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## Accepted Manuscript

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# Effects of co-inoculation and sequential inoculation of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* on soy sauce fermentation

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## Abstract

The use of *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* as starter cultures is essential for desirable volatiles production during moromi stage of soy sauce fermentation. In this study, the effect of simultaneous and sequential inoculation of cultures in moromi fermentation models, with respect to viability, physicochemical changes, and volatiles formation (using SPME-GC/MS) was investigated. Interestingly, an antagonism was observed as *T. halophilus* only proliferated (3 log increase) in the presence of *Z. rouxii*, while *Z. rouxii* growth was suppressed by 4 log in concurrence with pH increase to 7.31. Final content of reducing sugars, ethanol, acetic acid, and amino nitrogen did not differ significantly ( $p < 0.05$ ) between co-inoculation and sequential inoculation. However, *Z. rouxii* promoted alcohols formation and produced a more complex aroma profile under

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suppression. According to Principal Component Analysis (PCA), the inoculation sequence (co-inoculation and sequential) has impacts on volatile compound profiles during moromi fermentation.

Keywords: soy sauce, *Tetragenococcus halophilus*, *Zygosaccharomyces rouxii*, co-inoculation, sequential inoculation, volatile compounds, aroma, GC-MS

## 1. Introduction

Soy sauce is a fermented condiment originating from China, which is popular around the world due to its intense umami taste and distinct aroma. Two types of soy sauce can be distinguished based on the raw materials: The Chinese-type produced using predominantly soybeans and wheat, and the Japanese-type made using equal amounts of soybeans and wheat (Wanakhachornkrai & Lertsiri, 2003). The Chinese-type dominates Asian regions such as China, Indonesia, Malaysia, Philippines, Singapore, Thailand while the Japanese-type is more popular in Japan and western countries (Zhu & Tramper, 2013).

Soy sauce production involves a 2-step fermentation process, *koji* and *moromi*. In Japanese-type, *koji* is prepared by growing *koji* mould, such as *Aspergillus oryzae*, on an equal amount of cooked soybean and wheat flour mixture, followed by *moromi* fermentation by mixing the resulting *koji* with brine solution containing 18–22% NaCl (Yong & Wood, 1977). *Moromi* stage is mainly driven by halotolerant lactic acid bacteria (LAB) and yeast that grow spontaneously during conventional brewing. However, in recent years, the amount of NaCl in the final product has been reduced to approximately 8–11%, driven by industry and the World Health Organization (WHO) recommendation on reducing dietary intake of sodium salt. Studies focusing on salt reduction during *moromi* fermentation have demonstrated that despite a reduced salt concentration, sensory quality and safety of the final product could be preserved with the use of either mixed culture of lactic acid bacteria and yeast (Singracha, Niamsiri, Visessanguan, Lertsiri, & Assavanig, 2017) or mixed culture of indigenous yeast isolated from different stages during traditional *moromi* fermentation (Song, Jeong, & Baik, 2015).

*Moromi* stage is very crucial since key volatile compounds, taste active amino acids and peptides, and sugars that contribute to the final flavour of sauce are produced in this stage

(Harada et al., 2016; Zhao, Schieber, & Gänzle, 2016; Zhu & Tramper, 2013). Lactic acid bacterium *Tetragenococcus halophilus* and yeast *Zygosaccharomyces rouxii* compose the core microbial complex which drives the moromi fermentation, regardless of soy sauce origin and production procedure (Harada et al., 2016; Singracha et al., 2017) and therefore the sequence of proliferation of microbial species and their equilibria are paramount to the quality of the final product.

There are abundant secondary metabolites produced by *T. halophilus* and *Z. rouxii* via lactic acid and alcoholic fermentation, respectively, which are responsible for the flavour of the final product (Lee, Lee, Choi, Hurh, & Kim, 2013; Tanaka, Watanabe, & Mogi, 2012). Important aroma compounds in soy sauce, such as acetic acid, formic acid, benzaldehyde, methyl acetate, ethyl 2-hydroxypropanoate, 2-hydroxy-3-methyl-2-cyclopenten-1-one, and 4-hydroxy-3-methoxybenzaldehyde are produced by *T. halophilus* (Lee et al., 2013). Moreover, *Z. rouxii* plays an important role in the formation of ethanol, higher alcohols (isobutyl alcohol, isoamyl alcohol, 2-phenylethanol) (Jansen, Veurink, Euverink, & Dijkhuizen, 2003; Van Der Sluis, Tramper, & Wijffels, 2001), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) (Hauck, Brühlmann, & Schwab, 2003; Hecquet, Sancelme, Bolte, & Demuynck, 1996), and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) (Sasaki, 1996) during moromi stage, which are essential for the characteristic flavour in the final product. Studies showed that despite salt reduction, production of essential volatiles, such as ethanol, 2-methyl-1-propanol, HDMF, and maltol, was significantly higher than traditional moromi when a combination of *T. halophilus* and *Z. rouxii* was used (Singracha et al., 2017).

Since the activity of *T. halophilus* and *Z. rouxii* contributes to the aroma profiles of soy sauce, the utilisation of both cultures in the manufacturing process is important. *T. halophilus*, *Z. rouxii*, and co-culture of both were reported to cause physicochemical changes and affect aroma formation during moromi fermentation (Cui, Zheng, Wu, & Zhou, 2014;

Harada et al., 2016; Lee et al., 2013). Metabolomics analysis demonstrated different aroma profiles of moromi according to the types of microorganism added (Harada et al., 2016). *T. halophilus* TS71 and *Z. rouxii* A22 were also reported to enhance the aroma profile of moromi under a reduced-salt environment (Singracha et al., 2017). However, these studies did not investigate the impact of inoculation sequence of *T. halophilus* and *Z. rouxii*. Sequential growth of *Z. rouxii* occurs naturally during spontaneous fermentation of moromi because of lactic acid and acetic acid production by *T. halophilus* that reduces the pH. As the pH drops to <5.0, *Z. rouxii* starts to grow and begins the alcoholic fermentation (Yong & Wood, 1976; Van Der Sluis et al., 2001b; Röling et al., 1994). The inoculation method is important for production of desirable volatile compounds allowing full complexity to be achieved. In this study the effect of simultaneous and sequential inoculation of *Z. rouxii* as the pH drops to 5.0 was investigated for the first time in moromi models, with respect to microbial interactions, physicochemical changes, and formation of volatile compounds. Principal component analysis (PCA) was used to evaluate the influence of inoculum type (single culture or mixed culture) as well as the inoculation method on the volatile compound profile of soy sauce.

## 2. Materials and Methods

### 2.1 Materials

Soy flour, wheat flour, and sodium chloride (NaCl, extra pure) were purchased from Real Foods (Edinburgh, UK), Gilchesters Organics (Stamfordham, UK), and Acros Organics (Fairfield, NJ), respectively. *Aspergillus oryzae* 126842 was purchased from Centre for Agriculture and Biosciences International (Wallingford, 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. Microbiological growth media used were Czapek Dox agar (CDA; Oxoid Ltd., Basingstoke, UK), brain heart infusion agar (BHI; Oxoid Ltd), de Man, Rogosa, and Sharpe broth (MRS broth; Oxoid Ltd.), yeast malt agar (YM agar, Sigma-Aldrich, Gillingham, Dorset, UK), yeast malt broth (YM broth, Sigma-Aldrich). Bacteria and yeast growth were controlled using chloramphenicol (Oxoid Ltd.) and natamycin (Sigma-Aldrich), respectively. 1-octen-3-ol (purity  $\geq 98\%$ ) was purchased from Sigma Aldrich.

