Research Article

Characterization of Water Kefir from Broccoli Stem Extract with Addition of Palm Sugar

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ABSTRACT: *Water kefir is a fermented carbonated beverage and functional food with probiotic properties. Broccoli is an edible green plant which a rich source of vitamins A, B1, B2, B3, and C; it also contains isothiocyanate compounds that have anti-cancer and fiber for digestive system. Palm sugar is a sweetener obtained from concentrated coconut sap and is known to have a low glycaemic index. The purpose of this study was to investigate the effect of broccoli stem extract and palm sugar concentration on its biochemical and microbiological characteristics. Subsequently, the acceptance of the product by the panelists were evaluated through organoleptic tests. Water kefir with sugar content of 15% had the highest lactic acid, antioxidant activity, lactic acid bacteria, yeast, ethanol content, and water kefir with a concentration of 1:5 and sugar content of 15% found the highest acceptance by the panelists. Water kefir obtained in this study did not have contaminant bacteria. This product also met the codex standard.*

Keywords: *broccoli stem; functional food; palm sugar; water kefir*

1. Introduction

Functional foods are generally linked to health promotion, because their content of active components, can provide additional health benefits beyond their inherent nutrients [1]. Kefir is a fermented drink product authentically produced from kefir grains. Kefir grain, as a natural starter culture, contains numerous lactic acid bacteria, acetic acid bacteria, and yeasts within a polysaccharide structure. True kefir is a miraculous food in terms of its favorable contributions to human health. However, kefir starter cultures used in industrial kefir productions contain very few lactic acid bacteria and yeasts. These starter cultures do not contain characteristic kefir bacteria, such as *Lactobacillus kefiranofaciens*, *Lactobacillus kefiri*, and *Lactobacillus parakefiri* [2]. According to Slattery et al. [3], kefir is included in the functional food category and is considered as probiotic. According to codex standards, probiotics are living microorganisms recorded in sufficient quantities that provide positive value for digestive health. Water kefir has lower alcohol content, and the existing fat content is minimal, compared to kefir made from cow milk [4]. Thus, kefir water can be consumed by people with lactose intolerance [5].

Broccoli (*Brassica oleracea* L.) is a vegetable plant that belongs to the *Brassicaceae* species or cabbage. The market share of broccoli in Indonesia consistently increases by 15-20% per year [6]. Broccoli as a raw material for water kefir is favored because it is rich in nutrients, including vitamins A, B1, B2, B3, and C; it also contains isothiocyanate compounds that have anti-cancer activity [7]. Broccoli stems' hard texture is due to the cellular makeup of structural polysaccharides and lignin [8]. The stem contains a considerable amount of insoluble fiber that can bind to water [8]. This is beneficial for the digestive system of the body and reduces the risk of colon cancer; it can also maintain fat levels in the blood to reduce the risk of obesity, hypertension, and heart disease [9]. Indonesian mostly consume broccoli by separating it from the stem so that broccoli stems are discarded and become waste. According to Siti [10], broccoli stems and flowers have the same nutritional content. They contain antioxidants, provitamin A, vitamin C, and folic acid. Their content of sulforaphane can kill H. *pylori*-resistant bacteria that are suspected to cause stomach cancer. In addition, carotenoids, flavonoids, glucosinolate, and lutein are beneficial as inflammatory and cancer-fighting agents [11]. According to Siti [10] and Aisyah et al. [12], even though they are steamed, broccoli has not decreased a significant amount of beta carotene and antioxidant activity.

Palm sugar is obtained by concentrating coconut sap and produced in various forms [13]. Palm sugar from coconut sap has a light brown color, sweet taste, and cleaner than comparable refined sucrose. Coconut sugar has the advantage that it can be consumed as a natural sweetener by people who suffer from diabetes. Its glycaemic index (GI) falls into the low category (<55), while the GI of the granulated sugar is 100. In addition to low GI, palm sugar contains nutrients found only very little in the granulated sugar [14].

This study in contributes to formulate an alternative source of water kefir by using broccoli stem and combined with palm sugar. The best formula is identified by its biochemical and microbial character, and also the acceptance by organoleptic test.

