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Research

Probiotic Growth Pattern and Physicochemical Evaluation of Water Kefir Fermentation

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ABSTRAK

Probiotics are live-friendly microorganisms that can confer a health benefit on the host if it is consumed in sufficient amounts. Water kefir is a probiotic-rich fermented beverage that contains multi-species of live cultures. Brown sugar and palm sugar were used for water kefir fermentation due to their high sucrose and mineral contents. The objective of this study was to determine the probiotic growth pattern of water kefir and to evaluate the physicochemical parameters, including the pH changes, lactic acid content, reducing sugar content, and total soluble solids. The fermented water kefir was collected at every 6-hour interval, until the end of 72 hours of fermentation. The growth curve was determined by enumerated probiotics on De Man, Rogosa, and Sharpe (MRS) agar, Yeast Extract-Peptone-Dextrose (YPD) agar, and Gluconobacter (GM) agar plates, respectively. MRS, YPD, and GM agar plates were used to enumerate lactic acid bacteria, yeast, and acetic acid bacteria, respectively. The result showed increased probiotic growth as fermentation time increased with different phases observed from the growth curve. The stationary phase of probiotics was recorded at 30-42 h and was recommended as the optimal harvesting point. Besides, longer fermentation time produced lower pH values and lower total soluble solids while higher lactic acid and higher reducing sugars. At the end of fermentation, the concentration of lactic acid and reducing sugars were 2.16 ± 0.09 g/L and 13.66 ± 0.14 mg/mL, respectively. In conclusion, probiotics from water kefir fermentation are suggested to be best harvested between 30-42 hours and can be used for self-consume or downstream processing.

Key words: Brown sugar, fermentation, probiotics, palm sugar, water kefir

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INTRODUCTION

FAO/ WHO (2001) defines probiotics are live microorganisms that, administered in adequate amounts, confer a health benefit on the host. Probiotics such as *Lactobacillus Leuconostoc*, *Bifidobacterium,* and *Saccharomyces* strains have been widely incorporated into foods and beverages to increase nutritional values (Staniszewski & Kordowska-Wiater, 2021). Some fermented foods such as yogurt and cheese are found to naturally contain probiotics. The advantages of probiotics include regulation of intestinal microflora, inhibition of the growth of disease-causing bacteria, modulation of immune function, relief of constipation and irritable bowel syndrome, as well as treating diarrhea and reducing blood cholesterol levels (Azizi *et al*., 2021; Koirala & Anal, 2021). Besides, probiotics also have been scientifically proven to reduce the risk of skin cancer and colon cancer (Sharifi *et al*., 2017; Calatayud *et al*., 2021).

Probiotic beverages have been best known and consumed for over twenty decades. Kefir is one of the well-known fermented beverages that contain potent sources of probiotics. Traditionally, kefir drinks are produced using kefir grains as a starter culture, and fermented in milk or sucrose solution, producing milk kefir and water kefir, respectively. The production of water kefir as dairy-free fermented beverages is important to cater to the needs of individuals

with lactose intolerance and dairy allergies while promoting the health benefits of probiotics (Dahiya & Nigam, 2023). Water kefir constitutes of complex mixture of lactic acid bacteria (10^7- 10^8 CFU/g grain), yeast (10^6- 10^7 CFU/g grain), and acetic acid bacteria (10^6- 10^7 CFU/g grain) that is embedded in a resilient water-soluble branched glucogalactan matrix, namely kefiran (Pendón *et al*., 2021).

Sucrose is an important substrate for water kefir fermentation (Laureys *et al*., 2021). Brown sugar and palm sugar are generally used for water kefir fermentation (Pendón *et al*., 2021). Brown sugar contains high sucrose content, essential vitamins, and minerals as it does not go through a refinement process. Meanwhile, palm sugar occurs naturally and is minimally processed. It also has a low glycaemic index (GI) and contains high mineral sources (Srikaeo *et al*., 2019). In addition, palm sugar exhibited higher antioxidant activity compared to other types of cane sugars. The high antioxidant properties also contributed to the production of slightly acidic water kefir (Winarni *et al*., 2018).

Despite the extensive research investigated water kefir using different parameters (substrates, additives, and fermentation time) have been done (Çevik *et al*., 2019; Destro *et al*., 2019; Gamba *et al*., 2019; Dwiloka *et al*., 2020), however, neither study was conducted to determine the growth pattern for multi-strain probiotics in water kefir. The growth of probiotics in food can be predicted using the Baranyi-Roberts model, consisting of three phases: lag phase, exponential phase, and stationary phase (Rickett *et al*., 2015). Probiotic growth can be measured by the standard plate count method and turbidimetric measurement, in which the standard plate count method is the simplest and most sensitive way to determine cell population density. As microbial growth is affected by various environmental factors, thus the growth curve is important to determine the optimal harvesting point for maximal growth and the best performance of probiotics during downstream processing. In this study, the objectives were to determine the probiotic growth pattern in water kefir and to evaluate the physicochemical parameters, including the pH changes, lactic acid content, reducing sugar content, and total soluble solids from 0 to 72 hr of fermentation. The correlation between the parameters was also determined to understand the relationship between each other.

