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Starter cultures inoculation procedure changes microbial community structure during low-salt *moromi* fermentation

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Abstract

Soy sauce is a condiment made of soybeans fermented in high salt concentration (18-22% NaCl) called *moromi*. Efforts to prevent sodium overconsumption has led to salt reduction in *moromi*. However, it could alter the microbial community dynamics during fermentation, resulting in undesirable sensory changes. This study investigates the effect of *moromi* starter cultures, *Tetragenococcus* sp. and *Zygosaccharomyces rouxii*, on microbial population changes during reduced-salt *moromi* (6% NaCl) fermentation. Both microbes were inoculated at different times, concentrations, and ratios, and the changes in total bacterial and yeast population during fermentation were monitored by culture on tryptic soy agar (TSA) and potato dextrose agar (PDA), respectively. The results showed that the inoculum size could shorten the bacterial exponential growth as it increased. Moreover, increasing the proportion of *Tetragenococcus* or *Z. rouxii* by two-fold could enhance the yeast and bacterial population, respectively by ~3 log CFU/mL. Simultaneous inoculation suppressed bacterial growth, while sequential inoculation of *Z. rouxii* could maximize bacterial growth up to 7.38 log CFU/mL. However, a synergistic growth in simultaneous inoculation was observed when 5% inoculum with an equal ratio was used. This study could help researchers and manufacturers determine appropriate inoculation procedures to control fermentation better.

1. Introduction

Soy sauce is a liquid seasoning commonly used in Asia and becoming increasingly popular in the Western world. It is made by fermenting a mixture of soybean and wheat through a two-step fermentation process, called *koji* (solid-state fermentation) and *moromi* (brine fermentation). Multiple species of mould, bacteria, and yeast act in concert during the fermentation process to break down complex molecules into simpler ones, impacting the final product's palatability (Wei *et al*., 2013; Zhang *et al.*, 2016; Ito and Matsuyama, 2021; Liu *et al*., 2021). Mould, typically *Aspergillus oryzae* and *Aspergillus sojae*, are usually added during *koji* fermentation. After approximately three days of fermentation, *koji* is immersed in a brine solution containing high salt (18-22% NaCl) and the *moromi* fermentation begins. In this stage, the majority of volatile compounds that give soy sauce a unique taste and characteristics are produced.

Moromi fermentation is driven by a diverse microbial community rather than a monoculture. In the early stage of *moromi* fermentation, lactic acid bacteria (LAB) grow to produce organic acids (e.g., lactic acid and acetic acid) which contribute to acidic taste and aroma. The acids also bring the pH down to below 5.0, creating a more favourable condition for the yeast to grow. Yeast dominates the later stage of *moromi* fermentation and produces numerous essential alcohols and esters through alcoholic fermentation (Harada *et al*., 2016). Some members of the *Bacillus* group are also known to play an important role in breaking down protein and starch into smaller components which serve as precursors for flavour formation (Jiang *et al*., 2019).

The high salt content in soy sauce raises a health concern, as World Health Organization (WHO) recommends limiting daily sodium intake to two grams or equivalent to five grams of salt. The risk of cardiovascular diseases due to sodium salt overconsumption leads to increased consumer demand for a healthier version of soy sauce. Efforts have been done to reduce salt in soy sauce using reverse osmosis (Otomi *et al*., 1992; Wang *et al*., 2021), nanofiltration (Luo *et al*., 2009), partial NaCl substitution with KCl (Segawa *et al*., 1995), salt replacement with ethanol, sugars, and polyols (Chiou, 1999), saltiness-associated

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odorants (Zhou *et al*., 2021), and several other methods. However, for a commercial purpose, these methods appeared to be not feasible due to high production costs and undesirable sensory properties.