## 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. 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) 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 haemocytometer 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 Koji fermentation

Koji was prepared using the modified method of Su et al. (2005). Soy flour and wheat flour were sterilised 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* spores 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.

#### 2.4 Study of *T. halophilus* and *Z. rouxii* growth in moromi fermentation

Koji was transferred aseptically into flasks. Brine solution (10% w/v NaCl) was added to the koji with ratio 3:1 (brine:koji) to create mash (Wan, Wu, Wang, Wang, & Hou, 2013; Wu, Kan, Siow, & Palniandy, 2010). The relatively low salt concentration allows faster fermentation (Muramatsu, Sano, Uzuka, & Company, 1993; Van Der Sluis et al., 2001) and reflects the reduction of salt in the soy sauce industry.

Five types of soy moromi were prepared as follows: (i) uninoculated as control, (ii) inoculated with *T. halophilus*, (iii) inoculated with *Z. rouxii*, (iv) co-inoculated with *T. halophilus* and *Z. rouxii*, and (v) inoculated with *T. halophilus*, followed by sequential inoculation of *Z. rouxii* when the pH dropped to 5.0 (SevenCompact S220 pH meter; Mettler Toledo, Switzerland). After inoculation, the mash was homogenised with a vortex and incubated at 30 °C for 30 days. Samples were taken at Day 0, 5, 10, 15, 20, 25, and 30. *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.

#### 2.5 Physicochemical analysis

Before analysis, soy mash samples were treated at 100 °C for 2 min, to prevent assay interference by enzymes produced during moromi fermentation. Then samples were centrifuged at 10000 g for 10 min at 4 °C. The supernatant regarded as raw soy sauce was

transferred to microtubes and kept at  $-20^{\circ}\text{C}$  until analysis. Total reducing sugar (D-glucose and D-fructose), total lactic acid (L-lactic acid and D-lactic acid), acetic acid, primary amino nitrogen, and ethanol were analysed using an 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).

## 2.6 Flavour 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  $\times$  75.5 mm; Grace Alltech, UK) and the vials were sealed with magnetic cap (20 mm diameter, 5 mm centre, PTFE/silicone liner). Samples were allowed to equilibrate at  $22^{\circ}\text{C}$  for 30 min before analysis. Three replicates were prepared for all samples.

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

Chromatography was carried out using a Trace GC Ultra gas chromatograph (Thermo Electron Corporation, Hemel Hempstead, UK) equipped with a polar column ZB-Wax (30 m  $\times$  0.25 mm I.D.; film thickness: 1  $\mu\text{m}$ ) from Phenomenex (Torrance, CA). Mass spectrometry (MS) was performed with a DSQ mass spectrometer (Thermo Electron Corporation, Hemel

Hempstead, UK). The GC-MS was set according to a previous study (Gkatzionis, Linforth, & Dodd, 2009): The temperature of the injection port was 230 °C. Helium was employed as the carrier gas, at a constant pressure of 17 psi. The oven temperature program was as follows: an initial temperature of 40 °C was maintained for 2 min, increasing at a rate of 8 °C /min to a final temperature of 220 °C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250 °C. The mass spectrometer was operated in positive ionization electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2 scans/s) scanning from  $m/z$  20 to 250. Source temperature was 200 °C.

Compounds were identified by comparing their retention times and mass spectra with those of standards, or their retention indices (RI) with those published in databases and their mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library using XCalibur Software (Thermo Electron Corporation). The signal intensity for each compound was expressed relative to the signal observed when the headspace above a 0.1 µg/mL 1-octen-3-ol solution was sampled.

## 2.7 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 means  $\pm$  standard deviation. Significant differences among means were tested by one-way analysis of variance (ANOVA) using IBM SPSS Statistics Software version 21 at  $p < 0.05$  and Tukey's test was applied for comparison of means. Principal component analysis (PCA) was performed using XLSTAT<sup>TM</sup> version 2015.6.01.24027 (Addinsoft, Paris, France) to reduce the dimensionality of the dataset and show the differences in volatile compounds among the soy sauce samples. Observations/variables was chosen as data format and Pearson's correlation matrix was used as PCA type.

### 3. Results and Discussion

#### 3.1 The effect of *T. halophilus* and *Z. rouxii* interaction on growth during moromi fermentation

An antagonistic relationship between *T. halophilus* and *Z. rouxii* was evident in mixed culture fermentation regardless of the inoculation sequence. *Z. rouxii* viable cell counts markedly decreased to less than 2 log CFU/mL by the end of fermentation period while counts in single culture remained >6 log CFU/mL (Figure 1C and 1D). On the other hand, *T. halophilus* growth was stimulated in co-inoculation and sequential inoculation, to final counts of 8.62 log CFU/mL (Figure 1C) and 8.47 log CFU/mL (Figure 1D) respectively, compared to growth in single culture, which was constant (6.67 log CFU/mL; Figure 1A).

A different pattern was observed for *T. halophilus* growth between co-inoculation and sequential inoculation. *T. halophilus* in co-inoculation reached its stationary phase (~9 log CFU/mL) at Day 15, while in sequential inoculation its stationary phase was reached at Day 25 (Figure 1C and 1D). These results indicate that delaying *Z. rouxii* inoculation could slow down the growth of *T. halophilus*.

Marked increase in *T. halophilus* growth in mixed culture could possibly be due to metabolites production by *Z. rouxii*, such as pyruvate, amino acids, and vitamins, which are essential for bacterial growth (Sudun, Wulijideligen, Arakawa, Miyamoto, & Miyamoto, 2013). Moreover, the dynamics of *T. halophilus* and *Z. rouxii* viable cells were apparently influenced by soy mash pH values during moromi fermentation. Initially, the pH values of all moromi samples were similar (~5.5). When the pH was constantly low (~5.0) throughout the fermentation period, the viable cell counts of *T. halophilus* and *Z. rouxii* in single culture remained constant (Figure 1A and 1B). The final pH values of moromi inoculated with pure culture of *T. halophilus* and *Z. rouxii* were 4.83 and 5.38, respectively. In contrast, as the pH

of moromi increased ( $>6.0$ ), *T. halophilus* growth was enhanced in parallel with *Z. rouxii* growth declining (Figure 1C and 1D). The increase in pH could stimulate *T. halophilus* growth since the optimal pH for its growth is around 7.0 (Justéet al., 2008; Wilred F. M. Röling & Van Verseveld, 1997). On the other hand, since the optimal pH range for *Z. rouxii* is 3.5–5.0, the pH increase in moromi could inhibit *Z. rouxii* growth (Membré, Kubaczka, & Chéné, 1999). Moreover, *Z. rouxii* cannot maintain its salt tolerance when the extracellular pH goes above 5.5, due to loss of the proton gradient across the plasma membrane (Watanabe & Tamai, 1992). In addition, the growth retardation of *Z. rouxii* might also be influenced by the inoculum size and ratio in the mixed culture. According to the finding of Kedia, Wang, Patel, and Pandiella (2007), in a mixed culture with *Lactobacillus reuteri* (LAB), yeast growth was inhibited at LAB:yeast ratio of 1:1 and 2:1, but enhanced at the ratio of 1:2.