2. Materials and method

2.1. Materials

The starter kefir grain used in this study was obtained from the laboratory of Microorganism of the University of Surabaya. Broccoli stems were purchased at the Soponyono traditional market Surabaya and the coconut palm sugar powder was from Superindo. All the chemicals were of reagent grade and used without further purification: sodium chloride (Merck), de Mann Rogosa Agar (Merck), Rochelle's salt (Merck), ethanol 70%, lactic acid (Merck), DPPH reagent (Aldrich), H_2SO_4 concentrated, $CuSO_4$ (Merck), PHF reagent

(Merck), Hektoen Agar media (Merck), Lactose Broth media (Merck), Bismuth Sulphite Agar (Merck), Xylose Lysine Deoxycholate media (Merck), Brilliant Green media (Merck), and Lactose Bile (Merck).

2.2. Kefir grains production

The 30 gr kefir grain from laboratory was activated by inoculating it into 300 mL (15% w/v) sugar solution at room temperature for a short period (24-48 h).

2.3. Broccoli stem extraction

The outer skin of the hard broccoli stem was peeled to yield the greenish-white interior which was washed thoroughly with running water. It was sliced into smaller pieces, weighed, and steamed until soft (15 min), then processed in a blender. To obtain the juice, it was filtered. Water was added at a ratio of 1:3, 1:4, and 1:5 (v/v), respectively. To each juice sample, palm sugar was added at 5%, 10%, and 15% (w/v), respectively. The mixture was stirred and pasteurized for 15 min at 70°C, then cooled to 45°C. Each variation is shown in Table 1.

2.4. Refresh starter kefir grains

The 200 mL of sugar water sample was heated until completely dissolved and cooled to room temperature. Next, 10% kefir grain (w/v) was added and the sample was stirred. The mixture was then incubated for 24 hours at room temperature. It must be filtered to recycle the kefir grain for reuse [15]. This step was repeated 3 times.

2.5. Kefir fermentation

For every 9 treatments, 150 mL of broccoli stem extract as prepared before, 10% (w/v) grain kefir was added (15 g), the mixture of broccoli stem extract and kefir grain was placed in a sterile jar, incubated at room temperature for 24 h, and the pH checked with a pH meter.

2.6. Lactic acid test with PHF method

Standard solutions were made with 20, 40, 60, 80, and 100 ppm lactic acid, respectively. No deproteination was needed because the kefir sample did not contain any milk or protein. A total of 0.33 mL of sample solution was added to 2 mL concentrated H_2SO_4 and shaken before heating in boiling water for 10 min. The sample was cooled under running water. Then 33.33 µL 4% $CuSO_4$ and 66.67 µL p-Hydroxydiphenyl (PHF) were added and the solutions were shaken at room temperature for 30 min. The samples turned to a purplish color and their absorbance was observed at a wavelength of 570 nm with a UV-Vis spectrophotometer (Genesys 105 Uv-Vis).

Table 1. Description of each broccoli stem in water kefir palm sugar

Treatment	Description
B13P-5	Broccoli stem extract: water 1:3 with brown palm sugar 5% (w/v)
B13P-10	Broccoli stem extract: water 1:3 with brown palm sugar 10% (w/v)
B13P-15	Broccoli stem extract: water 1:3 with brown palm sugar 15% (w/v)
B14P-5	Broccoli stem extract: water 1:4 with brown palm sugar 5% (w/v)
B14P-10	Broccoli stem extract: water 1:4 with brown palm sugar 10% (w/v)
B14P-15	Broccoli stem extract: water 1:4 with brown palm sugar 15% (w/v)
B15P-5	Broccoli stem extract: water 1:5 with brown palm sugar 5% (w/v)
B15P-10	Broccoli stem extract: water 1:5 with brown palm sugar 10% (w/v)
B15P-15	Broccoli stem extract: water 1:5 with brown palm sugar 15% (w/v)
C	Control (without the addition of broccoli stem extract and sugar)

2.7. Antioxidant test with DPPH method

A 0.5 mL kefir sample was added to 0.5 mL 5×10^{-4} M DPPH (2,2-diphenyl-1-picrylhydrazyl) in a closed test tube. The samples were stored in a dark room at room temperature for 30 min. Subsequently, the absorbance was read with a UV-Vis spectrophotometer at a wavelength of 515 nm [16].