Spontaneous fermentation by indigenous microbes present in the raw material is still preferred in common practices since the resulting soy sauce has a more complex flavour and aroma. At the same time, the utilization of defined starter cultures is increasingly favoured due to better control of the fermentation process to obtain product with desirable quality (Lee *et al*., 2013; Liang *et al*., 2019; Liu *et al*., 2021). *Tetragenococcus* sp. and *Zygosaccharomyces rouxii* have been known to be predominant during *moromi* fermentation, as well as essential for the production of nearly 300 aroma compounds that embodies the complex sensorial properties of soy sauce (Lee *et al*., 2013; Devanthi, Linforth, Onyeaka *et al*., 2018; Devanthi and Gkatzionis, 2019). The utilization of both *Tetragenococcus* sp. and *Z. rouxii* as starter cultures have been reported to be able to compensate for the loss in flavour due to salt reduction during *moromi* fermentation (Harada *et al*., 2016; Singracha *et al*., 2017; Devanthi, Linforth, El Kadri *et al*., 2018). However, the introduction of starter cultures may cause biochemical and physical changes of *moromi*, affecting the existing microbial community structure and interactions, ultimately affecting the fermentation success. Furthermore, in addition to selecting appropriate starter cultures, the key to ensuring a balance between consistency and process complexity also lies in the inoculation sequence (Liu *et al*., 2021). Moreover, according to Bartle *et al.* (2019), the inoculation procedures (e.g. cell concentration, ratio, and inoculation time) could significantly influence the microbe-microbe interaction and sensory properties of the final product. This study aims to investigate the effects of inoculum concentration, ratio, and inoculation timing of *Tetragenococcus* sp. and *Z. rouxii* on reduced-salt *moromi*'s microbial community structure, comprising bacteria and yeast.

2. Materials and methods

2.1 Inoculum preparation

Aspergillus oryzae was obtained from American Type Culture Collection (ATCC; USA) 10124. *Tetragenococcus* sp. (InaCC B648) was obtained from the Indonesian Institute of Sciences (LIPI; Jakarta, Indonesia), while *Zygosaccharomyces rouxii* was obtained from National Collection of Yeast Cultures (NCYC; UK) 381. *Aspergillus oryzae* was grown on Potato Dextrose Agar (PDA; Merck, Germany) for 7 days at 30°C. The spores were harvested using a physiological solution (0.85% w/v NaCl (Merck, Germany)). *Tetragenococcus* sp. was grown in de Man Rogosa and Sharpe (MRS; Merck, Germany) broth supplemented with 7% w/v NaCl for 36 hrs at 37°C. Meanwhile, *Z. rouxii* was grown on Yeast Malt Broth (YMB; Himedia, India) medium supplemented with 5% w/v NaCl for 24 hrs at 30°C with agitation.

2.2 Koji and moromi preparation

Koji was made by mixing cooked soybeans and wheat flour with a ratio of 3:1, followed by inoculation with *A. oryzae* spores (10% v/v) at a 105 spores/mL concentration. After 3 days of incubation at 30°C, *koji* was mixed with 6% w/v NaCl solution with a ratio of 1:3 to create *moromi* mash. *Moromi* was then inoculated with *Tetragenococcus* sp. and *Z. rouxii* as described in section 2.3.

2.3 Study on the effect of inoculum size, inoculum ratio, and inoculation time interval on the growth of bacteria and yeast

The fermentation was conducted in a 500 mL glass jar containing 400 mL of *moromi*. Unless stated, *Tetragenococcus* sp. and *Z. rouxii* were added simultaneously at a concentration of 5% v/v and a ratio of 1:1. To study the effect of inoculum size, *Tetragenococcus* sp. and *Z. rouxii* were added at concentrations of 2%, 5%, and 10% v/v. To test the effect of the inoculum ratio, *Tetragenococcus* sp. and *Z. rouxii* were added at ratios of 1:1 (T1Z1), 1:2 (T1Z2), and 2:1 (T2Z1). The effect of inoculation time interval was tested by inoculating *Tetragenococcus* sp. at day 0, while *Z. rouxii* was inoculated on day 0, 7, and 14. The fermentation jars were placed in a static incubator at 30° C for 29 days. Samples were collected on day 2, 8, 22, and 29.