### 3.2 Physicochemical changes in moromi fermentation

The reducing sugar content in all models containing *Z. rouxii* decreased to undetectable levels 5 days after inoculation (Figure 2A). This reducing sugar depletion was due to sugar utilisation by yeast for propagation as well as ethanol conversion. Since *A. oryzae* in the moromi model was heat-inactivated before fermentation started, the mould could no longer perform starch hydrolysis resulting in lower availability of reducing sugar for *Z. rouxii*.

In contrast, the amount of reducing sugar in control and single culture of *T. halophilus* remained high throughout the incubation period. By the end of fermentation, 10.39 g/L and 10.42 g/L of reducing sugar were found in control and single culture of *T. halophilus*, respectively, which were relatively higher than the initial amount. Such increases could result from the hydrolysing activity of amylase produced by *A. oryzae*, which remains active during moromi fermentation (Chou & Ling, 1998; Cui et al., 2014). Higher amount of reducing

sugar in sample treated with *T. halophilus* also indicates limited ability of *T. halophilus* in utilizing D-glucose and D-fructose.

The changing reducing sugar content was associated with the ethanol production during moromi fermentation (Figure 2B). Ethanol was monitored since it is one of the main alcohols produced by *Z. rouxii* during brine fermentation *via* sugars conversion (Van Der Sluis, Tramper & Wijffels, 2001). Reducing sugar depletion in all *Z. rouxii*-containing samples occurred in parallel with maximum ethanol production 5 days after *Z. rouxii* was inoculated. As expected, ethanol was not detected in the sample treated with single culture of *T. halophilus* and control (Figure 2b), since the reducing sugar content remained stable. Once sugar in all *Z. rouxii*-containing samples was exhausted, ethanol content dropped to below detection level.

As expected, *T. halophilus* was found to play a major role in both lactic acid and acetic acid production (Figure 2C and 2D). Surprisingly, even though *T. halophilus* growth was enhanced, production of both acids was suppressed when *Z. rouxii* was present. In single culture, *T. halophilus* was able to produce lactic acid and acetic acid to a maximum level of 0.58 g/L and 0.26 g/L, respectively, which were the highest among all samples.

The amino nitrogen contents in all soy sauce samples were generally constant with some fluctuation throughout the fermentation process. Amino nitrogen changes might be due to the metabolic activity of *T. halophilus* or Maillard reaction (Cui et al., 2014), which is responsible for deep brown colour formation in soy sauce. This reaction involves condensation of carbonyls, such as reducing sugars and aldehydes, with compounds possessing a free amino group (Lertsiri, Maungma, Assavanig, & Bhumiratana, 2001; Martins, Jongen, & Van Boekel, 2000).

### 3.3 The effect of co-inoculation and sequential inoculation of *T. halophilus* and *Z. rouxii* on formation of volatile compounds

A total of 23 major volatile compounds was identified in the moromi samples, including eight alcohols, three acids, five aldehydes, two esters, two pyrazines, one furan, and two ketones (Table 1). Alcohols were found to be the most abundant compounds detected in all soy sauce samples which is in agreement with previous studies conducted under high (Feng et al., 2015) and low salt concentration (Singracha et al., 2017).

Higher amount of total alcoholic compounds was detected in the moromi treated with *Z. rouxii* either as single or mixed cultures, suggesting that *Z. rouxii* was mainly responsible for the alcohols formation. Fusel alcohols such as phenylethyl alcohol (floral, sweet), 3-methyl-1-butanol (malty, rancid, and pungent), and 2-methyl-1-propanol (bitter) were found to be the major alcohol components of the final moromi. Song et al. (2015a) also reported high production of the above fusel alcohols in reduced-salt moromi by adding indigenous yeast isolated from different stages during traditional moromi fermentation. These fusel alcohols could have been generated from their corresponding amino acids by *Z. rouxii* through the Ehrlich pathway (Jansen et al., 2003). Extracellular amino acids present during moromi fermentation can undergo deamination or transamination through the Ehrlich pathway, producing  $\alpha$ -keto acids. The  $\alpha$ -keto acids serve as the main key intermediates in the formation of fusel alcohols. Fusel alcohols are formed through decarboxylation and subsequent reduction of the corresponding  $\alpha$ -keto acids (Van Der Sluis et al., 2001).

Combination of *Z. rouxii* and *T. halophilus* significantly increased 3-octanol (mushroom-like) production compared with the single cultures. Ethanol was obtained in significantly lower amount ( $p < 0.05$ ) in single culture and co-culture compared to those treated with sequential

inoculation. Ethanol is generally produced by *Z. rouxii* from glucose through the glycolytic pathway and anaerobic fermentation (Benitez & Codon, 2004).

Other alcohols such as 2-furanmethanol (sugar burnt) and methanol were detected in all samples before fermentation started. All samples contained 1-octen-3-ol (mushroom-like), but its amount decreased due to microbial activity during fermentation. Although most alcohols in soy sauce are originated from sugars and amino acids metabolism, small amounts of alcohols are also produced by yeast through metabolism of related aldehyde compounds (Sun, Jiang, & Zhao, 2010).

Aldehydes (furfural, benzaldehyde, 2-methylpropanal, 3-methylpropanal, 3-methylbutanal, and 2-methylbutanal) were the second main group of aroma compounds found in the soy mash. They were present in soy mash from the beginning of fermentation. However, their amount decreased significantly due to the activity of *Z. rouxii*, especially in mixed culture. The decrease might be due to aldehyde conversion to its corresponding alcohol (Song et al., 2015a). This hypothesis is supported by the fact that the amount of alcohols obtained in the samples treated with *Z. rouxii* is higher than control and single culture of *T. halophilus*. These aldehydes are responsible for soy sauce malty and nutty aroma (Feng et al., 2015).

Acetic acid was found to be the major volatile in single culture of *T. halophilus*. Its amount was significantly ( $p < 0.05$ ) higher compared to other samples, since *T. halophilus* is known to produce acetic acid (Justé et al., 2012; Tanasupawat, Thongsanit, Okada, & Komagata, 2002). Acetic acid can give sour odour to the final product. Production of 3-methylbutanoic acid was favoured in single cultures of *T. halophilus* and *Z. rouxii*. It has been reported to be the major aroma compound in Chinese soy sauce and it is responsible for strong pungent, sweaty, and cheese-like odour notes (Sun et al., 2010). This branched-chain acid might be produced by *Z. rouxii* from branched-chain amino acids contained in soybean *via* the Ehrlich



pathway (Lee et al., 2013). The presence of mixed culture seemed to suppress the production of 3-methylbutanoic acid.

Acetate esters comprising isoamyl acetate (banana aroma) and 2-phenylethyl acetate (honey, rosy) were detected in soy sauce samples fermented with *Z. rouxii*. Mixed cultures significantly enhanced the production of isoamyl acetate ( $p < 0.05$ ). The production of 2-phenylethyl acetate was higher in samples treated with *Z. rouxii* either alone or in mixed cultures. Interestingly, sequential inoculation produces significantly higher amounts of 2-phenylethyl acetate than co-inoculation. Higher esters production in the presence of yeast could happen, since yeast is capable of esterifying alcohols with fatty acids (Van Der Sluis et al., 2001). Fatty acids can be produced through fungal lipase activity on the lipid contained in the soybean (Chung, 1999).