2.8. Ethanol level determination by gas chromatography

Ethanol was analyzed using a Gas Chromatograph (Perkin Elmer AutosystemxL). The system was equipped with a flame ionization detector (Perkin Elmer) with a 50 m CP WAX 52 capillary column (inner diameter, 0.32 mm; film thickness, 1.2 mm; Carbowax 20M, Varian). The chroma–tography was connected to an automatic headspace sampler (Model: Turbo Matrix 16, Perkin Elmer). The operating parameters of the chromatograph were as follows: 25 psi of head pressure, 95°C injector temperature, and 250°C detector temperature. The oven temperature was held at 70°C for 1 min; the temperature was increased in increments of 4°C/min up to 150°C with a total cycle time of 5.5 min. The parameters of the headspace sampler were as follows: 70°C sample temperature, 30 min thermostat time, 80°C needle temperature, 90°C transfer line temperature, and 3 min pressurization time, and 0.04 min of injection time and ethanol level was declared in percentage [17].

2.9. Presence of lactic acid bacteria and yeast

A 1 mL of kefir sample was placed into 9 mL of sterile 0.9% NaCl, then diluted to 10^{-6}, 10^{-7}, 10^{-8}, and 0.1 mL, respectively. The samples were planted in MRSA media and incubated for 24 h at 37°C. The number of Lactic Acid Bacteria (LAB) colonies was counted with the help of an Electric Bacteria Colony Counter [18]. To estimate the total yeast, 1 mL of the kefir sample was placed in 9 mL of 0.9% sterile NaCl solution. After diluting to 10^{-6}, 10^{-7}, and 10^{-8} and 0.1 mL respectively, the samples were planted on PDA media and incubated for 5 days at a temperature of 37°C and the growing yeast was observed [18].

2.10. Presence of coliform bacteria

Approximately, 0.1 mL of kefir was placed into each Lactose Broth (LB) media (Merck) and incubated at 37°C for 24 hours [18]. For the affirmation stage, the incubated samples that gave a positive test result, namely by forming bubbles in the Durhamm tube, were aseptically transferred with the help of an inoculating needle to the brilliant green lactose bile media. Each sample was incubated at 37°C for 24 h. The number of positive tubes in Brilliant Green Lactose Bile (BGLB) media was calculated with the Most Probable Number (MPN) table of coliform bacteria [18].

2.11. Presence of Salmonella spp.

The 10 mL samples were inoculated into 100 mL Tetrathionate Broth (TT Broth) media and then incubated for 24 h at a temperature of 37°C. Furthermore, 1 inoculating needle of the enrichment of Tetrathionate Broth (TT Broth), was streaked on the media Bismuth Sulphite Agar (BSA), Xylose Lysine Deoxycholate Agar (XLD), and Hektoen Enteric Agar (HE Agar), respectively. It was then incubated at 37°C for 24 h [18].

2.12. Organoleptic test

This organoleptic test has two stages: first, a hedonic rating test followed by a structured scale description test. A hedonic test was conducted, 30 panelists (college students) were selected to taste the samples and to give an assessment of the quality attributes of the water kefir, including taste, color, odor, and aftertaste. The best score on the hedonic test was followed by a structured scale description test. The parameters of the structured scale description test were color, aroma (sour), taste (sweet), taste (alcoholic), taste (sour), and aftertaste.

2.13. Experimental design & data analysis

This study used complete randomized design and minitab data analysis (version 18.1). Parametric data (lactic acid, antioxidant, and ethanol level test) was analysed using two-way ANOVA

and tukey post hoc method (α = 0.05). Kruskal-wallis test was performed to analyse non parametric data (α = 0.05).

3. Results

3.1. Biochemical and microbiological characte–ristic result

Changes in biochemical and microbiological characteristics of broccoli stem water kefir based on the concentration of the ingredients were assessed using several techniques such as lactic acid, antioxidant activity, ethanol, and total plate count (lactic acid bacteria and yeast). A summary of the results is shown in Table 2.