2.4 Microbiological and pH analysis

Samples were serially diluted using Phosphate Buffered Saline (PBS; Sigma-Aldrich, USA) followed by plating on agar media for colony counting. Total bacteria number was counted using tryptic soy agar (TSA; Merck, Germany) supplemented with 6% w/v NaCl and cycloheximide (100 µg/mL) (Sigma-Aldrich, USA). Total yeast was enumerated using PDA supplemented with 6% w/v NaCl and chloramphenicol (20 µg/mL) (Himedia, India). The agar plates were incubated at 30°C for 2-3 days. pH changes were measured using pH meter (Mettler Toledo, USA) in triplicates.

2.5 Statistical analysis

Each experiment was done in triplicates and the

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results were expressed as mean ± standard deviation. The data were analyzed with one-way analysis of variance (ANOVA) using XLSTAT™ version 2020.5.1 (Addinsoft, New York, NY, USA) at $p < 0.05$ and Tukey's test was applied for means comparison.

3. Results and discussion

3.1 Effect of Tetragenococcus sp. *and Zygosaccharomyces rouxii inoculum size*

Inoculum size can affect the rate of nutrient consumption and metabolite production, which can further cause changes in microbial population structure (Carrau *et al*., 2010; Jaronski and Jackson, 2012). In this experiment, the effect of inoculum size of *Tetragenococcus* sp. and *Z. rouxii* co-culture on total viable bacteria and yeast cell counts during *moromi* fermentation was evaluated. The results showed that the inoculum size had a significant effect ($p < 0.05$) on the bacterial and yeast population changes. The inoculum size affected the length of the bacterial exponential phase, which was extended as the inoculum size decreased (Figure 1a). When 10% inoculum was added, the bacterial exponential phase only lasted for 8 days, reaching a maximum population of 8.09 log CFU/mL. After that, the total viable cell count of the bacteria began to decline and reached a final concentration of 6.59 log CFU/mL. The bacterial exponential phase was extended to 22 days before it reached its maximum population (8.54 log CFU/mL) when 5% inoculum was added. After this period, the number then started to decline to a final population of 7.44 log CFU/mL. The bacterial population increased more slowly when 2% inoculum was added, and the peak was not observed until the fermentation ended. A shorter exponential phase was expected to occur with the increase in inoculum size. Inoculum size could trigger the bacterial population to grow more rapidly, resulting in faster nutrient depletion and toxic metabolite production (Robinson *et al*., 2001; Sood *et al*., 2011; Liu, 2017). Consequently, the bacterial population would enter the death phase earlier.

When 5% inoculum was added, yeast's exponential phase was extended to 22 days, allowing the yeast to reach a maximum concentration of 8.53 log CFU/mL (Figure 1b). A similar phenomenon was observed in a mixed population of LAB and yeast by Sieuwerts *et al*. (2018). The exponential phase only lasted for 8 days when 2% and 10% inocula were used, increasing the population to 6.81 log CFU/mL and 7.47 CFU/mL, respectively, before it started declining. The declining yeast population after it peaks on day 8 could be attributed to sugar depletion, as it is converted into biomass and ethanol (Devanthi, El Kadri, Bowden *et al*., 2018; Yao *et al*., 2020). In reduced-salt conditions, sugar depletion can occur faster as the yeast's metabolic activity occurs at a higher rate in low-salt environments (Jansen *et al*., 2003). However, such a decline in population could be avoided by adding 5% inoculum of *Tetragenococcus* sp. and *Z. rouxii*.