Pyrazines were detected in all samples and they might be produced through Maillard reaction between saccharide and amino residues (Sun et al., 2010). Trimethylpyrazine (burnt) and 2,5-dimethyl pyrazine (roasted nuts) were found in all samples, although 2,5-dimethylpyrazine was significantly higher in the mixed culture samples. The main furan compound detected was 2-pentylfuran and its levels were similar among samples. It is known as a singlet oxidation compound produced from linoleic acid (Lee et al., 2013).

Acetone seemed to be the major ketone found in all samples, which is in agreement with the findings of Lee et al. (2013), and Wanakhachornkrai and Lertsiri (2003). The formation of acetone is related to metabolism of microorganisms, especially yeast, *via* lipid oxidation (Lee et al., 2013; Song, Jeong, & Baik, 2015b). However, its amount was significantly higher at Day 0, suggesting that the acetone might have originated from koji. According to Feng et al. (2013), acetone found in koji could be derived from raw materials used in the koji making. Co-inoculation was found to significantly reduce the amount of acetone after 30 days'

fermentation. Acetoin contributing to buttery odour was detected in all samples and its amount did not significantly vary. Therefore, acetoin might be derived from koji fermentation (Lee et al., 2013).

### 3.4 Principal component analysis

PCA was performed in order to understand the relationship between formation of volatile compounds, microbial species, and their order of inoculation. According to the PCA score plot (Figure 3), all moromi samples were clearly differentiated, indicating that the microbial species and their order of inoculation contributed to different aroma profiles in each sample. Single culture of *Z. rouxii*, co-inoculation, and sequential inoculation were positioned in positive PC1 region. On the other hand, control and single culture of *T. halophilus* were located in the negative PC1, suggesting that *T. halophilus* alone is not sufficient to develop complex aroma profiles. The PCA loading plot (Figure 4) shows this separation to be driven by a group of compounds dominated by alcohols (3-octanol, 3-methyl-1-butanol, 2-phenylethanol, 2-methyl-1-propanol, 2-phenylethyl acetate, isoamyl acetate, trimethylpyrazine and 2,5-dimethylpyrazine) while aldehydes are positioned on the left side of the plot (PC1; 44.95%). This is in agreement with the observed physicochemical changes discussed in Section 3.2, where higher amount of alcohols were detected in all moromi containing *Z. rouxii*.

Interestingly, sequential inoculation was distinguished from co-inoculation on PC2 (23.60%). Co-inoculation was distinguished by pyrazines and random groups of compounds. However, sequential inoculation accounted for significantly higher numbers of volatile compounds (13 out of 21) indicating production of more complex flavor profiles. PCA results demonstrated that simultaneous or subsequent inoculation of *Z. rouxii* matters to the development of flavour characteristics in moromi fermentation.

#### 4. Conclusion

The inoculation sequence demonstrated the antagonistic relationship between *T. halophilus* and *Z. rouxii*, which also affected the physicochemical and volatile profile changes during reduced-salt moromi fermentation. Although the antagonistic interaction occurred in both co-inoculation and sequential inoculation, it resulted in different physicochemical changes, which seemed to favour the aroma profiles in the case of sequential inoculation. Since the microbial proliferation and activity is very important to the development of moromi, understanding microbial interactions can assist strategies for controlling microbial release and activity (e.g., encapsulation) or modulation of the inoculation time. Furthermore, varying the inoculum size and ratio between *T. halophilus* and *Z. rouxii* should also be investigated, in order to fully explore the interaction between the two microbes.

