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Formulation of Fruit-Based Probiotic Drink From Snake Fruit (*Salacca Zalacca*) and *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13

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Abstract

Snake fruit contains monosaccharides, mainly fructose and glucose, which are indispensable substrates for the growth factors of probiotic bacteria. Therefore, this study aims to develop the Fermented Snake Fruit Juice (FSFJ) using the local probiotic bacteria *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13. The results showed that the optimal fermentation time was 24 hours, with a viable cell count of 2.7×10^8, pH 3.77, and total acid of 0.33%. The glucose and fructose content in FSFJ were decreased during fermentation. The addition of sucrose at 0%, 3%, and 6% showed that different sucrose concentrations were statistically insignificant to the viable cell count, pH, and total acid. A hedonic test was conducted, where the sample with a 6% sucrose level was the most preferred by the panelists hence, deemed as the best formulation. Furthermore, the optimal formulation sample was stored at 4°C for 30 days, and the result indicated that the viable cell count did not present a significant difference. The pH value was decreased from 3.68 to 3.60 and the total acid was increased from 0.42% to 0.56%. The volatile compounds of FSFJ were dominated by compounds responsible for snake fruit character, such as methyl 4-methyl-2-pentanoate and methyl β-methyl valerate, with some fermentation-related volatile compounds. In conclusion, Snake Fruit Juice (SFJ) is a suitable carrier medium for probiotic bacteria and remains of sufficient quality after 30 days of cold storage.

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Introduction

Maintaining a healthy body relies primarily on consuming nutritious and functional foods. One of the functional foods shown to provide health benefits to the human body is probiotic products. A probiotic is a group of live microorganisms beneficial to the human body when consumed in sufficient quantities.[1] They play a role in maintaining the balance of the human gut microflora in the digestive tract. To obtain this beneficial trait, it is recommended that probiotic be consumed at a minimum rate of 10^6 CFU/mL in a product.[2] *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 (known as *Lactobacillus plantarum* Dad-13) is a local probiotic strain that has been isolated from fermented buffalo milk.[3] The safety assessment test showed that this probiotic strain was safe for human consumption.[4]

The local strain, *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 can be applied to different food products.[5,6] Probiotic are promising in fruit juice since few consumers are familiar with dairy products due to lactose intolerance or diet preferences. Previous studies have shown that probiotic fermented beverages such as pineapple,[7] cashews,[8] and apple juice.[9] One of the exciting fruit to be used as the main ingredient for probiotic fermented beverages is snake fruit (*Salacca zalacca*).

Snake fruit is a sweet-sour taste fruit with many antioxidant compounds such as caffeic, ferulic, and p-coumaric acids. Due to its high concentration of bioactive compounds, this fruit provides several health benefits, such as anti-cholesterol, anti-diabetic, anti-hyperuricemic, and anti-tyrosinase properties.[12] According to data from the Central Institution Statistics of Sleman Regency, Yogyakarta, Indonesia,[10] the total production of snake fruit, especially the Pondoh variety, reached 73,005,300 kg in 2016, indicating its relative abundance with low economic value. Product diversification is an urgent need to boost the local economy, and Snake Fruit Juice (SFJ) is a potential ingredient for probiotic fermented beverages. Fruit contains monosaccharides such as glucose and fructose, used as a substrate for probiotic strains during fermentation.[11] Based on another study, which examined probiotic fermented beverages from pomegranate juice, the strain of *Lactobacillus plantarum* could utilize glucose and fructose during the fermentation process.[13] Since snake fruit contains valuable nutrients, it may provide a perfect carrier for probiotic. Therefore, it is necessary to study a probiotic fermented beverages from SFJ using a local probiotic strain, namely *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13.

In developing FSFJ beverages, it is necessary to determine the optimal fermentation time and the correct formulation preferred by adding different sucrose levels during formulation. Therefore, this study examines the physicochemical, sensory, and microbiological characteristics of probiotic fermented beverages of SFJ using a local probiotic Dad-13 on the effect of fermentation time, variations in sucrose, and product stability during storage at cold temperatures.