Statistical analysis of the parameters displayed in Table 2 revealed that the sugar concentration of both broccoli stem extract and palm sugar had a significant impact.

3.2. The microbial contaminant test result of broccoli stem water kefir

Contaminant tests on broccoli stem water kefir included coliform contaminants and *Salmonella* sp. The Coliform test was performed using Lactose Broth (LB) media with concentrations of 1× strong and 3× strong. This was done using selective media, and the result was listed in Table 3.

3.2.1. Salmonella sp. and coliform contamination tests.

All kefir water samples tested negative for coliform contamination (Figure 1) as well as for the presence of *Salmonella* sp. bacteria (Figure 2) performed in Xylose Lysine Deoxycholate agar, Bismuth Sulphite agar, and Hektoen agar, respectively.

3.3. Organoleptic tests

The parameters of the organoleptic test included color, odor, taste, and aftertaste. The parameters of the structured scale description test were color, aroma (sour), taste (sweet), taste (alcoholic), taste (sour), and aftertaste. The results are compiled in Figure 3. The highest result was shown in B15P-15, and the descriptive result was listed in Table 4.

4. Discussion

The biochemical and microbiological characteristics of water kefir from broccoli stems and palm sugar are listed in Table 2.

Table 2. Biochemical and microbiological characteristics of kefir water from broccoli extract

Parameters	Concentration of palm sugar (%)	Broccoli stem extract : water (v/v)		
		1:3	1:4	1:5
Δ Lactic acid (%)	5	0.14± 0.000	0.14±0.000	0.14±0.000
	10	0.15± 0.000	0.14± 0.000	0.14± 0.001
	15	0.15± 0.000	0.15± 0.001	0.15±0.000
Δ Antioxidant activity	5	14.66^{c} ± 0.160	13.27^{d} ± 0.120	10.53^{e} ± 0.060
	10	17.54^{b} ± 0.220	15.01^{c} ± 0.110	13.27^{d} ± 0.160
	15	20.76^{a} ± 0.590	17.23^{b} ± 0.110	14.53^{c} ± 0.330
TPC (Lactic acid bacteria) (on log 10 CFU/ mL) (T24)	5	24.33^{c} ± 1.150	17.33^{d} ± 2.080	11.67^{e} ± 0.580
	10	32.67^{b} ± 1.530	27.67^{c} ± 0.580	19.33^{d} ± 0.580
	15	44.33^{a} ± 2.520	36.33^{b} ± 2.080	25.67^{c} ± 2.080
Ethanol (T24) (%)	5	14.37^{d} ± 0.060	13.20^{e} ± 0.100	10.20^{f} ± 0.170
	10	17.43^{b} ± 0.120	15.47^{c} ± 0.060	13.30^{e} ± 0.170
	15	20.53^{a} ± 0.210	17.40^{b} ± 0.100	14.67^{d} ± 0.06

Δ is the average difference before and after fermentation, letter annotation expressesa significant difference based on the Tukey test with α = 0.05. No statistical tests were performed on ethanol parameters.

Table 3. Microbial contaminant coliform test result

Sample	*Coliform*			MPN/ml
	Results of positive tube			
	LB 3x (10 ml)	LB 1x (1 ml)	LB 1x (0,1 ml)	
1:3 (5%)	0	0	0	<3
1:3 (10%)	0	0	0	<3
1:3 (15%)	0	0	0	<3
1:4 (5%)	0	0	0	<3
1:4 (10%)	0	0	0	<3
1:4 (15%)	0	0	0	<3
1:5 (5%)	0	0	0	<3
1:5 (10%)	0	0	0	<3
1:5 (15%)	0	0	0	<3

