted in triplicate and repeated in two independent experiments. The results were presented as mean ± standard deviation. Significant differences among means were tested by one-way analysis of variances (ANOVA) using IBM SPSS Statistics Software Version 21 at $p < 0.05$ and Tukey's test was applied for means comparison. Principal component analysis (PCA) was performed using XLSTAT^{TM} version 2015.6.01.24027 (Addinsoft, New York, NY) to reduce the dimensionality of the dataset and show the differences in volatile compounds among the soy sauce samples. Observations/variables were chosen as data format and Pearson's correlation matrix was used as PCA type.

3. Results and Discussion

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

DEs were formulated using ingredients relevant to moromi constituents and soybean oil was used as the oil phase. Since the reduced-salt moromi contained 6% NaCl, the internal W_1 and external W_2 phase of DE also contained 6% NaCl. This aimed to balance the osmotic pressure between the two phases, thus reducing instability of DE due to water movement across the oil phase (Mezzenga, Folmer, & Hughes, 2004).

In order to describe the relationship between the viscosity of moromi and DE stability, moromi formulations with different viscosities were tested by varying the ratio of koji:brine (1:3, 1:5, and 1:7). The Power-Law model was used to describe the flow curves of the moromi. The rheological parameters of this model are presented in Table S1. All the moromi formulations exhibited non-Newtonian behavior at shear rates ranging between 0.1 and 100 s^{-1} at 30 °C (Figure S1a). Moreover, the plot of the viscosity against shear rate of the koji and brine mixtures yielded a flow index (n) of less than 1 (shear thinning), indicating that their

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flow behavior had a non-Newtonian profile. Similar non-Newtonian behavior has been reported for semi-solids of similar composition to koji which could be attributed to the presence of high molecular weight components, such as proteins or dextrin (Manohar, Manohar, & Rao, 1998).

DE maintained its microstructure after 4 weeks of fermentation (Figure 2a). However, the oil globule size significantly decreased from 27.88 μm to 11.40 μm (Figure 2b and 2c). This could be attributed to the high viscosity of the moromi system. The viscosity increased when the amount of brine added was decreased (Figure S1a). After incorporation into the moromi system, the DE stability was determined by observing its microstructure (i.e. inner W_1 phase) using microscopy and monitoring the oil globule size. The initial oil globule size (31.84 μm) decreased immediately after incorporation into the moromi slurry and during storage (Figure S1b and S1c). However, the decrease in koji:brine 1:3 was more noticeable compared to those with higher fractions of brine. By the end of storage, the oil globule size of DE in koji : brine 1:3, 1:5, and 1:7 was 6.84 μm, 18.02 μm, and 15.29 μm, respectively. Moreover, all the oil globules in koji:brine 1:3 completely lost their inner phase, while in koji:brine 1:5 and 1:7, the DE structure was maintained (Figure S1d). These data indicate that DEs were destabilized in the moromi system; however, the destabilization was not proportional to the viscosity of the moromi.

3.2 The effect of salt reduction and inoculation sequence on the growth of T. halophilus and Z. rouxii

Salt concentration is a significant parameter that determines soy sauce fermentation process by affecting microbial growth. High salt concentration is typically used in soy sauce fermentation, in order to suppress the growth of undesirable microorganism as well as improving the organoleptic properties of the final product. *T. halophilus* growth was

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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

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with *T. halophilus* and *Pichia guilliermondii* was shown to increase the total population of lactic acid bacteria and yeast in reduced-salt moromi fermentation. Since *Z. rouxii* grows optimally at low pH, *Z. rouxii* would be better added at the later stage of fermentation, once moromi is acidified due to organic acids production by *T. halophilus*. In the present study, *Z. rouxii* sequential inoculation ($C2_{[6\%]}$and $C3_{[6\%]}$) and gradual release in DE ($C4_{[6\%]}$) did not have significant effect on growth, as this seemed to depend primarily on the salt formulation and less on inoculation sequence (Figure 3b).