Materials and Methods

Materials

Snake fruit (*Salacca zalacca*) cv. Pondoh was obtained from CV. Mitra Turindo, Turi, Sleman, Yogyakarta, Indonesia, and sucrose (Gulaku brand) was produced by Sugar Group Co., Ltd. Mineral water (Aqua brand) by Aqua Golden Mississippi Co., Ltd. The local probiotic powder *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 was obtained from FNCC (Food and Nutrition Culture Collection), Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. The materials for analysis included MRS broth (Merck), bacteriological agar (Oxoid), Calcium carbonate (Merck), NaCl (Merck), distilled water, NaOH (Merck), standard oxalic acid (Sigma-Aldrich) and phenolphthalein indicator (Sigma-Aldrich).

Sample Preparation

The preparation of SFJ started with weighing, peeling the skin, removing the seeds, and washing the pulp. Subsequently, the pulp was soaked in citric acid solution (3 gr/L) for 30 minutes, rinsed in running water, and cut into small pieces. The mineral water was added to the pulp at 1:1, blended using Niko NK-210SP Blender, and filtered with a filter cloth to extract the juice. The juice was centrifugated at 4°C, 3500 rpm for 30 minutes using LR6M Cold Centrifuge and filtered by a filter cloth to separate the remaining sediment from fruit juice. The free-sediment of SFJ was pasteurized using GFL–1003 water bath at 80°C for 5 minutes before cooling down to room temperature.

Fermentation Process

After the SFJ reached room temperature, probiotic powder (2x107 CFU/gr) was added at 0.1 g for 100 ml of fruit juice and was homogenized using Thermo Scientific–Maxi Mix II Vortex Mixer. The fermentation process was conducted using Memmert UL 50/600 Incubator at 37°C with time variations of 0, 6, 12, 18, and 24 hours stored in a cold room (4°C). During fermentation, the viable cell count, pH, total acid, and monosaccharides content were evaluated, and the time was selected based on the highest cell count, lowest pH, and highest total acid.

Formulation Process

Different sucrose levels at 0%, 3%, and 6% were added before the pasteurization process, and the fermentation was carried out for 24 hours. The dilution plating method was applied for the enumeration analysis of probiotic, and the viable cell count, pH, and total acid were evaluated.

Sensory Evaluation

Sensory evaluation of FSFJ with variations of sucrose (0%, 3%, and 6%) was performed by 83 non-trained panelists. Meanwhile, probiotic snake fruit fermented juice has been approved for a permit with protocol number KE/FK/0949/EC/2022 from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada-Dr. Sardjito General Hospital, Yogyakarta, Indonesia. About 20 mL of the sample was poured into a plastic cup and presented randomly, and mineral water was provided to neutralize the taste bud during the test. The panelist preference test evaluated the taste, aroma, color, viscosity, and overall acceptance using a seven-point hedonic scale of 1 to 7 for intensely disliked, moderately disliked, slightly disliked, standard, slightly liked, moderately liked and enormously liked.14 The average value of all attributes was calculated and plotted.

Storage Evaluation

The most preferred sample by the panelists SFJ fermented with 6% sucrose was continued for storage at cold temperatures of 4°C for 30 days. The samples were tested on days 0, 10, 20, and 30, and the viable cell count, pH, and total acid were evaluated.

Profiling of Volatile Compounds

GC-MS analyzed the volatile compounds extracted with a headspace solid-phase micro-extraction (HS-SPME-GC–MS) method with certain modifications.[14] For extraction, a 5 mL sample in a 22 mL SPME vial was heated at 35°C for 45 minutes using SPME fiber DVB/CAR/PDMS 2 cm. The tested components were separated on the DB-Wax column (30 mx250 µmx0.25 µm) with a 250 C splitless injector and using Helium as the carrier gas at the flow rate of 1 mL/min with GC Agilent 7890A and MS detector Agilent 5975C XL EI/CI. The starting temperature of the oven was 40°C for 3 min and was increased to 120°C, 160°C, and 220°C at 3°C/min, 4°C/min, and 6°C/min. The Mass Spectra (MS) device was conditioned with the interface of 250 C, MS Source of 230 C, MS Quad 150 C, scan mass of 29-550 amu, and library of NIST14.

