Potential of snake fruit (Salacca zalacca (Gaerth.) Voss) for the development of a beverage through fermentation with the Kombucha consortium

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1. Introduction
Snake fruit (Salacca zalacca (Gaerth.) Voss) is a tropical fruit widely found in South East Asia. It is known by various names such as Salak (Indonesia, Malaysia and Philippines), Rakam (Thailand), Sa Laka and She Pi Guo Zong (China), Schlagenfrucht and Zalak (Germany), Sarakka Yashi (Japan), Salaca (Span) and Yingan (Myanmar) (Mohd Zaini et al., 2013). It has a white firm pulp with a sweet, slight acidic and astringent taste. Snake fruit is a good source of vitamins, minerals, dietary fiber, and bioactive compounds with antioxidant activities (Aralas et al., 2009; Suica-Bunghez et al., 2016). There are many snake fruit cultivars or genotypes worldwide, and each cultivar has unique physical, chemical and sensory characteristics. The ripe fruit is mostly eaten fresh, but it can also be processed into canned fruits, juices, dried fruits, pickles, syrups, and fermented products. Some studies revealed the suitability of sugared snake fruit juice for wine and vinegar fermentations, as it is a good medium for relevant microorganisms (Gunam et al., 2009; Zubaidah et al., 2017). This favourable fermentability property of the sugared snake fruit can be explored for other fermented products with functional properties.

Kombucha (tea fungus) is a refreshing health-promoting beverage of sugared tea infusion fermented by a symbiotic consortium of yeast species and acetic acid bacteria. Various yeasts (e.g. Pichia, Candida, Zygosaccharomyces, Brettanomyces, and Saccharomyces species) and Acetobacter xylitum have been identified in Kombucha fermentation (Jayabalan et al., 2014). The beverage possesses functional properties such as antimicrobial, antioxidant, anticancer, and antidiabetic, and is beneficial in treating gastric ulcer (Jayabalan et al., 2011; Aloulou et al., 2012; Bhattacharya et al., 2013; Banerjee et al., 2011; Chakravorty et al., 2016). Specifically, Kombucha is reported (Sreeramalu et al., 2000; Jayabalan et al., 2014) to inhibit a broad spectrum of Gram-positive (e.g. Staphylococcus aureus and Bacillus cereus) and Gram-negative bacteria (e.g. Escherichia coli and Pseudomonas aeruginosa). Moreover, total phenolic compounds and radical scavenging activities of Kombucha increase with fermentation time (Jayabalan et al., 2014). In view of the functional properties of Kombucha, substrates other than tea had been studied, including Jerusalem artichoke, echinacea, mentha, eucalyptus, sour cherry, grape, orange, and blackcurrant (Yavari et al., 2010, 2011; Jayabalan et al., 2014; Gamboa-Gómez et al., 2016; Lobanova et al., 2016; Ayed et al., 2017).
However, we are unaware of the fermentation of snake fruit juices with the Kombucha consortium, and in view of the global availability of snake fruits, a study along these lines would provide another product to health-conscious consumers particularly in areas where snake fruits are abundant. Therefore, using different cultivars of snake fruit to understand cultivar effects, the objectives of this research were to:

(a) Investigate fermentation of snake fruit with the Kombucha consortium.
(b) Evaluate physicochemical and sensory properties of the fermented products.
(c) Assess antioxidant and antibacterial activities of the most promising cultivar, with its bioactive compounds.

2. Materials and methods

2.1. Materials

Snake fruits of commercial maturity were obtained from plantations in Malang, Jombang and Kediri, East Java, Indonesia, and were of cultivars Salak Doyong, Salak Madu, Salak Pondoh, Salak Segaran, and Salak Sunawar. Commercial Kombucha starter was purchased from a local distributor, while cane sugar was bought from a local supermarket.

2.2. Snake fruit juice preparation and Kombucha fermentation

The snake fruits were peeled, washed, and cut into small sizes, from which 400 g was mixed (1:1, w/w) with water, juiced (blended) and filtered (cheese cloth). The snake fruit juices were sweetened (1:10, w/v) with the cane sugar, pasteurized (Waterbath Memmert, Germany) at 65 °C for 30 min and cooled to room temperature before storing in a sterile jar and refrigerating until used. The juices were sweetened or sugared to provide additional sugars and facilitate fermentation as reported elsewhere (Sreeramulu et al., 2000; Jayabalan et al., 2014). The sugared juices were inoculated (1:10 w/w) with the Kombucha starter and incubated for 14 days at room temperature. Samples of the fermenting broth were taken at days 0, 7 and 14 for analysis.