3.3 Physicochemical changes during fermentation

The changes in pH, reducing sugar, lactic acid, ethanol, and glutamic acid were measured to monitor the fermentation progress, as they are associated with the growth of microorganisms (Figure 4). Besides increasing in population during soy sauce fermentation, LAB also utilize and convert carbohydrates into organic acids, which can bring the pH down. Reduction in pH can also occur due to the accumulation of free fatty acids, amino acids, and peptides containing carbonylic side chains, resulting from other microbial activities and raw materials hydrolysis (Hoang et al., 2016; Van Der Sluis et al., 2001; Yanfang & Wenyi, 2009). As shown in Figure 4a, pH of all moromi samples decreased from ~5.3 to final pH of ~4.8, which was similar to values reported in previous studies of traditional Korean (Song, Jeong, & Baik, 2015b) and reduced-salt soy sauce (Singracha et al., 2017). The pH decreased within two weeks and then remained constant throughout the fermentation period, except for $C1_{[6\%]}$, where pH increased to 5.49. The reduction in pH was associated with the increase in the lactic acid amount produced by *T. halophilus* (Figure 4c). Although lactic acid production was greatly suppressed by 18% NaCl, the reduction in pH was unaffected, which could be due to production of other organic acids. Although *T. halophilus* is known as homofermentative, some strains are regarded as heterofermentative and they are able to produce acetic acid (Justé et al., 2012). Moreover, homofermentative strains of *T. halophilus*

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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

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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,

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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-

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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.

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Replacing NaCl with KCl decreased the amount of 2-phenylethyl acetate, which contributes to honey, rosy odor. However, this could be compensated for by adding *Z. rouxii* at Week 1 of the fermentation process. In our previous study, it was found that the production of 2-phenylethyl acetate could be enhanced by adding *Z. rouxii* sequentially rather than simultaneously (Devanthi et al., 2018). *Z. rouxii* enhances the production of esters (Van Der Sluis et al., 2001), although the production of isoamyl acetate (banana aroma) was significantly enhanced in reduced-salt moromi. This was only observed in $C1_{[6\%]}$, while the amount of isoamyl acetate in $C2_{[6\%]}$, $C3_{[6\%]}$, and $C4_{[6\%]}$ was similar to that at high salt concentration ($A_{[18\%]}$and $B_{[6:12\%]}$).

The only furan and phenol compounds detected in all moromi samples were 3-acetyl-2,5-dimethylfuran and 2-methoxy-5-methylphenol, respectively. These were produced in negligible amount when either salt or NaCl were reduced, except when *Z. rouxii* was added at Week 1. Several ketones, such as 3-methyl-2-pentanone and acetoin, were produced at significantly higher concentrations in reduced-salt moromi. The amounts of these compounds were similar to moromi containing high salt ($A_{[18\%]}$ and $B_{[6:12\%]}$) when *Z. rouxii* was added sequentially, with or without DE.

3.5 Principal component analysis

PCA analysis was conducted, in order to gain more understanding on the relationship between the fermentation conditions and profiles of volatile compounds. The first (PC1) and second principal component (PC2) accounted for 30.60% and 21.43% of the total variance, respectively (Figure 5a-b). The PCA score plot demonstrates distinct separation of some moromi samples (Figure 5a). In the case of co-inoculated samples, low salt moromi sample ($C1_{[6\%]}$) was differentiated from high salt moromi sample ($A_{[18\%]}$) while reduced NaCl sample (substituted with 12% KCl; B) was positioned in the middle of PC1. This indicates that salt

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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

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possibility of producing soy sauce in a low salt environment with a volatile profile pattern identical to the original high-salt soy sauce. The results obtained in this study provide the soy sauce industry with a new technique for standardizing the microbial activity and aroma development, which also offers health benefits to the consumers, due to low salt content in the final product. However, since modulating the release has a great impact on the aroma formation, further study is needed in order to tailor the physicochemical properties of DE, therefore enabling the cell release in a more controlled manner.

5. Acknowledgement

The authors are grateful to Indonesia Endowment Funds for Education (LPDP), Ministry of Finance for funding this work as part of a doctorate program.