Determination of pH, Total Acid, and Viable Cell Count

The pH was measured using the ST20 OHAUS Pen pH meter, and the total acid content was determined using the titration method with 0.1 M NaOH, which had been standardized with a standard oxalic acid solution. The results were determined based on the percentage of lactic acid and expressed as % Titratable Acidity (TTA).15 The total number of viable cell counts was determined by the standard serial dilution method using a sterile sodium chloride solution. Furthermore, aliquots (1 mL) of diluted samples were pour-plated in triplicate into MRS agar media (Merck) and incubated at 37°C for 48 h. Plates containing 30–300 colonies were counted, and the results were expressed as Log CFU/mL.[9]

The Monosaccharides Content

The glucose and fructose as the monosaccharides content were evaluated by the HPLC method. The sample was diluted before centrifugation and filtered with a 0.45 µM Millex filter. A volume of 20 µL of the filtered sample was injected into the High-Performance Liquid Chromatography (HPLC) system that was equipped with a Metacharb H plus column, measuring 7.8x300 mm. Furthermore, H_2O was employed as the eluent, and the flow rate was set to 0.6 mL/min at a temperature of 70°C with a Refractive Index Detector (RID).[16]

Statistical Analysis

R-studio 2022.07.2 software was used for all statistical analyses, and the experiments were conducted in triplicate and expressed as mean. The one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) was used to assess statistical significance with a p-value<0.05.

Results and Discussion

SFJ Fermentation Evaluation

The result of the fermentation evaluation of SFJ is presented in Figure 1, where viable cell count, pH, total acid, and monosaccharides content were evaluated.

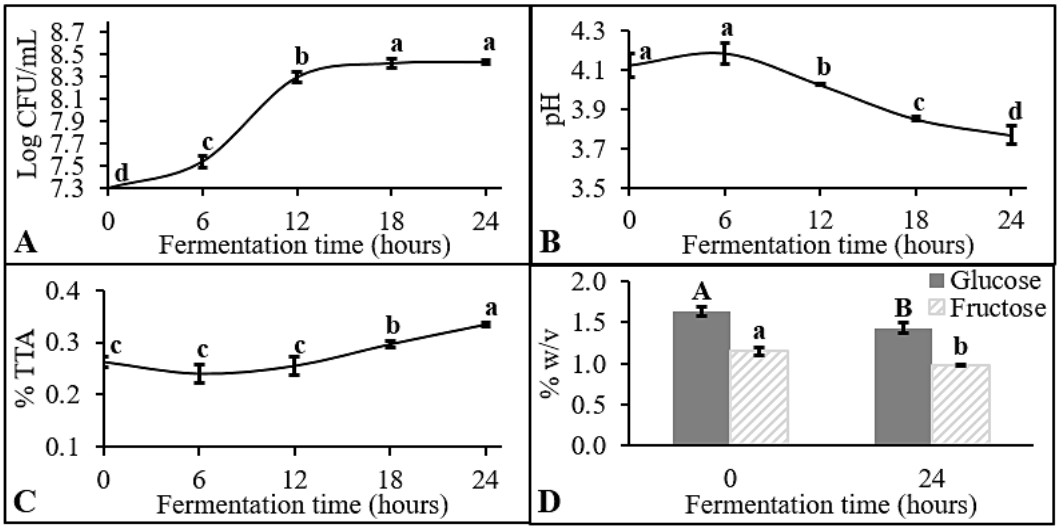

Fig. 1: Fermentation evaluation. Viable cell count (A), pH (B), total Acid (C), and monosaccharides content (D) during the fermentation process. Means followed by a different letter in each row showed significantly different (p-value<0.05).