2.3. Media and chemicals

Culture media, nutrient agar (CM0003 Oxoid™) and potato dextrose agar (CM0139 Oxoid™) were products of Thermo Fischer Scientific, USA. Sodium hydroxide (Merck 106462, Germany), oxalic acid (Merck 100495, Germany), anthrone reagent (Merck 101468, Germany), sulfuric acid (Merck 100731, Germany), glucose (Merck 108337, Germany), gallic acid (Sigma-Aldrich, Germany), Folin-Ciocalteau phenol reagent (Sigma F9252, Sigma-Aldrich, Germany), tannic acid (Sigma-Aldrich, Germany), quercetin (Sigma-Aldrich, Germany), sodium carbonate (Merck 1063950500, Germany), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) used, were products of the indicated suppliers, and were of an analytical grade, as well as other minor reagents.

2.4. Physicochemical analysis

Color was measured by a color reader (Konica Minolta CR-10, Japan) and pH by a pH meter (Hanna, Thermo Fischer Scientific, USA). Total sugar was determined by the anthrone method (Islam et al., 2013) with modifications, whereby 1 mL of the fermented or non-fermented juices was transferred to a test tube and mixed with 5 mL of the anthrone reagent (0.05 g anthrone in 50 mL of concentrated H2SO4). The test tube were held at 100 °C for 12 min and cooled before measuring the absorbance at 630 nm in a spectrophotometer (Spectro 20D Plus, Labomed, USA) with a glucose solution as the standard. Total acidity was measured according to Ranggana (1977), whereby 10 mL of the fermented or non-fermented juices was mixed with 100 mL of distilled water and some drops of 1% phenolphthalein indicator, and then titrated with 0.1 M NaOH. Total soluble solids of the samples were measured by refractometry (Atago handheld refractometer N-1E, Japan). One drop of sample was placed on the prism, closed the day-light plate over the sample, then read through the focussable cushioned-rubber eyepiece.

2.5. Microbiological analysis

Yeast and bacteria counts of the Kombucha samples were done using the standard plate count procedure according to BAM-FDA protocol (Maturin and Peeler, 2001) that involved mixing 1 mL of the samples with 9 mL of sterile 0.1% peptone water (10−1 dilution). Aliquots of the mixture and subsequent dilutions were further diluted with the peptone water to have serial decimal dilutions from 10−2 to 10−8. One milliliter of each dilution was transferred into duplicated petri dishes, into which 15 mL of sterile nutrient agar (50 °C) containing cycloheximide (4 g/L) to prevent yeast growths was poured, mixed immediately and left to solidify before incubation at 37 °C for 24 h., after which the growths were counted. For yeast counts, the same procedure was followed without the addition of cycloheximide, but with the potato dextrose agar as the medium and the incubation was at 30 °C for 48 h.

2.6. Antioxidant and antibacterial activities

Antioxidant activity was measured in vitro by using the DPPH radical scavenging activity method (Hatano et al., 1988), whereby 1 mL of 0.2 mM DPPH solution was mixed with 2 mL of the snake fruit Kombucha. The mixture was incubated in a dark room for 30 min and the absorbance was measured at 517 nm with the control being the sample blank. Changes in the absorbance were measured, and antioxidant activity was expressed as % DPPH radical scavenging ability.

Antibacterial activity assay against pathogenic bacteria Staphylococcus aureus and Escherichia coli was conducted by using the agar diffusion technique of Wolf and Gibbon (1996). One milliliter of the indicator bacteria culture with 106 colony forming units (cfu) per mL was transferred to a sterile petri dish, into which 10 mL of sterile nutrient agar was poured, and upon solidification, six mm-diameter wells were made by a sterile perforator. An appropriate volume (100 μL) of sterile snake fruit Kombucha was transferred into the well and incubated at 37 °C for 24 h. The diameter of the inhibition zone was measured with a micrometer.

2.7. Bioactive compounds analysis

Total phenolic content was determined with the Folin-Ciocalteau reagent (Yang et al., 2007) using gallic acid as the standard. One milliliter of the methanolic extract of the fermented or non-fermented juices was placed into a test tube and vortexed (Nissin mixer N-20 M, USA) for 15 s with 1.5 mL of the Folin-Ciocalteau reagent, and allowed to stand at room temperature for 5 min, before adding 1.5 mL of 0.57 M Na2CO3 and incubated for 90 min at room temperature. Absorbance was measured at 750 nm using the spectrophotometer, with the same mixture except the sample extract was replaced by methanol as the blank. Total phenolic content was expressed as mg GAE (Gallic Acid Equivalent)/L.