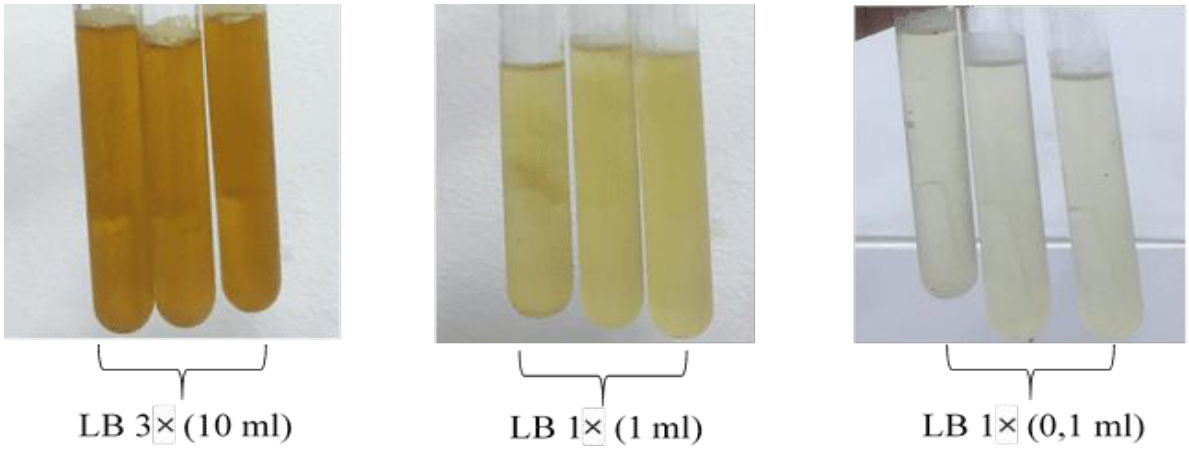

Figure 1. The results of the coliform contaminant test water kefir broccoli stem on Lactose broth media

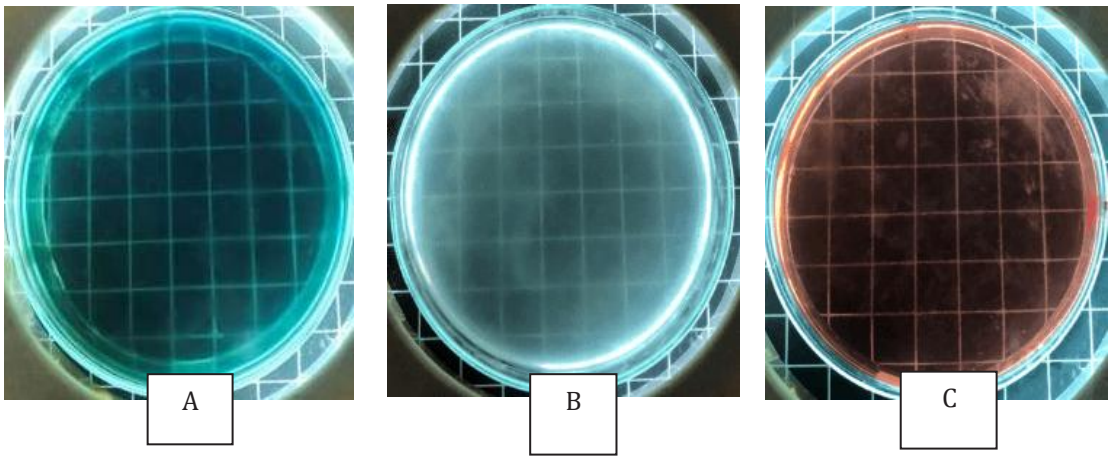

Figure 2. The results of the *Salmonella sp* contaminant test on water kefir broccoli stem on HE (A), BSA (B), XLD (C)