Growth of Dad-13 shows a significantly different result (p-value<0.05) during the fermentation process in SFJ, where there was an increase in the number of cells from 2×10^7 (0 h) to 2.7×10^8 (24 h). This result indicates that the compositions in SFJ can support the growth of Dad-13. Snake fruit contains glucose and fructose, which can be used as probiotic growth substrates[11,17] and the pH during fermentation presents different results (p-value<0.05). At the beginning of the fermentation, the pH of the SFJ was 4.13 and decreased to 3.77 within 24 hours of fermentation. The decrease in SFJ indicates that Dad-13 produces metabolites, such as organic acids, affecting the pH of the sample. Dad-13 strain is categorized as LAB, which can produce metabolites such as lactic acid during fermentation.[6]

Total acid during fermentation (Figure 1C) shows significantly different results (p-value <0.05). There was an increase in total acid during the fermentation process from 0.26% (0 h) to 0.33% (24 h). During the process, probiotic form organic acids from metabolism. Dad-13 belongs to the group of a homofermentative lactic acid bacteria (LAB) that dominantly produces lactic acid.[18] Therefore, the organic acids formed during the fermentation process are dominated by lactic acid.

The content of monosaccharides shows significantly different results (p-value<0.05). There was a decrease in glucose and fructose content from 1.64% to 1.43% w/v, and 1.15% to 0.98% w/v. This result indicates that Dad-13 uses glucose and fructose in SFJ as a substrate for growth during fermentation. The result is also in line with other study on pomegranate juice fermentation, where *Lactobacillus plantarum* bacteria use glucose and fructose.[13] These sugars can be used by *Lactobacillus plantarum* as a substrate through the Embden-Meyerhof Parnas (EMP) pathway to produce lactic acid.[19,20]

The 24-hour fermentation was selected as the optimal result in fermentation time, producing the lowest and highest pH to represent the product and the total acids.

Formulation

The addition of variation in sucrose (0, 3, and 6%) in SFJ was applied to achieve the balance of sourness and to add more flavors to SFJ beverages with the intention of consumers' acceptance. Furthermore, the viable cell count, pH, and total acid were evaluated.

Based on the result of formulation, there was no significant difference in the number of viable cell counts, pH, and total acid between the sucrose concentrations. The results obtained from the analysis with concentrations of 0%, 3%, and 6% were found to be similar. Specifically, the viable cell count was measured at 8.41, 8.37, and 8.40 Log CFU/mL for sucrose solutions with 0%, 3%, and 6% concentration, respectively. The pH values recorded for the same solutions were 3.81, 3.81, and 3.80. Finally, the total acid content was determined to be 0.34%, 0.34%, and 0.31%, for the % concentration. This data indicates that sucrose was not consumed as a substrate for Dad-13 growth during fermentation. Meanwhile, LAB used monosaccharides such as glucose and fructose in the medium for their growth.[13] Based on the carbohydrate pathways used by *Lactobacillus plantarum* strains REB1 and MLB LP1, fructose and glucose can metabolize directly, while sucrose ought to be breakdown first.[21] Even though there were no microbiological or chemical changes, variations in sucrose concentration provided a different sweet taste to each sample.

Sensory Evaluation

In determining the optimal formulation of the sample, the hedonic test was used by involving the panelists to assess their preferences for each sample, as shown in Figure 2.