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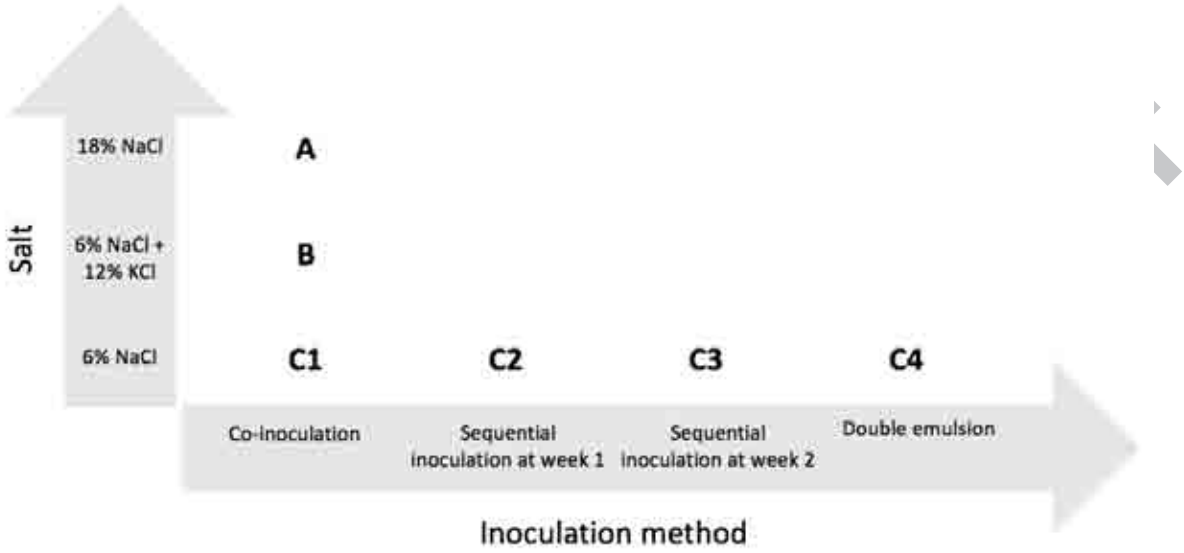

593 Figure 1. Set of moromi samples varying in salt composition and inoculation method

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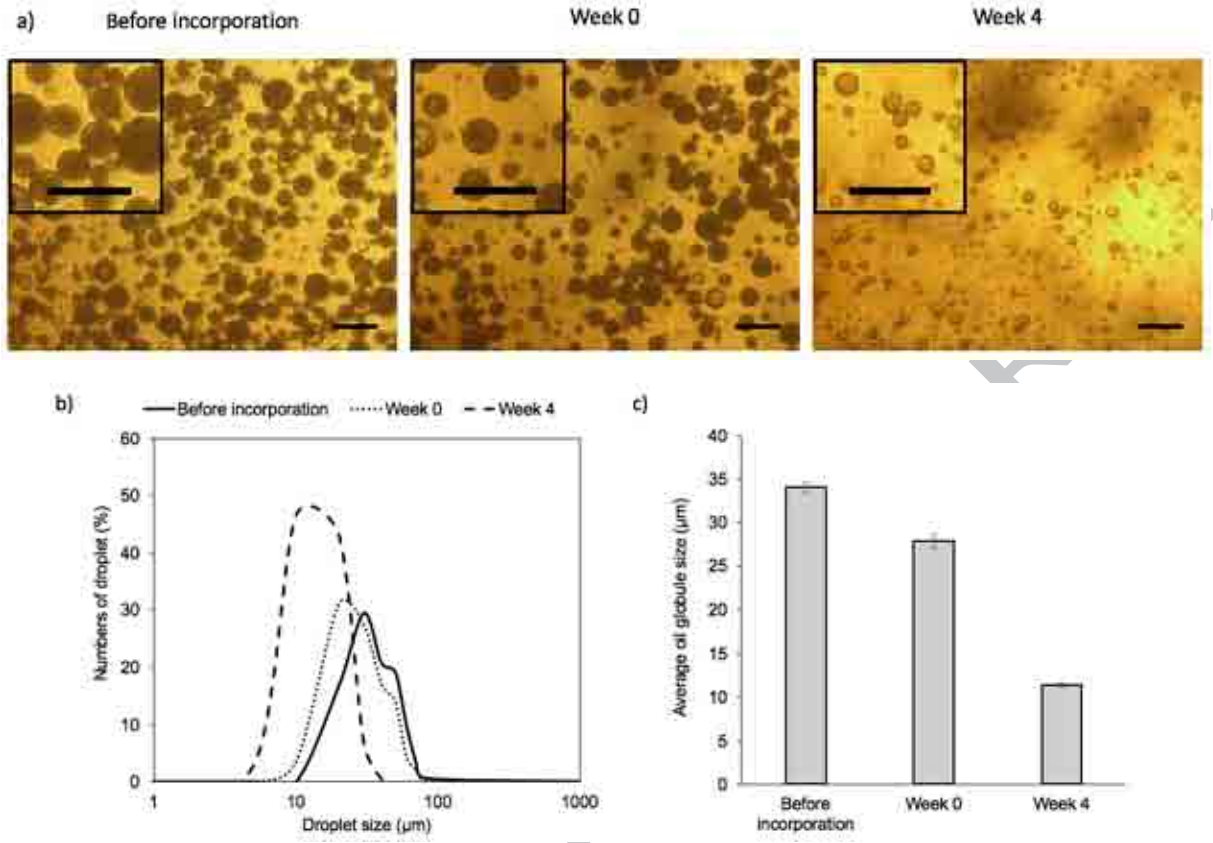