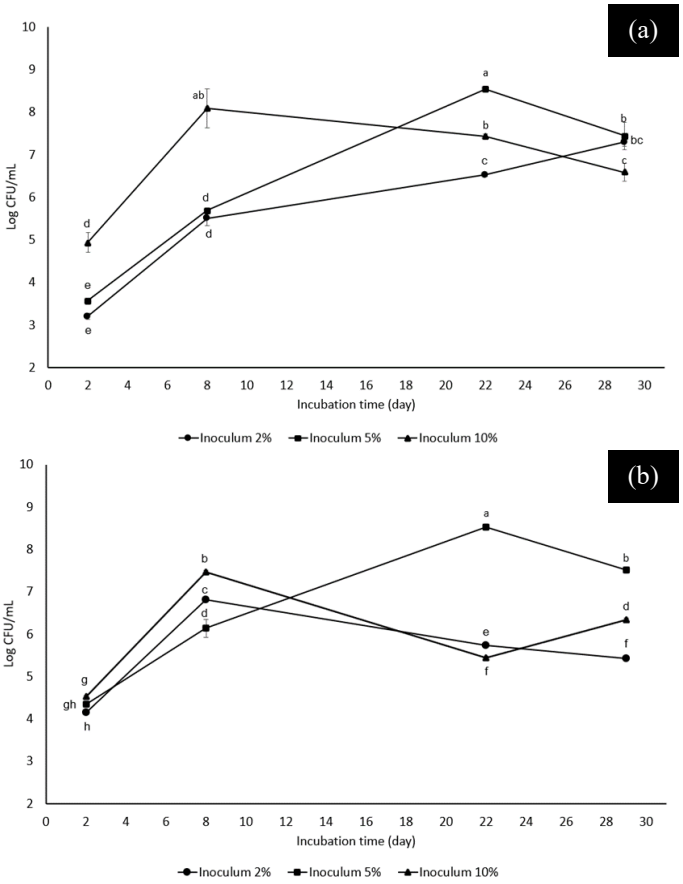

Figure 1. Changes in total population of (a) bacteria and (b) yeast during reduced-salt *moromi* (6% NaCl) fermentation at 30°C after the addition of *Tetragenococcus* sp. and *Z. rouxii* varying in inoculum sizes (2%, 5%, and 10%). Both cultures were added simultaneously at a ratio of 1:1. Means with different letters are statistically significant different ($p<0.05$).

3.2 Effect of Tetragenococcus sp. *and Zygosaccharomyces rouxii ratio*

Several studies have investigated how inoculum ratios play an important role in the interaction between bacteria and yeast populations during the fermentation process, which then affects the quality of the final products (Kedia *et al*., 2006; Blanco *et al*., 2020). The results showed that the ratio of *Tetragenococcus* sp. and *Z. rouxii* significantly affected ($p < 0.05$) the total bacterial and yeast populations. The bacterial population increased more rapidly during the first 8 days as the proportion of *Z. rouxii* increased (T1Z2), reaching 8.12 log CFU/mL (Figure 2a). During the same period, lower bacterial populations were observed in samples T1Z1 (5.73 log CFU/mL) and T2Z1 (6.95 log CFU/mL). Similarly, the total yeast population increased faster with the increased amount of *Tetragenococcus* sp. added (T2Z1), reaching its peak (8.34 log CFU/mL) on day 8

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(Figure 2b). Yeast population in sample T1Z1 and T1Z2 also reached its peak on day 8, however, the cell counts were lower by 0.76 log CFU/mL and 2.1 log CFU/mL, respectively, compared to T2Z1. The results of this study also demonstrated bacterial suppression whenever yeast population was enhanced, and vice versa, indicating an antagonistic interaction between them, as previously reported by Devanthi, Linforth, Onyeaka *et al*. (2018). The results of this experiment demonstrate that the degree of inhibition depends on the ratio of starter cultures added.