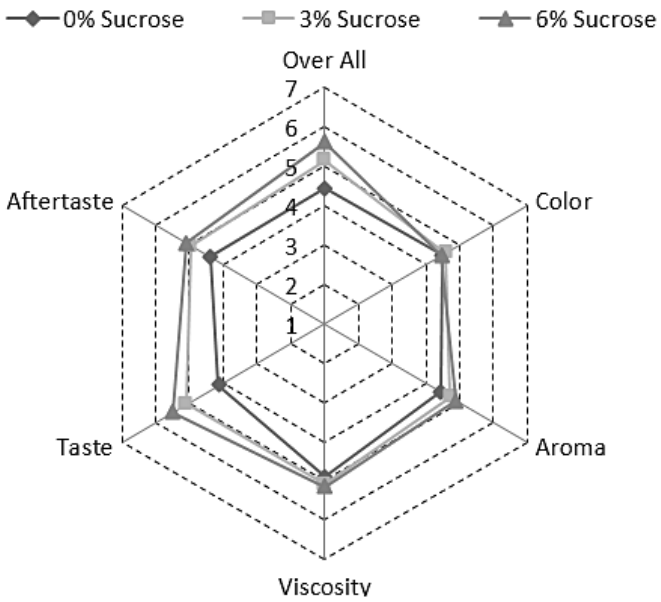

Fig. 2: Sensory evaluation. Hedonic scale 1 intensely disliked, 2 moderately disliked, 3 slightly disliked, 4 standards, 5 slightly liked, 6 moderately liked, and 7 enormously liked.

The result showed a significant difference (p-value <0.05) in the panelists' preference for each taste and aftertaste of each sample. "For the attribute of aftertaste, the panelists preferred formulation containing 3% and 6% sucrose, with preference levels of 4.95 (standard) and 5.08 (slightly preferred) on the preference scale, respectively. In terms of taste and overall attributes, the panelists' most

desired sample was the addition of 6% sucrose with 5.49 (slightly liked) and 5.59 (slightly liked) scale levels of preference. Sucrose acts as a sweetener in fermented products to cover or balance the sour taste of the fermentation process.[22]

The sample with the addition of 6% sucrose emerged as the optimal formulation as the most preferred by the panelists, based on the highest scale level of aftertaste, taste, and overall preferences.

Storage Evaluation

The survival of Dad-13 in FSFJ, pH, and total acidity during cold storage at 4°C was evaluated for 30 days using samples with the optimal formulation, as shown in Figure 3.

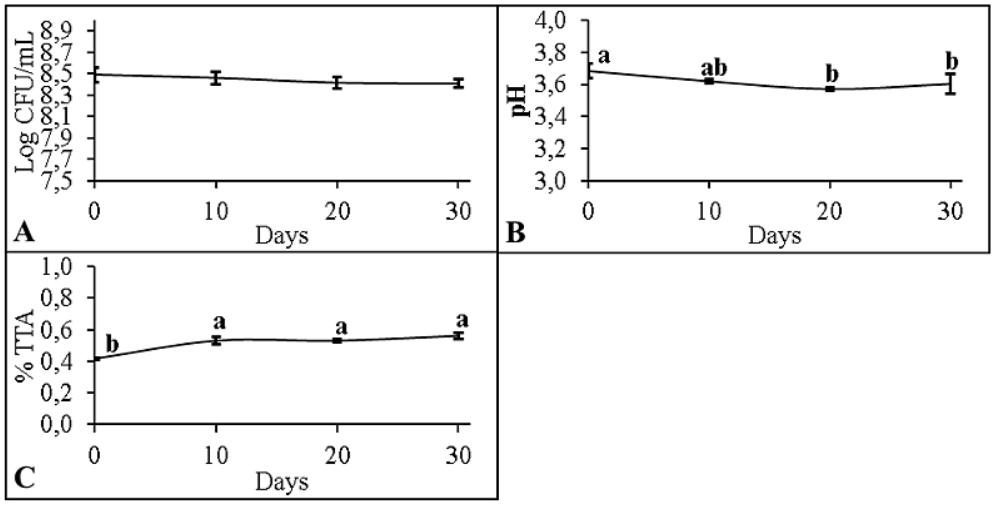

Fig. 3: Storage evaluation. Viable cell count (A), pH (B), and total Acid (C) of the best formulation (6% sucrose) during 30 days of cold storage (4°C). Means followed by a different letter in each row showed significantly different (p-value<0.05).