MATERIALS AND METHODS

Activation of water kefir grain and fermentation

The water kefir grains were purchased from My Kefir World, Kuala Lumpur, Malaysia. The dehydrated water kefir (40 g) was activated before the fermentation. Briefly, 40 g of kefir grains (starter culture) were weighed and used for water kefir fermentation.

Firstly, the sugar solution was prepared in 1 L Schott bottle by mixing 5% (w/v) brown sugar and 2.5% (w/v) palm sugar in 2:1 ratio. The sugary solution was mixed followed by the inoculation of water kefir grains. The solution was closed to create an airtight environment. The bottle was left at room temperature for 72 hr. The mixture of brown sugar and palm sugar in this ratio was chosen due to brown sugar might contribute certain flavors and nutrients, while palm sugar could offer distinct microbial substrates and fermentation properties.

Growth curve determination

Approximately 1 mL of fermented water kefir was collected every 6 hr, up to 72 hr. The growth of probiotic bacteria was determined by the standard plate count method (Arepally et al., 2020). In detail, a ten-fold serial dilution was performed by mixing 9 mL of 0.9 % saline water with 1 mL of fermented water kefir. A 0.1 mL of sample was aseptically transferred to De Man, Rogosa and Sharpe (MRS; Oxoid, Hampshire, UK) agar supplemented with 0.5 mg/mL cycloheximide stock solution, Yeast Extract-Peptone-Dextrose (YPD; Solarbio, Beijing, China) agar supplemented with 0.1 g/L chloramphenicol (Nacalai tesque, Kyoto, Japan), and Gluconobacter (GM) agar supplemented with 0.5 mg/mL cycloheximide (Goldbio, United States) respectively. MRS, YPD, and GM agar plates were used to enumerate lactic acid bacteria, yeast, and acetic acid bacteria, respectively. MRS and YPD agar plates were incubated anaerobically at 30°C for 72 hr while GM agar plates were incubated aerobically at 30°C for 72 hr. All microbiological analyses were conducted in triplicate and the data were presented as colony-forming units per milliliter (CFU/mL).

pH measurement

The pH values of fermented water kefir were determined using a pH meter (FiveEasy, Mettler Toledo, USA), according to AOAC method 981.12 (AOAC, 2012). To ensure accurate results, the pH meter was calibrated for 2 points with standard buffer solutions pH 4.0 and pH 7.0 before measuring the fermented water kefir.

Titratable acidity

The content of lactic acid in fermented water kefir was measured using the direct titratable acidity method, according to AOAC method 947.05 (AOAC, 2012). Fermented water kéfir (1 mL) was diluted with 9 mL of distilled water in a ratio of 1:9 (v/v). Then 0.1 N NaOH in the presence of 0.1% (w/v) phenolphthalein was titrated into the solution until a faint pink formed or the pH of the solution reached an endpoint of pH 8.1. The titratable lactic acid of fermented water kefir was calculated using the formula below, and the result was reported as g/L titratable lactic acid.

$$\text{Titratable lactic acid } \left(\frac{g}{L}\right) = \frac{\text{volume of titrant} \times 0.1 \text{ N NaOH} \times 90 \frac{g}{mol}}{\text{volume of sample}}$$

Measurement of total soluble solid content (TSS)

The measurement of total soluble solids content (TSS) was performed according to the AOAC method 932.12 (AOAC, 2012). TSS was determined using a portable refractometer (PAL-1, Atago, Japan) and the result was reported as degree Brix (°Br).

Estimation of reducing sugar (RSC) content

The reducing sugar content was determined by the DNS (3, 5-dinitrosalicylic acid) method according to Miller (1959). Glucose standard solutions with a concentration of 10 g/L were prepared. A 0.1 mL of fermented water kefir was diluted and mixed with 0.9 mL of distilled water. Then, 1 mL of DNS reagent was added to each test tube containing 1 mL sample solution. The opening of each test tube was covered with aluminum foil. The mixture in the test tubes was vortexed for 10 sec and heated for 5 min to develop a red-brown color. Then, the mixture was cooled under running water followed by the addition of 8 mL of distilled water to stabilize the colour. The absorbance reading was read at 540 nm.

Statistical analysis

Experiments were carried out with at least two replications and each sample was analyzed in triplicates. Data was analyzed by statistical test using SPSS Version 22 (IBMTM Company, USA). All data were expressed as mean ± standard deviation (SD) and were tested for normality using the Kolmogorov-Smirnov at a 95% confidence interval. Two-tailed Pearson correlation coefficients were calculated to determine the association between the probiotic growth with the physicochemical parameters.

RESULTS AND DISCUSSION

Microbiological analyses

The growth curve data were obtained by taking mean values of colonies number into account and were presented in Figure 1.