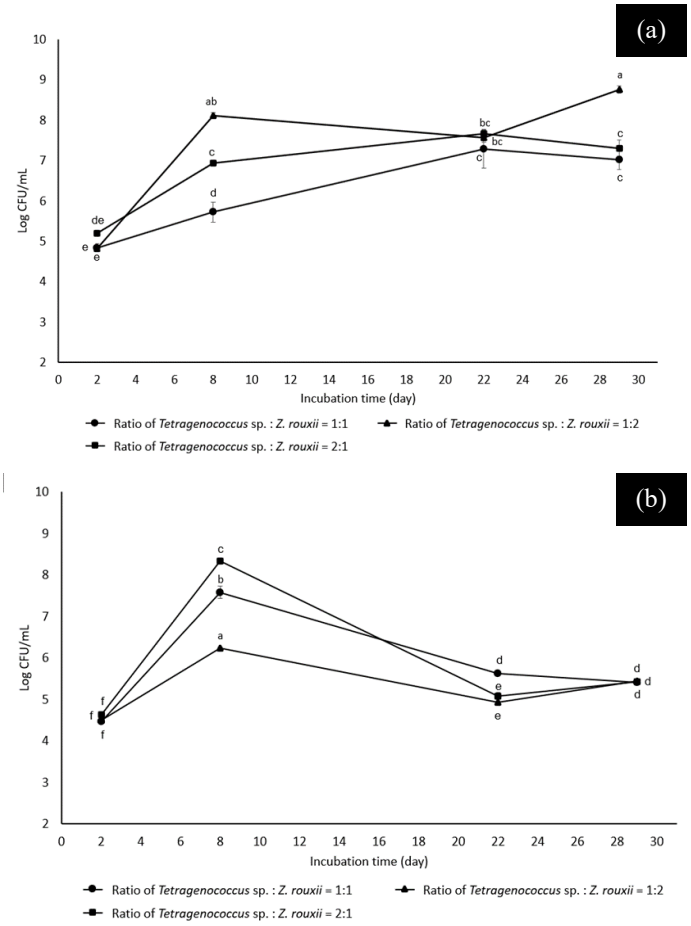

Figure 2. Changes in total population of (a) bacteria and (b) yeast during reduced-salt *moromi* (6% NaCl) fermentation at 30°C after the addition of *Tetragenococcus* sp. and *Z. rouxii* varying in inoculum ratios (1:1, 2:1, and 1:2). Both cultures were added simultaneously at an inoculum concentration of 5% v/v. Means with different letters are statistically significant different ($p<0.05$).

3.3 Effect of Tetragenococcus sp. *and Zygosaccharomyces rouxii time interval*

Tetragenococcus sp. and *Z. rouxii* inoculation time interval was previously found to affect the aroma formation during reduced-salt *moromi* fermentation (Devanthi, Linforth, Onyeaka *et al*., 2018; Devanthi, Linforth, El Kadri *et al*., 2018). In the present study, the effect of *Tetragenococcus* sp. and *Z. rouxii* inoculation time intervals on total bacterial and yeast population growth were evaluated. As shown in Figure 3a, sequential inoculation of *Z. rouxii*, regardless of time interval, could enhance the bacterial population by ~ 3 log CFU/mL during the first 8 days of fermentation, which is then followed by a plateau (Figure 3a). Contrastingly, simultaneous inoculation caused the bacterial population to remain constant (~ 5 log CFU/mL) throughout the fermentation period. To see the effect of inoculation time intervals on yeast growth, the yeast cell counts were monitored after *Z. rouxii* was introduced into the *moromi*. The highest yeast population observed was on day 8 (7.50 log CFU/mL) when *Tetragenococcus* sp. and *Z. rouxii* were inoculated simultaneously, followed by a sudden decrease to 4.91 log CFU/mL on day 22 (Figure 3b). Regardless of the inoculation time intervals, by the end of the fermentation period, the final yeast cell count in all samples remained at ~ 4 log CFU/mL. The results of this study suggest that simultaneous inoculation of *Tetragenococcus* sp. and *Z. rouxii* may have an inhibitory effect on the total bacterial population growth, as the bacterial population remained constant when both cultures were inoculated simultaneously. A similar finding was reported by