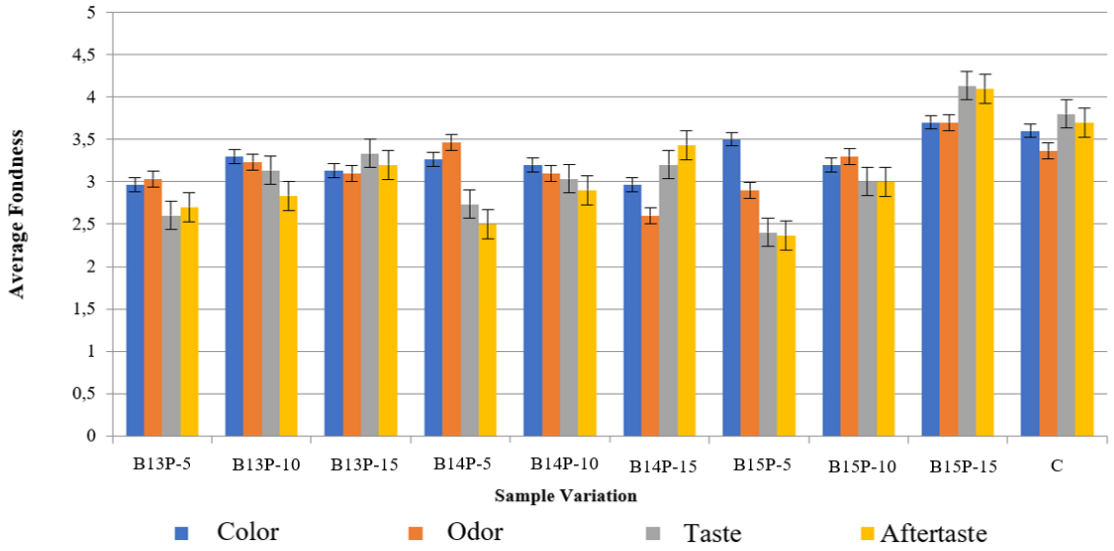

Figure 3. Result of organoleptic test of hedonic water kefir broccoli stem. Rating score description of average fondness: 1. Very bad, 2. Bad, 3. Quite good, 4. Good, 5. Very good

Table 4. Result of the descriptive test on a structured scale of B15P-15 water kefir

Parameter	Description
Color	Brown
Odor (Sour)	Quite Sour
Taste (Sweet)	Quite Sweet
Taste (Alcoholic)	Low alcohol
Taste (Sour)	Quite Sour
Aftertaste	Not Bitter

4.1. Lactic acid production

The result in Table 2 indicates that for higher palm sugar concentration, the lactic acid level increased. This result is similar to Lestari et al. [19] that higher sugar concentration increased lactic acid level. The process of breaking down the sugar by LAB into lactic acid is carried out through glycolysis during fermentation [20]. Variations in palm sugar concentration and broccoli stem extract concentration not directly correlate based on statistical tests. It seems there is no association between the two factors in regard to influencing lactic acid levels. For higher concentrations of broccoli stem extract, the lactic acid level is increased, because in the broccoli stem carbohydrates (polysaccharides) can be converted into simple sugars during fermentation [21]. According to Atmojo [22], a portion of sugar will be converted into lactic acid, but not all of them.

4.2. Antioxidant activity levels

The antioxidant activity was also found to increase for concentration improvement of broccoli stem extract. Steamed broccoli is rich in vitamin C (43,56 mg/100 g) as an antioxidant [23]. Ascorbic acid can inhibit DPPH free radicals. This result is similar to Randazzo et al., [24] research that state higher kiwi fruit concentration (rich vitamin C) produced higher antioxidant activity. Variations in the content of palm sugar affect the antioxidant activity, where higher concentrations of coconut sugar lead to higher antioxidant acti–

vity. According to Susilowati et al. [25] palm sugar contains antioxidants in the form of melanoidin pigments. The addition of more palm sugar may cause more melanoidin pigments to form in broccoli stem water kefir so that the antioxidant activity becomes higher. Variations of palm sugar concentration and broccoli stem extract concentration showed some correlation based on statistical analysis of the data, indicating that both factors impact antioxidant activity.

4.3. Total plate count (TPC)

The difference in palm sugar concentration affected total lactic acid bacteria. Higher palm sugar concentrations caused an increase in total lactic acid bacteria. Following Dhana & Puspitarini [26] research, the more sugar is present, the more nutrients are available, and the higher growth of lactic acid bacteria resulted. Varying the concentration of broccoli stem extract also showed an effect on total lactic acid bacteria. Greater amounts of broccoli stem extract resulted higher total lactic acid bacteria. The growth rate and viability of lactic acid bacteria in the fermentation process are determined by the suitability and nutritional content of the fermentation media [27]. Variation in palm sugar and broccoli stem extract concentrations showed a correlation between the two variables as both impacts the growth of lactic acid bacteria.