Tannin content was determined with the Vanillin-HCl method as described by Price et al. (1978) with minor modifications, whereby 1 mL of the test juice was mixed with 5 mL of Vanillin/HCl mixture in a test tube and held for 20 min at room temperature. The formed color was determined at 500 nm wavelength. The tannic acid was used as the standard, and tannin content was expressed as mg TAE (Tannic Acid Equivalent)/L.

Total flavonoids were estimated according to the method of Nabavi.
et al. (2008), and this involved adding 0.4 mL of the test juice to 4 mL of distilled water, before 0.3 mL of 0.72 M NaNO₃ was added. After 5 min, 0.3 mL of 0.75 M AlCl₃ was added, after 6 min, 2 mL of 1 M NaOH was added and the total volume was measured at 510 nm against a reagent blank. Quercetin was used as the standard, and total flavonoid content was expressed as mg QE (Quercetin Equivalent)/L.

Organic acid composition of the Kombucha was analysed by using HPLC (Jayaban et al., 2007), and this involved centrifuging (Minispin) the content was expressed as mg QE (Quercetin Equivalent)/L.

2.8. Sensory evaluation

Sensory evaluation of the snake fruit Kombucha was conducted by using hedonic method (Stone and Sidel, 2004). A 5-point scoring was used with 1 representing extremely dislike and 5 representing extremely like. Thirty untrained panelists participated in the sensory evaluation, and had no previous or present taste or smell disorders. Kombucha samples were labeled with three-digit codes and randomly presented to avoid bias of order of presentation. The panelists scored for color, aroma and taste of the 14-day fermented snake fruit Kombucha from the five cultivars. The study was in accordance with the Declaration of Helsinki (Anon, 2017).

2.9. Statistical analysis

All the analyses above were conducted at least in triplicate. Data were analysed with the Analysis of Variance (ANOVA), and further analysis was conducted with the Least Square Difference (LSD), and both were at a 5% significant level (Granato et al., 2014). The Multiple Attribute Analysis was used to determine the most promising snake fruit cultivar Kombucha (Zeleny, 1982).

3. Results and discussion

3.1. Physicochemical characteristics of the snake fruit juice and Kombucha

Table 1 shows the chemical characteristics of the snake fruit juices from the five cultivars. It can be observed that there were no cultivar differences for pH and total acidity, but the Salak Suwaru cultivar had the highest sugar and phenolic contents (Table 1).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Total acidity (%)</th>
<th>pH</th>
<th>Total sugar (%)</th>
<th>Phenolic content(mg GAE/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salak Doyong</td>
<td>0.43 ± 0.03⁷</td>
<td>4.19 ± 0.11⁴</td>
<td>3.57 ± 0.09⁸</td>
<td>250 ± 7.05⁵</td>
</tr>
<tr>
<td>Salak Madu</td>
<td>0.33 ± 0.06⁶</td>
<td>4.38 ± 0.07⁹</td>
<td>3.68 ± 0.07⁹</td>
<td>178 ± 5.07⁴</td>
</tr>
<tr>
<td>Salak Fondoh</td>
<td>0.35 ± 0.05⁵</td>
<td>4.05 ± 0.08</td>
<td>3.64 ± 0.12</td>
<td>229 ± 9.01⁴</td>
</tr>
<tr>
<td>Salak Seguran</td>
<td>0.52 ± 0.07⁷</td>
<td>4.13 ± 0.07⁹</td>
<td>3.41 ± 0.11⁷</td>
<td>202 ± 5.05⁶</td>
</tr>
<tr>
<td>Salak Suwaru</td>
<td>0.31 ± 0.04⁴</td>
<td>4.42 ± 0.13⁸</td>
<td>4.11 ± 0.08⁸</td>
<td>264 ± 8.05⁶</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations, and values in a column with the same letters are not significantly (p > 0.05) different. This applies to all tables, where they appear.

This possibly indicated that the highest acid production occurred in the Salak Suwaru cultivar, and it might be connected with the Salak Suwaru juice nominally having the highest total sugar prior (Table 2) to fermentation. This is consistent with Blanc (1996), who directly related metabolites present in Kombucha to the amount of sugar. However, comparing the sugar levels (natural and added) in the fermented (Table 2) and non-fermented (Table 1) products, the Kombucha fermentation led to about the same (45–47%) metabolized sugar percentages in the cultivars. Hence, although its highest total acidity ought to be accompanied by lowest pH and sugar, the highest pH (significant) and total sugar (nominal) measured with the Salak Suwaru Kombucha could indicate the cultivar had inherent high acid production properties that could make the cultivar a strong potential for developing appropriate Kombucha products.