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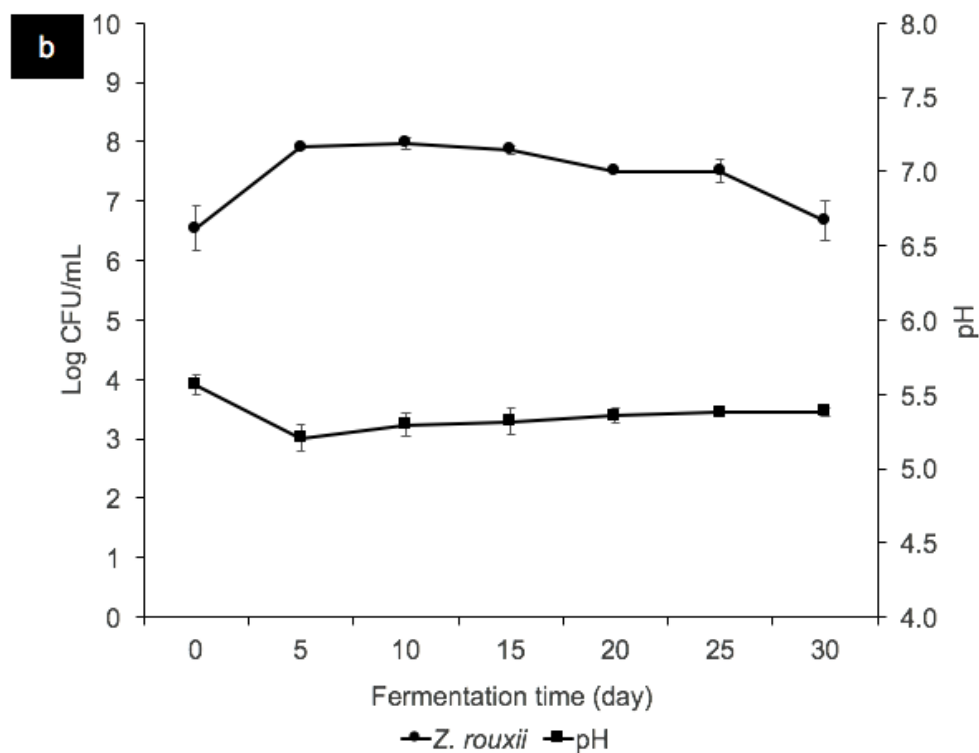
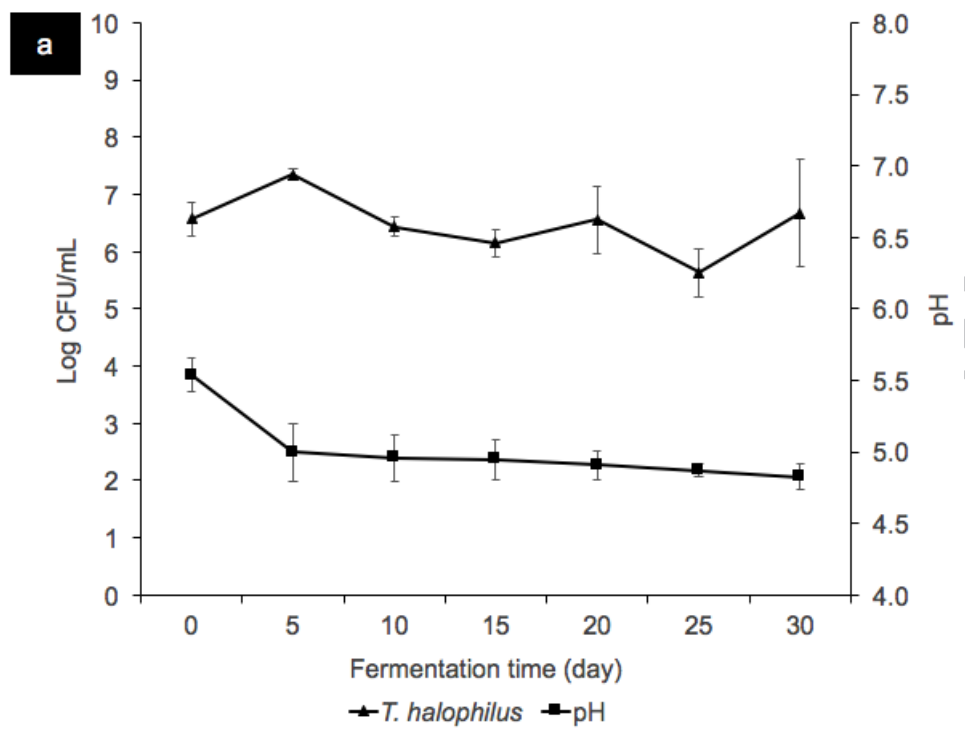
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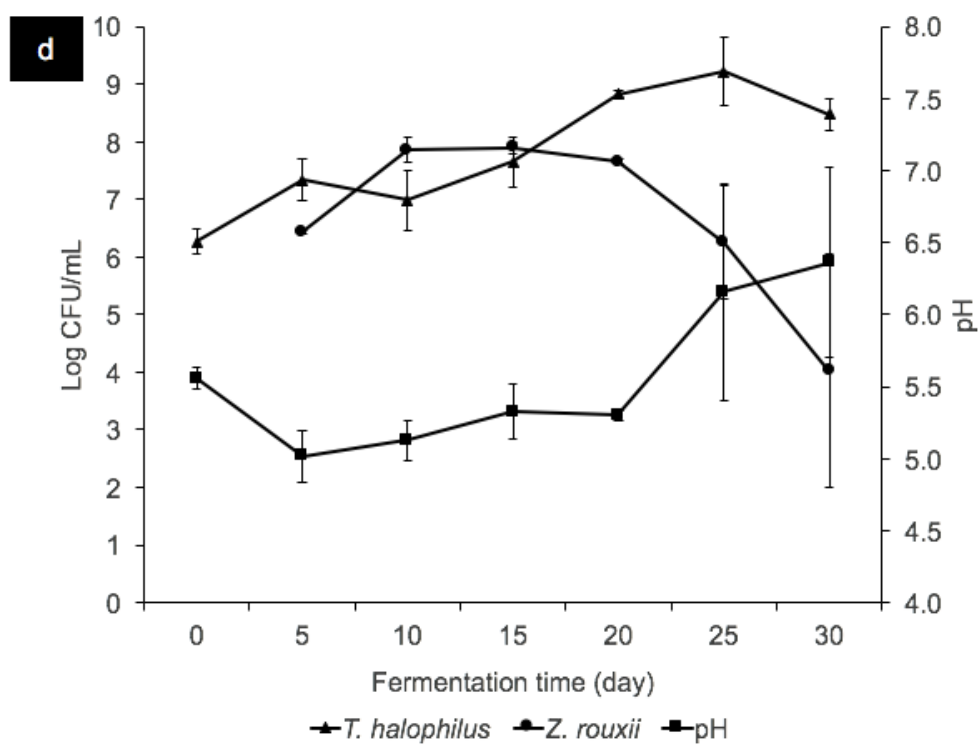
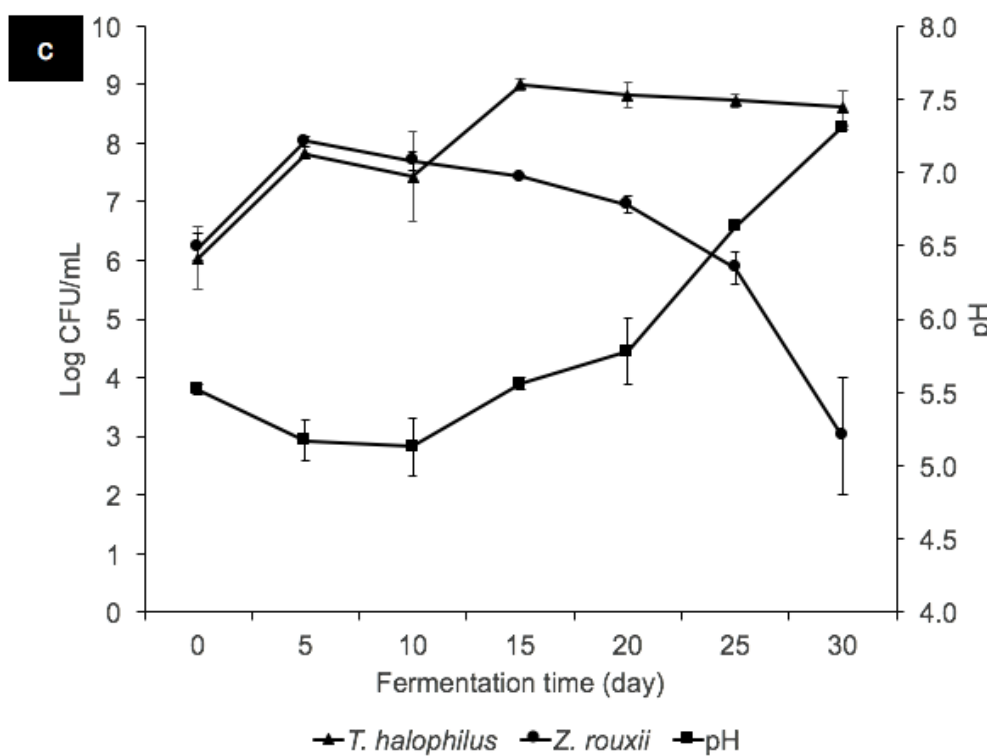
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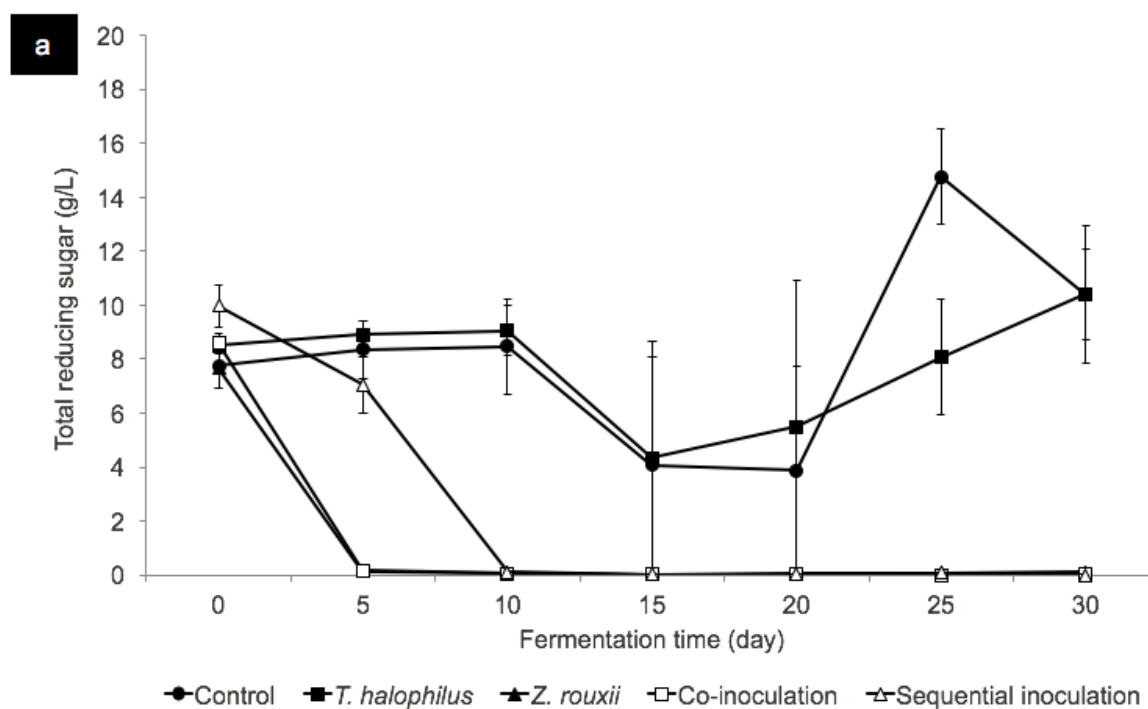
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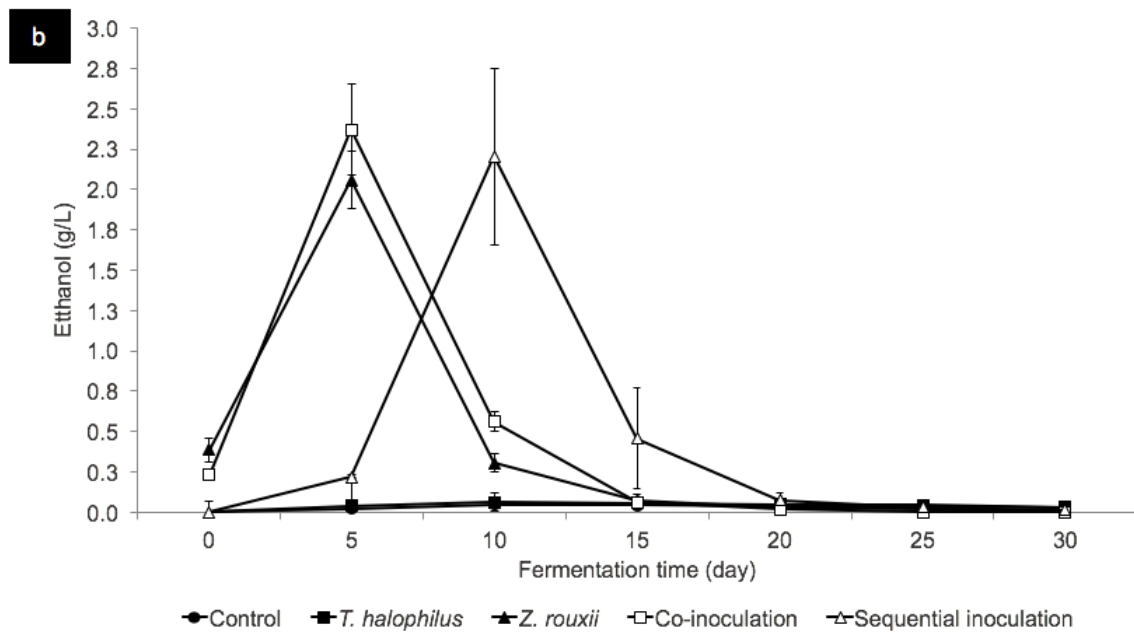