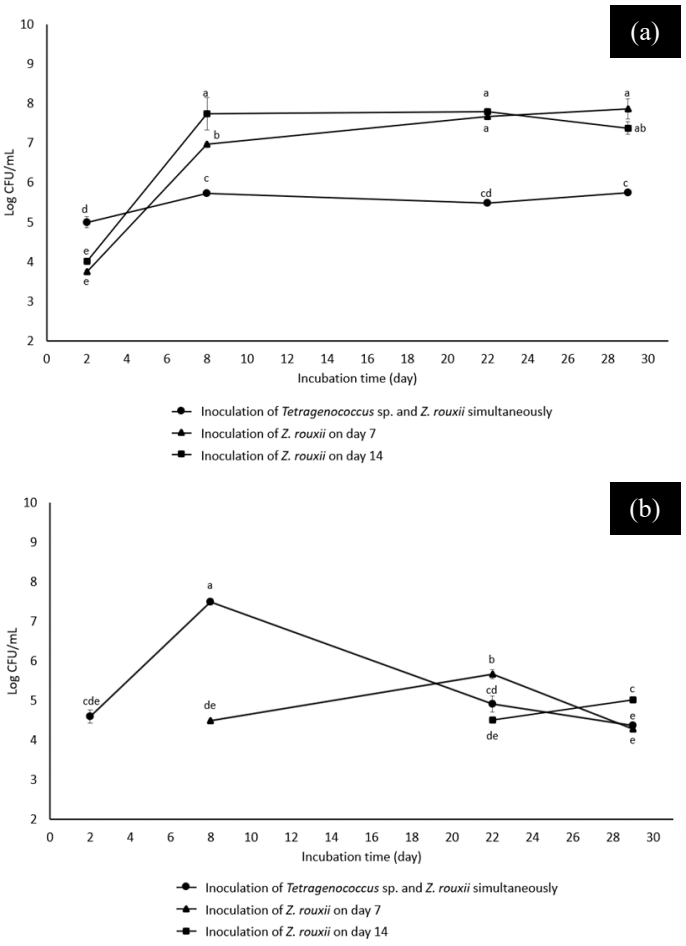

Figure 3. Changes in total population of (a) bacteria and (b) yeast during reduced-salt *moromi* (6% NaCl) fermentation at 30°C after the addition of *Tetragenococcus* sp. and *Z. rouxii* both simultaneously and sequentially. Both cultures were added at an inoculum concentration of 5% v/v and ratio of 1:1. Means with different letters are statistically significant different ($p<0.05$).

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Devanthi, El Kadri, Bowden *et al*. (2018), which demonstrated an inhibitory effect on *Tetragenococcus halophilus* upon simultaneous inoculation of *T. halophilus* and *Z. rouxii*. The authors also suggested that *Z. rouxii* could cause a rapid depletion of reducing sugars accompanied by ethanol formation, which might have detrimental effects on bacterial growth, which could be reduced by introducing *Z. rouxii* at a later stage.