3.1.1. Microbial and chemical changes during the Kombucha fermentation

As expected and irrespective of the snake fruit cultivars, there were changes to the microbial and chemical characteristics of the juices during the Kombucha fermentation (Fig. 1), as the yeasts and bacteria metabolized the fermentable constituents of the juices. The yeast and bacteria growth patterns were similar among the snake fruit cultivars, with the counts increasing during the first seven days of fermentation, before decreasing, which had been associated with acid shocks on the microorganisms. This trend agrees with the studies of Sreeramulu et al. (2000) on the Kombucha fermentation of sugared tea infusion. We observed that the microbial growths led to decreases of the total sugar,
with concomitant increases in the total acidity, as the yeasts in the Kombucha consortium symbiotically hydrolyzed sucrose to glucose and fructose to produce ethanol, which the acetic acid bacteria subsequently transformed to acetic and other organic acids. Consequently, the total acidity increased and the pH reduced, as widely reported for fermentations (Jayabalan et al., 2010; Veličanski et al., 2014; Gamboa-Gómez et al., 2016; Chakravorty et al., 2016), but there are secondary effects of fermentation that manifest in changes in or enhancement of sensory properties.

Table 2  
Physicochemical properties of the snake fruit Kombucha.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Color</th>
<th>Total sugar (%)</th>
<th>Total soluble solid (%)</th>
<th>Total Acidity (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salak Doyong</td>
<td>30.9 ± 0.77^a</td>
<td>13.7 ± 0.22^a</td>
<td>7.21 ± 0.17^a</td>
<td>10.6 ± 0.77^a</td>
<td>1.32 ± 0.04^c</td>
</tr>
<tr>
<td>Salak Madu</td>
<td>31.6 ± 0.53^a</td>
<td>13.8 ± 0.50^a</td>
<td>7.19 ± 0.35^a</td>
<td>10.9 ± 0.60^a</td>
<td>0.92 ± 0.03^a</td>
</tr>
<tr>
<td>Salak Pondoh</td>
<td>31.3 ± 0.67^a</td>
<td>13.3 ± 0.58^a</td>
<td>7.44 ± 0.41^a</td>
<td>10.8 ± 0.25^a</td>
<td>1.23 ± 0.04^b</td>
</tr>
<tr>
<td>Salak Seguran</td>
<td>30.5 ± 0.93^a</td>
<td>13.7 ± 0.49^a</td>
<td>7.34 ± 0.12^a</td>
<td>10.5 ± 0.61^a</td>
<td>1.44 ± 0.05^d</td>
</tr>
<tr>
<td>Salak Suwaru</td>
<td>30.8 ± 0.30^a</td>
<td>13.2 ± 0.73^a</td>
<td>7.54 ± 0.46^a</td>
<td>11.3 ± 0.76^a</td>
<td>1.65 ± 0.06^c</td>
</tr>
</tbody>
</table>

Table 3  
Preference scores of the snake fruit Kombucha.*

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Color</th>
<th>Taste</th>
<th>Aroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salak Doyong</td>
<td>3.93 ± 0.60^a</td>
<td>3.60 ± 1.50^ab</td>
<td>3.53 ± 1.02^a</td>
</tr>
<tr>
<td>Salak Madu</td>
<td>3.53 ± 0.51^a</td>
<td>4.13 ± 1.57^c</td>
<td>3.83 ± 0.91^b</td>
</tr>
<tr>
<td>Salak Pondoh</td>
<td>3.50 ± 0.80^a</td>
<td>4.07 ± 1.48^bc</td>
<td>3.70 ± 0.65^b</td>
</tr>
<tr>
<td>Salak Seguran</td>
<td>4.17 ± 1.01^a</td>
<td>3.20 ± 1.13^bc</td>
<td>3.07 ± 0.71^a</td>
</tr>
<tr>
<td>Salak Suwaru</td>
<td>3.90 ± 1.12^a</td>
<td>3.70 ± 1.29^abc</td>
<td>3.80 ± 0.83^b</td>
</tr>
</tbody>
</table>

* Extremely like = 5 and Extremely dislike = 1.