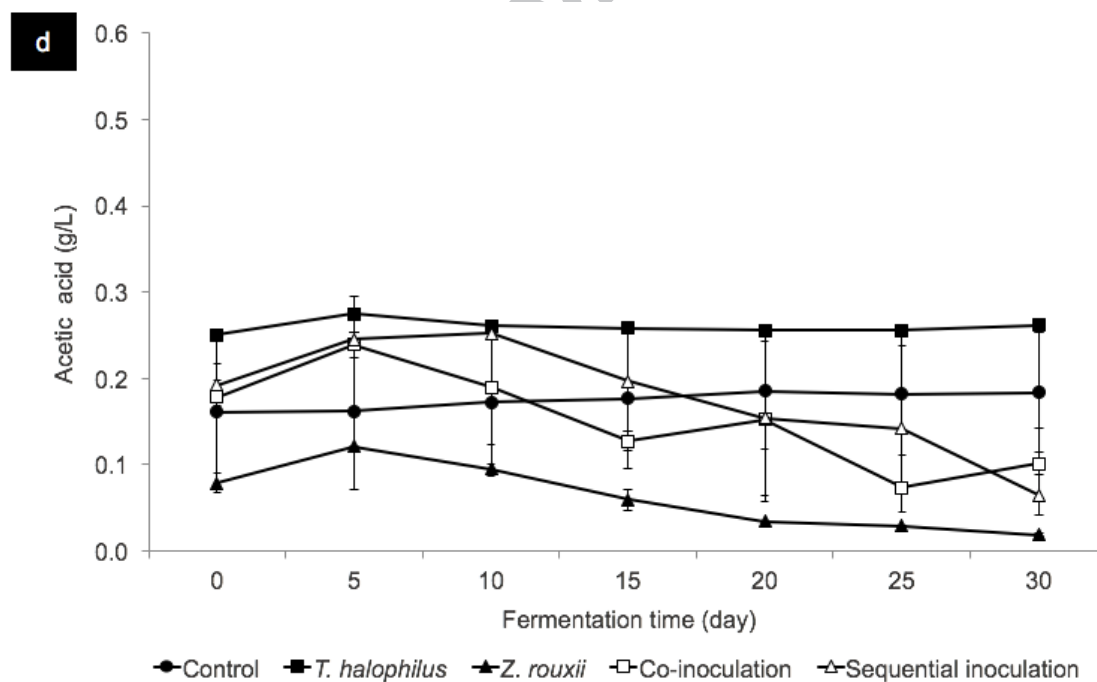
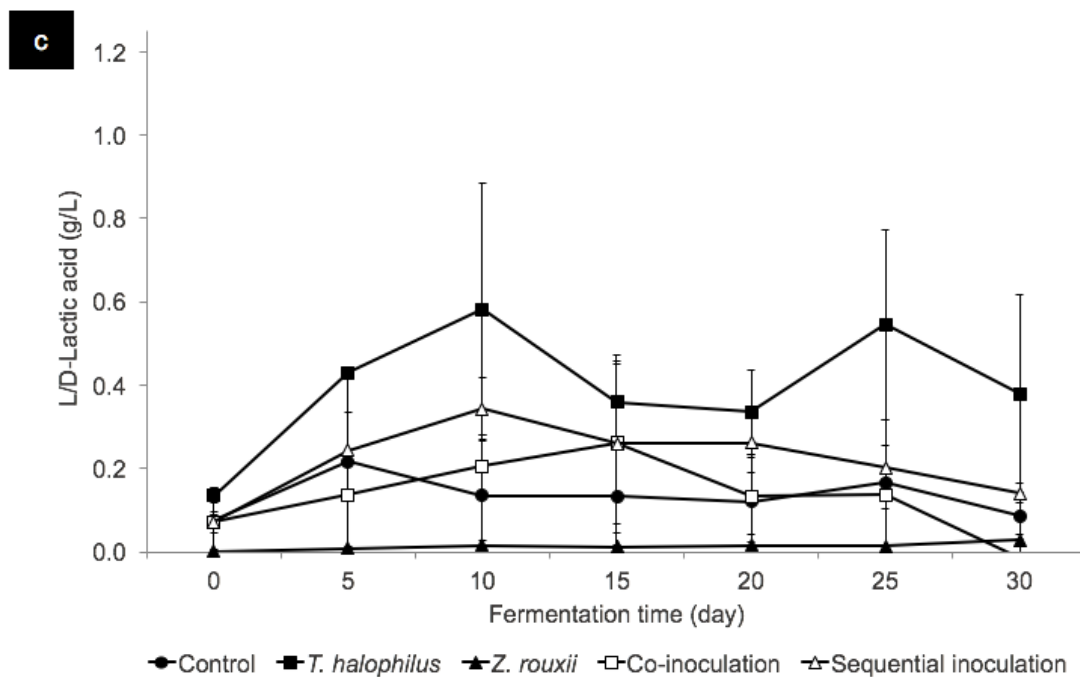