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606 Figure 2. (a) Optical micrograph of $W_1/O/W_2$ DE before and after incorporation into moromi,
607 and after 4 weeks of fermentation. Scale bar: 100 μm. (b) Oil globule size distribution before
608 and after fermentation. (c) Average oil globule size before and after fermentation.

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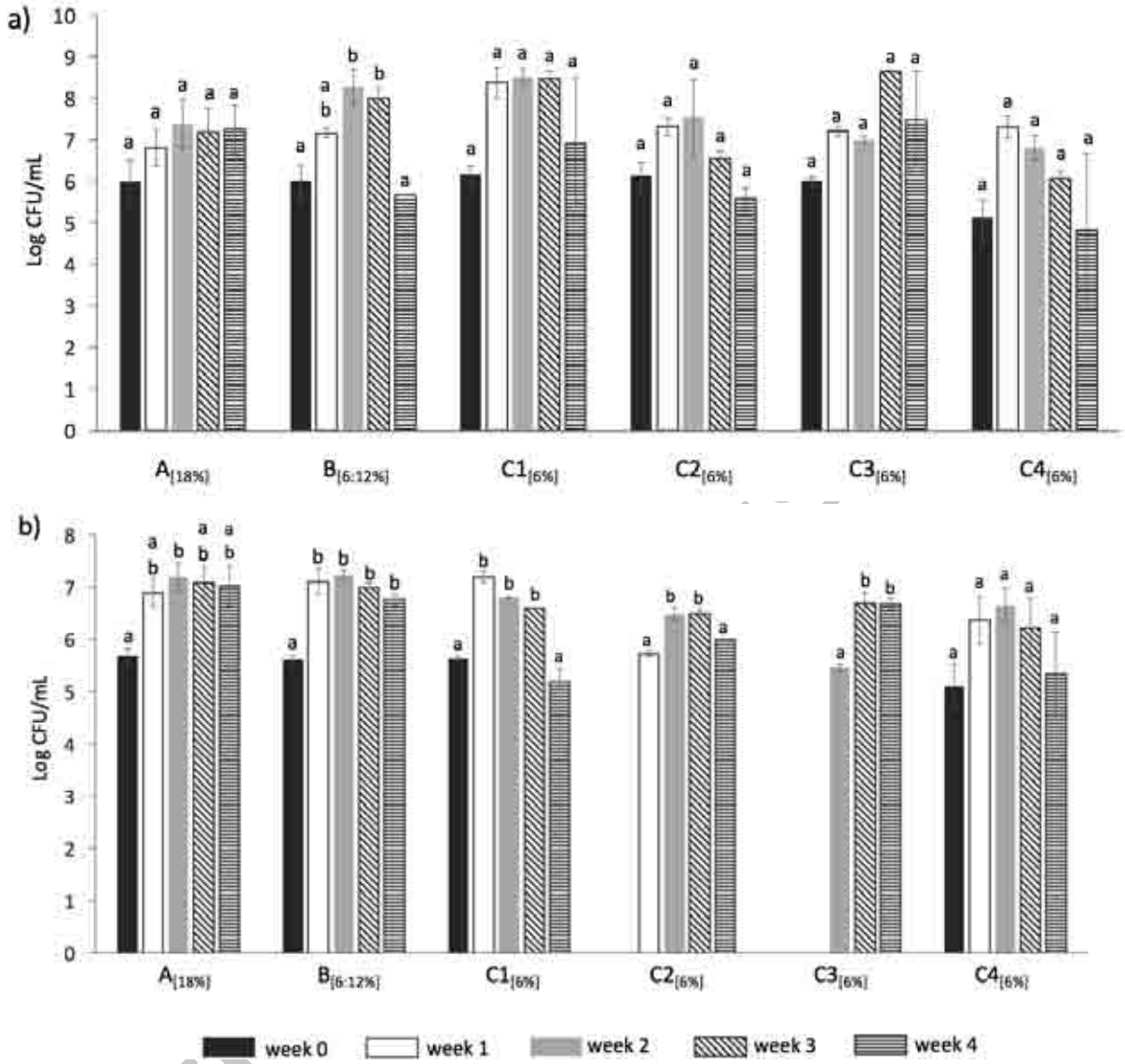