Fig. 1. Microbial growth and chemical changes during the Kombucha fermentation of the snake fruit cultivars. (A) Salak Doyong, (B) Salak Madu, (C) Salak Pondoh, (D) Salak Seguran, (E) Salak Suwaru. • Yeast count, ■ Bacteria count, ▲ Total sugar, ▼ Total acidity.
3.1.2. Sensory evaluation of the snake fruit Kombucha

Table 3 shows the sensory evaluation results of the snake fruit Kombucha, indicating that the panelists generally liked the products in all the assessed parameters. Preference scores for the color were not significantly different among the cultivars, in accordance with the instrumental results shown in Table 2. However, the preference scores for taste and aroma showed significant (p < 0.05) differences among the cultivars, as inherent properties of the cultivars manifested, with the Salak Segaran Kombucha being the least preferred based on the two attributes.

3.1.3. Bioactivities and bioactive compounds of the Salak Suwaru snake fruit Kombucha

The Multiple Attribute Analysis was applied to choose the most promising cultivar by determining the ideal value on each parameter of the Kombucha snake fruit characteristics. The best treatment selection result shows that the Salak Suwaru Kombucha has the highest value compared to the other cultivars. Further studies were, therefore, conducted on the Salak Suwaru Kombucha to fully understand its potential for the health-promoting beverage. Table 4 shows the antioxidant and antibacterial activities of this snake fruit Kombucha.

In vitro DPPH scavenging ability of the Salak Suwaru Kombucha slightly increased with fermentation to about 80%, a result that compares with tea Kombucha (Chakravorty et al., 2016; Lobo et al., 2017). The antioxidant activity demonstrated is thought to be due to the various bioactive compounds (phenolics, tannins, and flavonoids), naturally present in the cultivar (Table 4), and DPPH radical scavenging ability is mainly by electron transfers, and to a lesser extent by hydrogen donating abilities (Huang et al., 2015). The fermentation was beneficial to the release of these bioactive compounds, possibly as their complex forms were broken down into simpler forms, as reported by various authors (Chu and Chen, 2006; Jayabalan et al., 2008; Bhattacharya et al., 2013; Gamboa-Gómez et al., 2016; Bhattacharya et al., 2016). Compared to tea Kombucha (Lobo et al., 2017), the phenolic content of the sample is lower, but its flavonoid content is higher.

Table 4 shows that the snake fruit Kombucha inhibited Staphylococcus aureus (Gram-positive bacteria) and Escherichia coli (Gram-negative bacteria), with the former being more inhibited. This indicated that natural bioactive compounds of the snake fruit possessed antibacterial activity, which agrees with the observations of Chusnie and Lamb (2011) and Daglia (2012), as phenolic compounds and flavonoids are referred to as potent antimicrobial agents against pathogenic bacteria. Possibly, as the bioactivity was enhanced by the fermentation, the inhibition was pronounced and enhanced by the presence of organic acids produced by acetobacteria in the Kombucha consortium. Fig. 2 shows the organic acid profile of the Salak Suwaru Kombucha, and this revealed acetic acid (retention time of 16.6 min) as the major organic acid, in addition to others. The antimicrobial activity of tea Kombucha is largely attributable to the presence of organic acids, particularly acetic acid, which can inhibit a number of Gram-positive and Gram-negative microorganisms (Sreeramulu et al., 2006; Jayabalan et al., 2014). The antimicrobial activity of weak organic acids, including acetic acid, is multifactorial and includes the ability of the undissociated acid to diffuse freely across lipid bilayers and liberate protons in the cytoplasm, lowering the cytoplasmic pH, the intercalation of the undissociated acid into the lipid bilayers at low external pH and the consequences of anion accumulation. Acidification of bacteria cytoplasm may prevent growths by inhibition of glycolysis, prevention of active transport or interference with signal transductions (Lambert and Stratford, 1999; Roe et al., 2002).

4. Conclusions

Sugared snake fruit juices from five Indonesian snake fruit cultivars (Salak Doyong, Salak Madu, Salak Pondoh, Salak Segaran, and Salak Suwaru) can potentially be used for fermented beverages with the Kombucha consortium. The fermentation affected physicochemical and sensory properties of the juices. With a focus on the most promising cultivar, the Kombucha fermentation enhanced the antioxidant and antibacterial activities of the resulting products, so also were the bioactive compounds, with desirable overall properties of the fermented beverage. Snake fruits, therefore, have the potential for functional food development by fermentation with the Kombucha consortium.

Conflict of interest

The authors declare no conflict of interest.
References


Gunam, L., Wrasiati, L.P., Setioko, W., 2009. The influence of the type and amount of antioxidants, toxi-senes, toxi-}