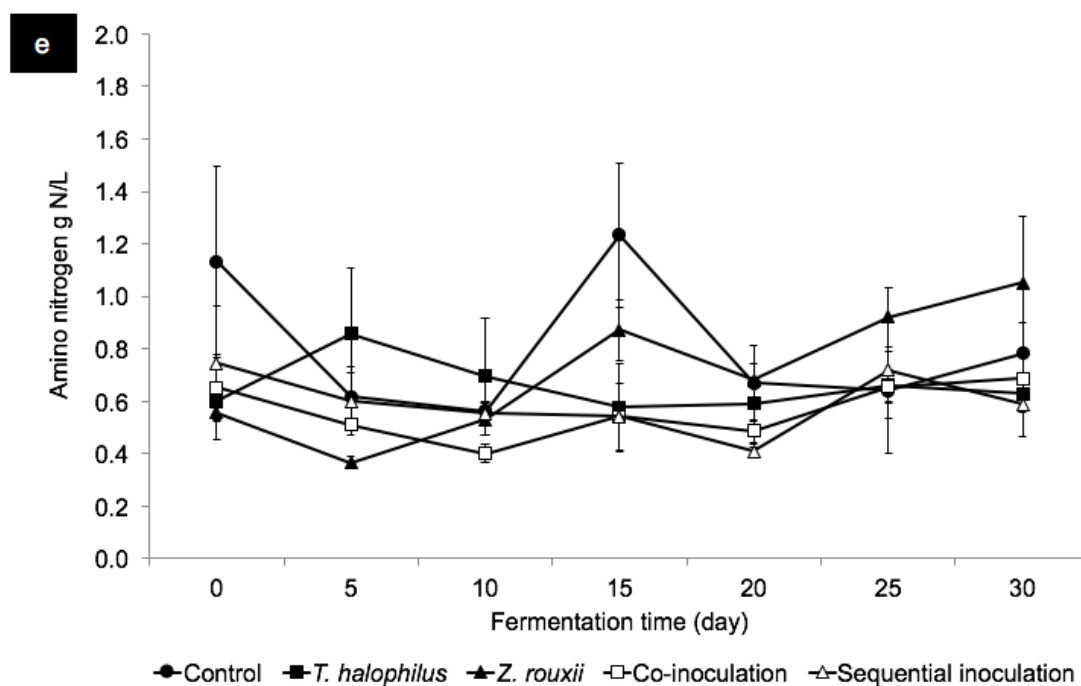




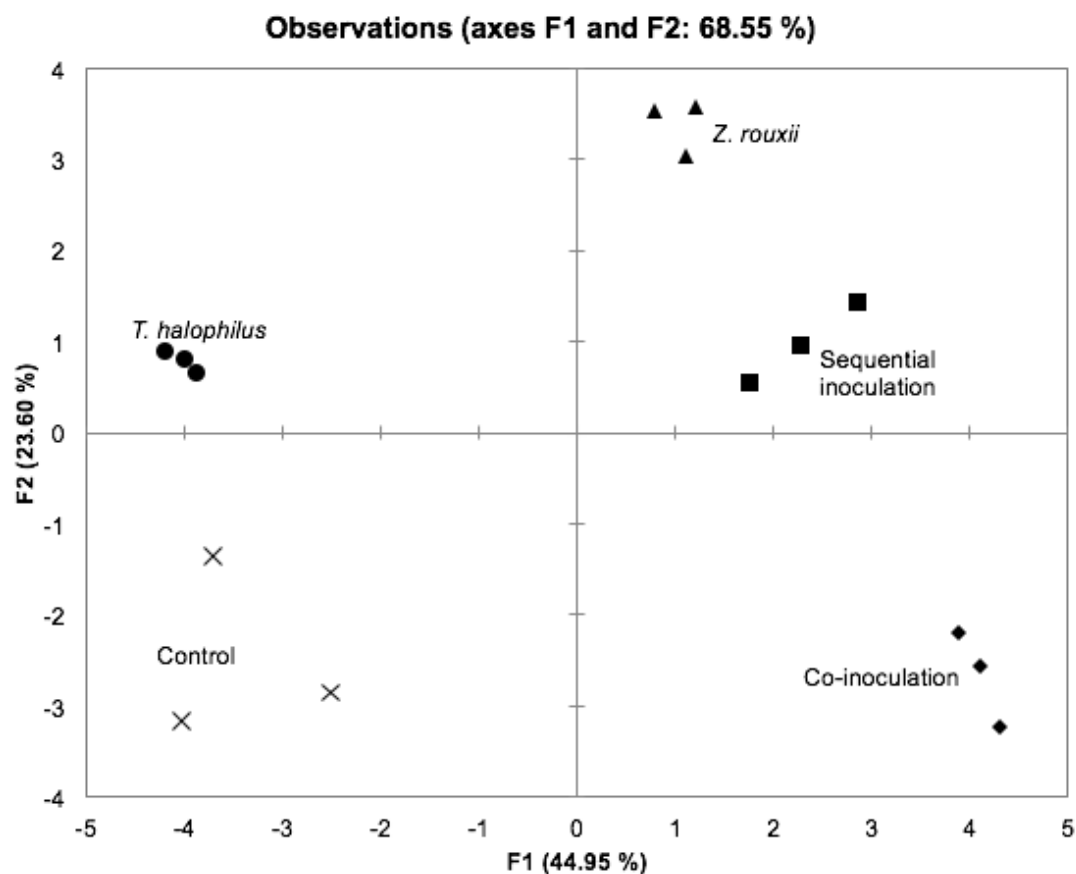
**Fig. 1.** Changes in pH and growth (cfu) of *T. halophilus* and *Z. rouxii* growth during moromi fermentation with pure culture of *T. halophilus* (a), pure culture of *Z. rouxii* (b), co-inoculation (c), and sequential inoculation (d).