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617 Figure 3. Changes in population of (a) *T. halophilus* and (b) *Z. rouxii* during fermentation of
618 low and high salt moromi at 30 °C. The samples contained co-inoculated *T. halophilus* and *Z.*
619 *rouxii* in 18% NaCl ($A_{[18\%]}$), 6% NaCl and 12% KCl ($B_{[6:12\%]}$), 6% NaCl ($C1_{[6\%]}$), and
620 sequentially inoculated *T. halophilus* and *Z. rouxii* at week 1 ($C2_{[6\%]}$), week 2 ($C3_{[6\%]}$), or
621 with DE ($C4_{[6\%]}$). The addition time of *Z. rouxii* cells for sequential inoculation is indicated
622 by the asterisk mark (*). Means within the same group with different letters (a, b, c) are
623 significantly different ($p<0.05$).

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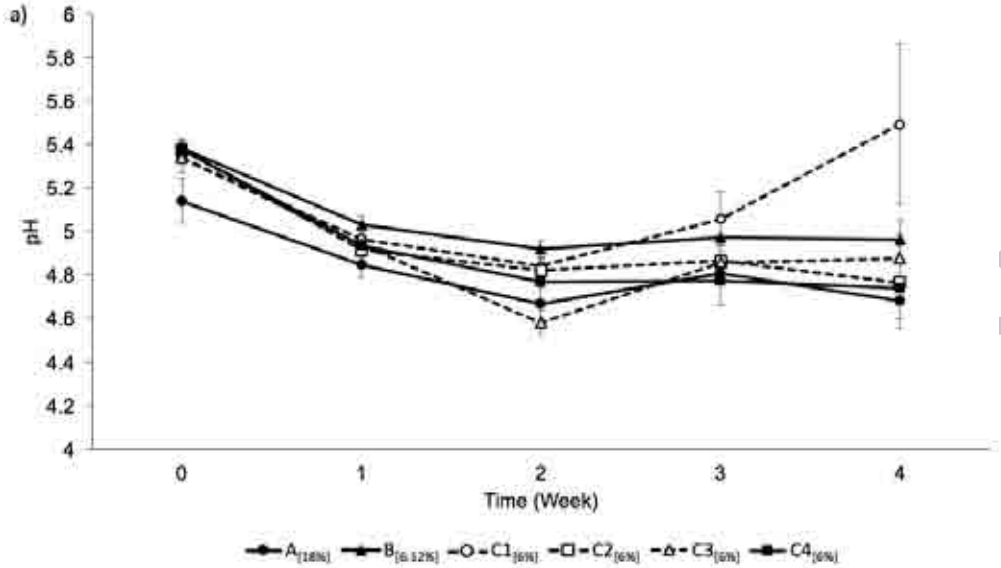