A similar trend also developed in yeast growth which could be due to the availability of high sugar content in broccoli stems utilized by yeast as nutrients. Varying the palm sugar concentration also affected the total yeast based on statistical test. As could be expected, higher palm sugar concentration led to higher total yeast. Insaniet et al. [28] stated that sugar (sucrose, glucose, lactose, and fructose), apart from being a source of sweetness, is also a good source of energy for microorganisms in the breeding process. The results of statistical tests indicate a correlation between the two factors.

In addition, according to the Codex Standard for Fermented Milk 243 of 2010, probiotic drinks must have bacteria (starter) of 10^7 CFU/mL or a minimum of 7 log10 CFU/mL of probiotic bacteria. Based on Table 1, water kefir from broccoli stems meets the quality of codex standard of probiotic drinks.

4.4. Microbial tests

Based on the SNI 2009, the maximum coliform contaminant bacteria is 10 AMP/gram (10 MPN/g). The test results showed that there were no gas bubbles in the LB media, which means that the Coliform bacteria test was negative (Table 3) or could be expressed as an AMP value < 3 per mL. While for *Salmonella* sp., the three differentials media XLD, HE, BSA were evaluated. Test results were also negative with no colony of *Salmonella* sp. seen, so that broccoli stem water kefir is declared safe for consumption.

4.5. Organoleptic evaluation

Based on the results of the Kruskal-Wallis statistical test on color, a significant effect was observed related to the concentration of palm sugar and broccoli stem juice on the color of the resulting water kefir (p-value < 0.05). The panelists preferred the color of water kefir with a concentration of 15% palm sugar and a concentration of 1:5 broccoli stem extract (Quite good). The color strongly depends on the amount of palm sugar added. The results of the Kruskal-Wallis statistical test regarding the odor also showed that the concentration of palm sugar and broccoli stem extract had a significant effect on the odor of water kefir (p-value < 0.05). Water kefir with a concentration of 15% palm sugar and a concentration of 1:5 broccoli stem extract was again the most preferred (Quite good). This may be due to the distinctive odor of broccoli (unpleasant), which caused many panelists dislike the product [29]. Also, for the taste parameters, the Kruskal-Wallis statistical test showed that varying concentrations of palm sugar and broccoli stem juice had a significant effect (p-value < 0.05). Water kefir with a concentration of 15% palm sugar and a concentration of 1:5 broccoli stem extract had the

highest level of acceptance. The higher the palm sugar concentration was, the sweeter the water kefir tasted which was obviously the preference of the panelists. Lower broccoli concentrations of 1:5 found higher acceptance since the broccoli stem had a less pronounced (unpleasant) taste [30]. Regarding the aftertaste (p-value <0.05) again water kefir with 15% palm sugar and a concentration of 1:5 broccoli stem juice had the highest level of preference (Good). The concentration of palm sugar affected the panelists' preference level, the higher the palm sugar concentration, the higher the panelist acceptance level. In summary, the total score of the hedonic rating test showed that water kefir broccoli stem 1:5 with a concentration of 15% palm sugar (sample code B15P-15, Table 1) obtained the best total score with the average score for the parameters of odor (3.7), taste (4.13), color (3.7), and aftertaste (4.1) (Figure 3). Then, a structured scale description test was conducted from the sample with the highest score (sample code B15P-15) as shown in Table 4.

5. Conclusion

The best result of lactic acid production was found in broccoli stem extract with a content of 1:4 and a concentration of 15% palm sugar. The best result of antioxidant activity was found in broccoli stem extract with a content of 1:3 and a concentration of 15% palm sugar. The best results (highest consumer acceptance) werefound for broccoli stem extract with a content of 1:5 and a concentration of 15% palm sugar. The produced water kefir also meets the codex standards regarding lactic acid bacteria, yeast, and contaminants.

6. Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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