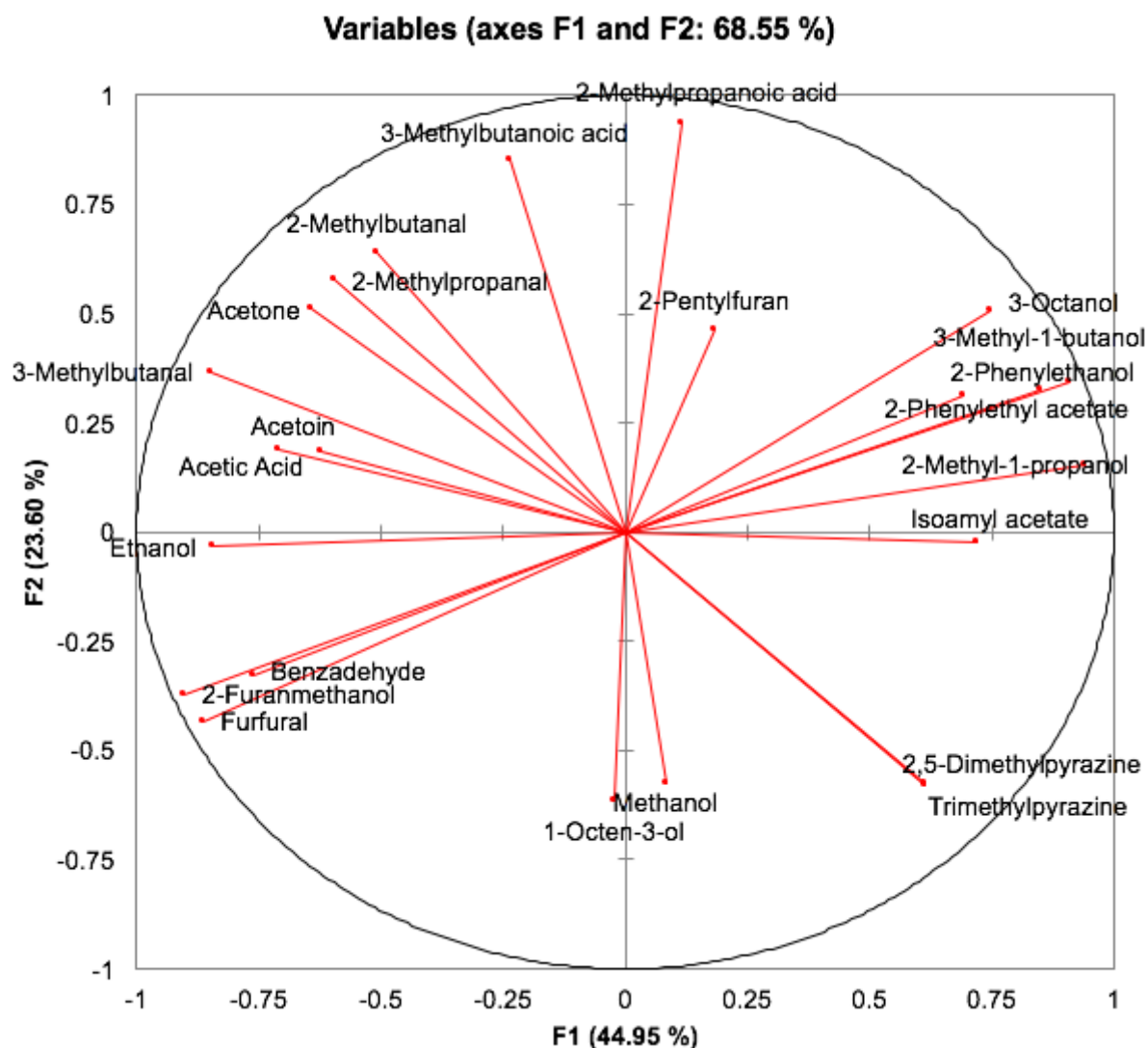




**Fig. 2** The changes of total reducing sugar (a), ethanol (b), L/D-lactic acid (c), acetic acid (d), and amino nitrogen (e) during 30-day period of moromi fermentation at 30°C with different combinations of microorganisms.



**Fig. 3** PCA score plot of five moromi samples after 30-day fermentation. The scores are based on three replicates of each sample. The identical symbols represent triplicate measurements.



**Fig. 4** PCA loading plot of the aroma compounds detected in moromi after 30-day fermentation with either single culture of *T. halophilus* and *Z. rouxii*, co-inoculation, and sequential inoculation.



**Table 1.** Aroma compounds found in moromi before and after 30 days of fermentation with different combinations of microorganisms. The values are relative to the peak area observed when the headspace above a 0.1 µg/mL 1-octen-3-ol solution was analysed. Each value is based on three replicates.

compound	LRI <sup>1</sup>	Day 0		Day 30						
				control		<i>T. halophilus</i>		<i>Z. rouxii</i>		ino
		mean	SD	mean	SD	mean	SD	mean	SD	mean
alcohols										
methanol	915	0.67a	0.12	0.82a	0.14	0.78a	0.08	0.75a	0.07	1.01
ethanol	952	0.48a	0.10	4.86b	0.95	4.70b	0.40	1.51ac	0.19	0.29
2-methyl-1-propanol	1117	0.00a	0.00	0.01a	0.00	0.01a	0.00	0.06b	0.01	0.08
3-methyl-1-butanol	1227	0.03a	0.00	0.87a	0.17	0.88a	0.06	14.93b	1.03	13.3
3-octanol	1408	0.01a	0.00	0.00a	0.00	0.00a	0.00	0.07b	0.02	0.04
1-octen-3-ol	1468	0.65a	0.06	0.28b	0.07	0.16b	0.02	0.18b	0.03	0.23

2-phenylethanol	1961	0.06a	0.05	0.06a	0.04	0.07a	0.06	19.50b	1.96	16.4
2-furanmethanol	1693	0.26a	0.03	0.23a	0.03	0.25a	0.03	0.01b	0.00	0.04
<b>acids</b>										
acetic acid	1486	0.12a	0.09	0.02a	0.04	14.34b	0.64	0.39a	0.31	0.09
2-methylpropanoic acid	1599	0.01a	0.01	0.00a	0.00	0.17a	0.01	0.42b	0.01	0.04
3-methylbutanoic acid	1700	0.05a	0.06	0.04a	0.04	2.03b	0.11	1.71b	0.14	0.14
<b>aldehydes</b>										
2-methylpropanal	825	0.31a	0.06	0.06bc	0.03	0.09b	0.01	0.11b	0.01	0.00
2-methylbutanal	930	0.18a	0.05	0.02b	0.01	0.03b	0.00	0.05b	0.01	0.00
3-methylbutanal	934	0.14a	0.02	0.06b	0.03	0.07b	0.01	0.05b	0.00	0.00
furfural	1502	1.11a	0.18	0.23b	0.04	0.14b	0.01	0.01b	0.00	0.01
benzaldehyde	1570	0.31ab	0.05	0.44a	0.09	0.17bc	0.03	0.14c	0.02	0.03

**esters**

isoamyl acetate	1140	0.00a	0.00	0.01a	0.00	0.03a	0.00	0.05a	0.01	0.27
2-phenylethyl acetate	1860	0.01a	0.00	0.00a	0.00	0.00a	0.00	0.17b	0.03	0.15

**pyrazines**

2,5-dimethylpyrazine	1353	0.03a	0.00	0.06a	0.01	0.17a	0.02	0.05a	0.01	2.00
trimethylpyrazine	1434	0.05a	0.01	0.05a	0.01	0.05a	0.01	0.05a	0.01	0.15

**furan**

2-pentylfuran	1251	0.07a	0.02	0.05a	0.04	0.08a	0.01	0.08a	0.01	0.07
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**ketones**

acetoin	1321	0.15a	0.02	0.16a	0.02	1.23a	0.09	0.14a	0.01	0.00
acetone	830	1.47a	0.23	0.62b	0.16	0.63b	0.09	0.59 b	0.01	0.15

<sup>1</sup>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 ( $p < 0.05$ )

ACCEPTED MANUSCRIPT

## Highlights

- *T. halophilus* and *Z. rouxii* interaction was driven by antagonism.
- *Z. rouxii* growth was inhibited by *T. halophilus* proliferation.
- *Z. rouxii* under suppression resulted in more complex volatile profiles.
- Moromi aroma profiles were distinguished based on sequence of inoculation.