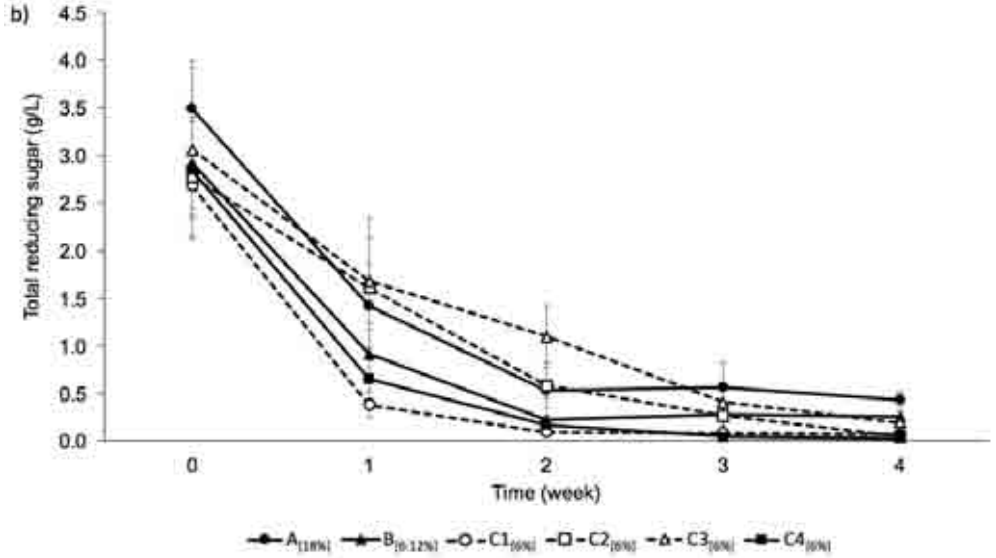

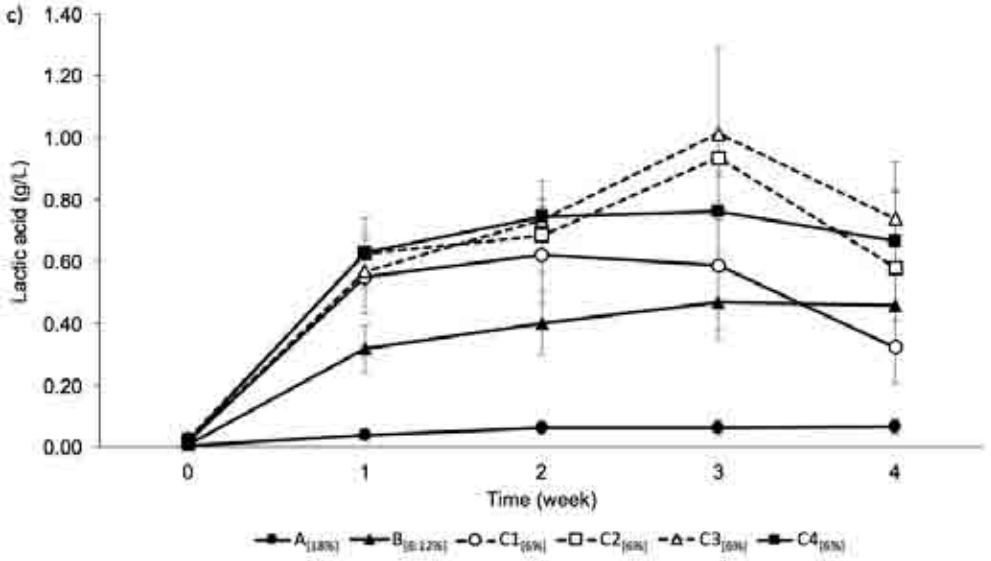

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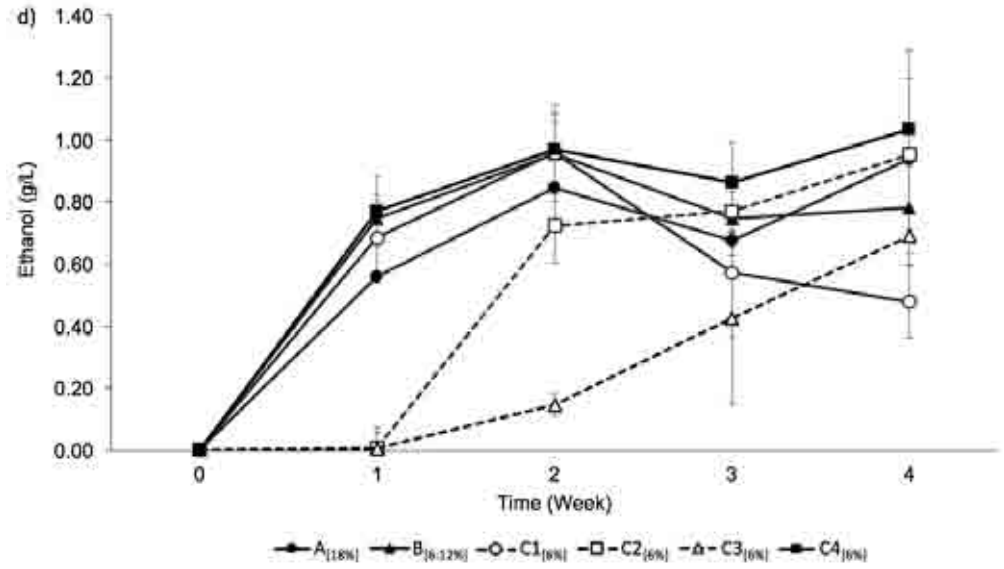

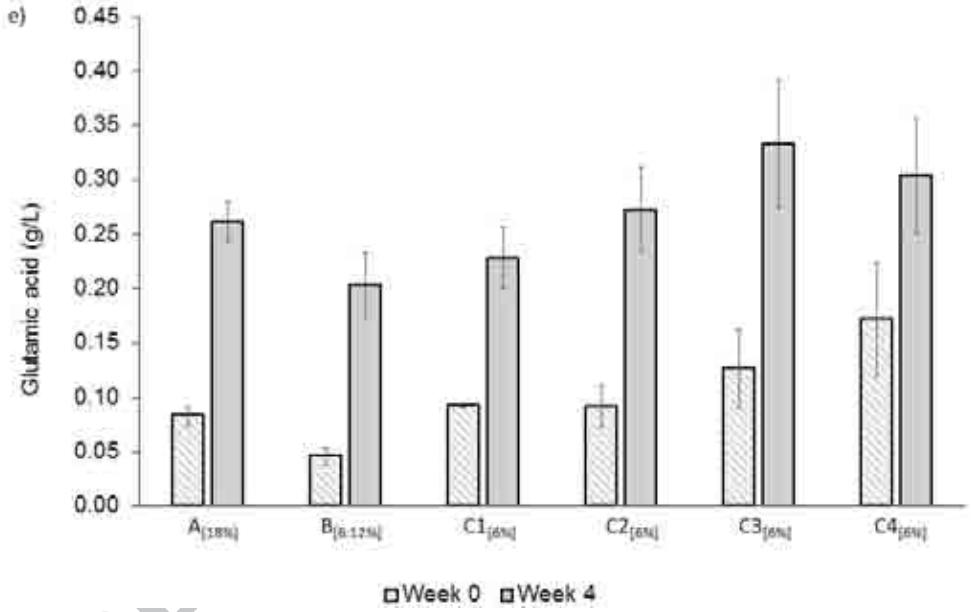

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626 Figure 4. Changes in (a) pH, (b) total reducing sugar, (c) lactic acid, (d) ethanol, and (e)
627 glutamic acid during fermentation of low and high salt moromi at 30 °C.