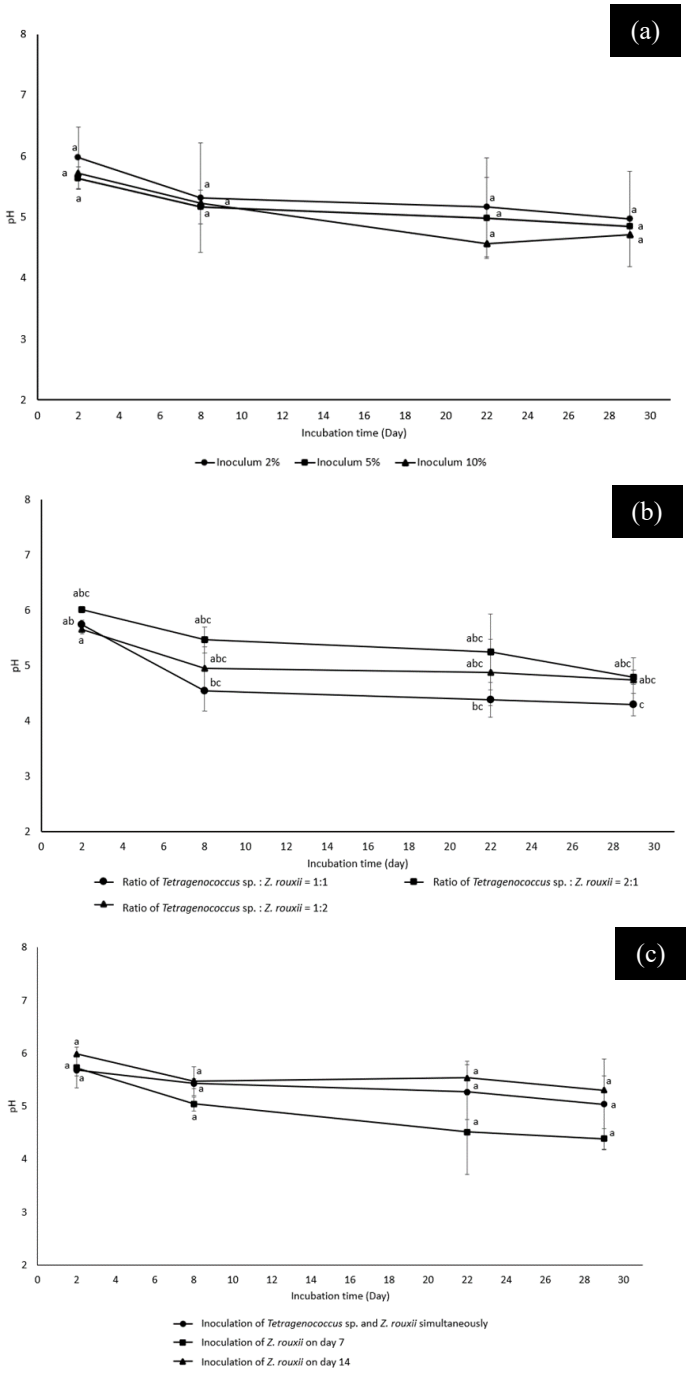

Figure 4. Changes of pH during reduced salt *moromi* (6% NaCl) fermentation at 30°C using mixed cultures of *Tetragenococcus* sp. and *Z. rouxii* varying in (a) inoculum sizes, (b) inoculum ratios, and (c) inoculation time interval. Means with different letters are statistically significant different ($p<0.05$).

3.4 Changes of pH during moromi fermentation

pH is one of the crucial factors affecting the growth of the microbial population during fermentation and it could be used to identify the progress of fermentation. *Moromi* fermentation typically begins with an initial pH of 6.5–7.0 which then decreases to 5.5-4.7 by the end of the fermentation period (Hamada *et al*., 1989; Sulaiman *et al*., 2014; Hoang *et al*., 2016). Such pH decreases results from the acid production by bacteria, especially LAB. The acidic environment (pH < 5.0) is essential for creating a favorable environment for the yeast to grow and perform alcoholic fermentation to generate various essential aroma compounds (Der Sluis *et al*., 2001; O'toole, 2019). In this study, the initial pH of all samples was ranging from 5.6-6.0, which is suitable for the halotolerant bacteria, especially LAB, to proliferate rapidly during the early stages of fermentation (Figure 4). Regardless of the inoculum size, ratio, and inoculation time interval, the pH gradually decreased throughout the fermentation, resulting in a final pH of 4.3-5.0, indicating the production of acid by the bacteria.

4. Conclusion

This study concludes that the growth of the total bacterial and yeast population during reduced-salt moromi fermentation is affected by the inoculum size, ratio, and inoculation time interval of *Tetragenococcus* sp. and *Z. rouxii* co-culture. A possible antagonistic interaction between the bacteria and yeast population was observed, as one's population growth was suppressed when the other's enhanced. However, a synergistic growth occurred when equal amounts of *Tetragenococcus* sp. and *Z. rouxii* were added simultaneously at an inoculum size of 5%. Furthermore, the impacts on pH changes were also observed, and it was found that the pH of all samples decreased from 5.6-6.0 to 4.3-5.0, regardless of the inoculum size, ratio, and inoculation time interval. This study provides valuable insights into mixed starter cultures inoculation procedures to control the fermentation better.

Conflict of interest

The authors declare no conflict of interest.

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