Table 1: The top dominant volatile compounds of SFJ and FSFJ

No	Volatile Compounds	Abundance (%)		Flavor Quality	Citation
		SFJ (Before)	FSFJ (After)		
1	Methyl 4-methyl-2-pentenoate	64,40	62,59	Snake Fruit Character	[11]
2	2-Hexanone, 5-methyl-	11,37	0,02	Fruity Odor	[25]
3	Methyl β-methyl valerate	10,58	9,64	Snake Fruit Character	[11]
4	Dimethyl 2-hydroxy-2-methyl succinate	1,91	2,67		
5	Acetaldehyde	1,51	2,14	Pungent, Fresh, and Green	[26]
6	2-Methyl butyric acid	1,13	4,26	Fermented, Sour	[27]
7	Methyl (3E)-2-methyl-3 -pentenoate	0,82	1,01		
8	Methyl caproate	0,09	8,12	Fruity Odor	[28]

The number of viable cell counts in Figure 3A shows no significant difference during storage, and from the observations, the number of Dad-13 viable cells was stable at around Log 8.4 CFU/mL. Therefore, Dad-13 can survive in SFJ for 30 days in cold storage, and the Fermented Snake Fruit Juice

(FSFJ) can be considered a probiotic drink. In the study of apple juice fermentation using a strain of *Lactobacillus* casei, cold storage for 28 days maintained a cell count of around Log 8 CFU/mL.[23] Furthermore, for the pH (Figure 3B) and total acid (Figure 3C), the results were significantly different (p-value<0.05). There was a decrease in pH from 3.68 to 3.60 and an increase in total acid from 0.42% to 0.56% during 30 days of storage, but the changes were not drastic. Changes in pH and total acid might occur because Dad-13 as a LAB group remains in the metabolic state despite having a low chance of growth during cold storage.[24] The result of metabolism is the production of lactic acids, which could lower the pH and increase the total acid in the sample.[18]

Profiling of Volatile Compounds

The flavor is an essential sensory property of food products and can be improved through probiotic fermentation. For the samples before and after fermentation, SFJ without added sucrose and FSFJ with 6% sucrose were used. The top dominant volatile compounds of SFJ and FSFJ is presented in Table 1.

Based on the data, the most dominant volatile compounds of SFJ and FSFJ were methyl 4-methyl-2-pentenoate and methyl β-methyl valerate with a snake fruit character flavor.[11] Volatile compounds that decreased after fermentation was methyl 4-methyl-2-pentenoate, 2-Hexanone,5-methyl-, and methyl β-methyl valerate, and the fermentation process caused a decrease in character and odor flavor.[11,25] After fermentation, the detected escalation volatile compounds were dimethyl 2-hydroxy-2-methyl succinate, acetaldehyde, 2-methyl butyric acid, methyl (3E)-2-methyl-3-pentenoate, and methyl caproate. These components improved pungent, fresh, green, sour, and fruity odor flavor characteristics.[26,27,28]

The volatile compound 2-methyl butyric acid is a metabolite produced from the amino acid leucine during the nutrition starvation time of bacteria.[27] In this study, the compound increased due to the growth of Dad-13 during fermentation. The result is consistent with a previous study,[29] where tomato juice fermented with *Lactobacillus plantarum* strain could produce 2-methyl butyric acid as a volatile compound.

Methyl caproate or hexanoate is an ester fatty acid derived from hexanoic acid in the metabolites produced by *Saccharomyces cerevisiae,* acting as a flavoring agent.[30] The results have shown an increase in the compound during the fermentation process. The previous study on the production of cream cheese by combining two probiotic cultures (L. *plantarum* Dad-13 and L. *plantarum* Kita-3) yielded the dominant volatile fatty acid content such as decanoic and hexanoic acids. This compound is produced due to lipolytic activities and amino acid breakdowns by bacteria fermentation.[31]

Conclusion

SFJ is a potential medium carrier for local probiotic *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13. The optimal treatment for developing a probiotic fermented beverages from SFJ was fermentation for 24 hours with 6% sucrose. Dad-13 survived in FSFJ for 30 days of cold storage at 4°C, and the process led to the change of flavor character.

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Conflict of Interest

The authors declare no conflict of Interest.

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