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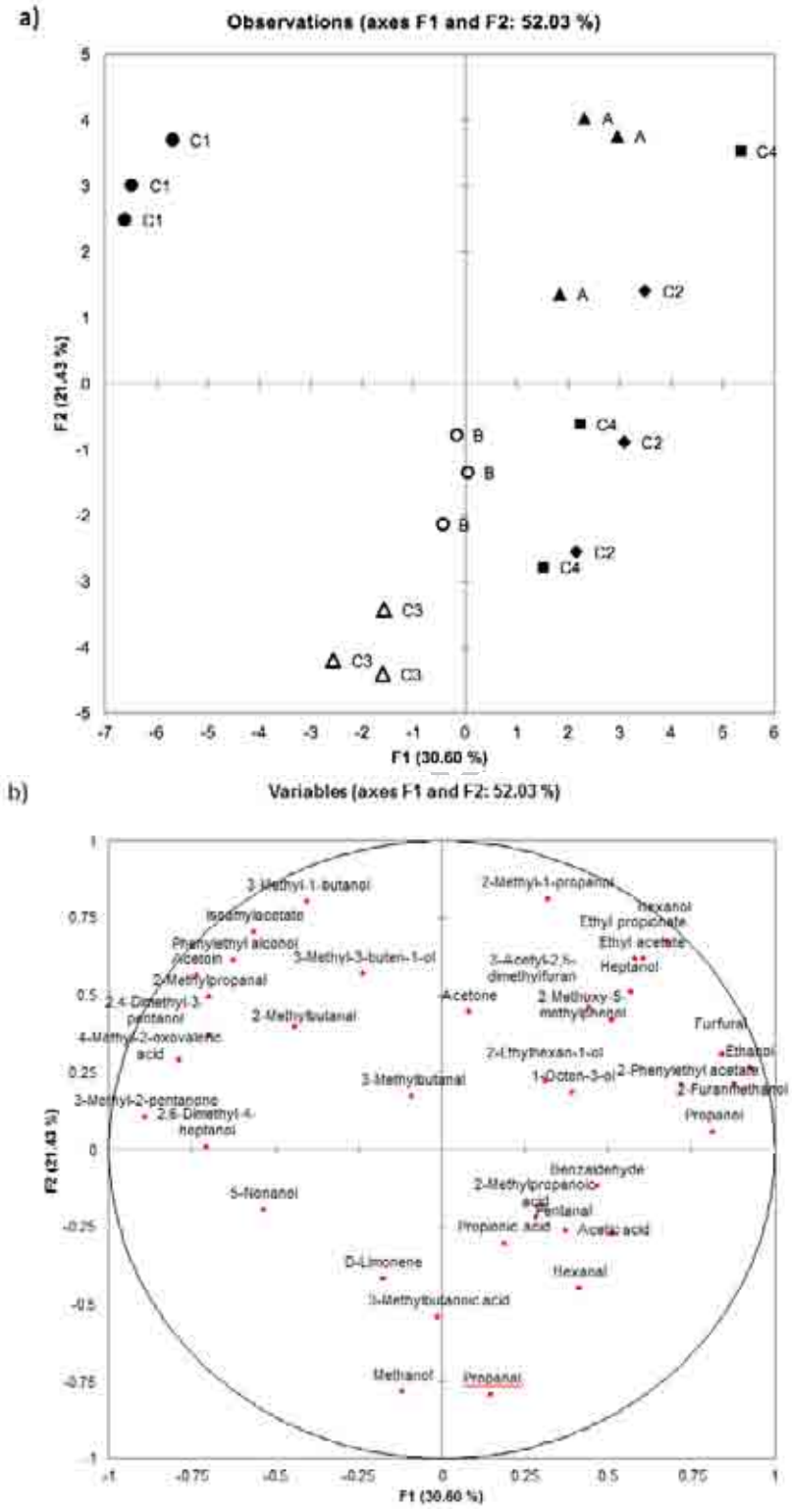

628 Figure 5. (a) PCA score plot of six moromi samples after 4-week fermentation. The scores
629 are based on three replicates of each sample. The identical symbols represent triplicate
630 measurements. (b) PCA loading plot of the aroma compounds detected in moromi after 4-
631 week fermentation.

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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

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acid													
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

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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

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Others

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

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

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

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

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

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

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

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

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

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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

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