At initial fermentation (0 hr), the total cell count was 7.33×10^6 CFU/mL, with both LAB and AAB count recorded at 2.33×10^6 CFU/mL and yeast count recorded at 2.67×10^6 CFU/mL, respectively. At 6 hr, the total cell count showed a slight increase. Starting from the 12 hr, all bacteria and yeasts showed a sharp increase until the 48 hr, with total cell count increased from 8.77×10^7 CFU/mL to 2.06×10^8 CFU/mL. An interesting phenomenon was observed, where the LAB and AAB reached a peak at 48 hr, whereas yeasts reached their peak at 30 hr. Meanwhile, the growth of probiotics seemed to reach a plateau between 30 hr and 42 hr, although a slight decline in growth was observed as compared to the growth at 48 hr. After 48 hr, the growth of all probiotics was seen to reduce and fluctuate. Overall, the total cell counts of 1.68×10^8 CFU/mL were recorded at the end of 72 hr fermentation, with the LAB, yeast, and AAB count was 4.57×10^7 CFU/mL, 7.47×10^7 CFU/mL, and 4.77×10^7 CFU/mL respectively. In addition, approximately an 18.44% reduction in total cell count was recorded from 48 hr (2.06×10^8 CFU/mL) to 72 hr (1.68×10^8 CFU/mL). Among the three probiotics, the yeast count was recorded as the highest, as compared to LAB and AAB during the entire fermentation.

Based on the growth curve, four phases of probiotic growth were observed over 72 hr of fermentation. At the beginning of fermentation (0 hr), the concentration of total cell count was marked as 10^6 CFU/mL. The growth of probiotics was maintained at 10^6 CFU/mL even after 6 hr of fermentation. After 6 hr of fermentation, slow probiotic growth was observed. Beginning from 12 to 48 hr, a sharp increase was observed, with all bacteria and yeasts increased by 1 log CFU/mL. During the exponential phase, probiotics undergo rapid cell division in the presence of sucrose, thus the number of probiotics

increases logarithmically. A similar statement was reported by Veselovsky *et al*. (2022) for the growth of *Bifidobacterium longum*. Since different strains have different growth rates, therefore different peaks of growth were observed at different fermentation times (Ram *et al*., 2019). For instance, a different growth pattern was observed when *Bifidobacterium*, *Lactobacillus,* and *Saccharomyces* strains were grown individually in a similar growth medium (Kareena *et al*., 2022).

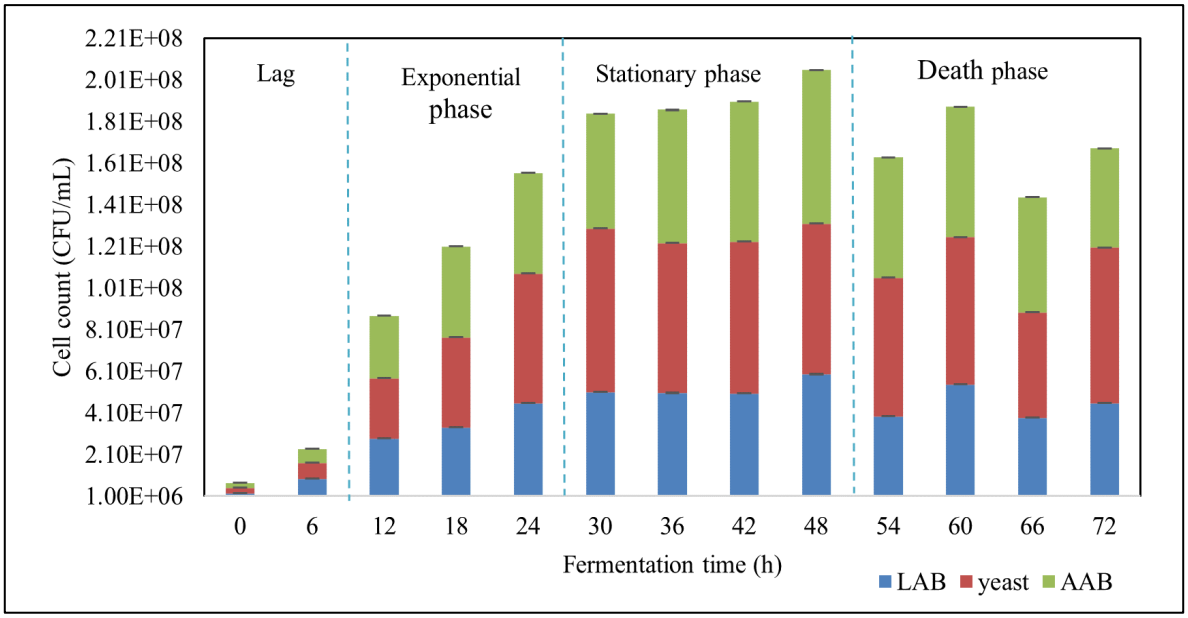

Fig. 1. Growth pattern of lactic acid bacteria (LAB), yeast, and acetic acid bacteria (AAB) over 72 hr of fermentation based on cell count (CFU/mL) on De Man, Rogosa and Sharpe (MRS) agar, Yeast Extract-Peptone-Dextrose (YPD) agar, and Gluconobacter (GM) agar plates, respectively.

Based on Figure 1, it is observed that the stationary phase happened between 30 to 42 hr since the medium showed a constant microbial population. As the nutrients gradually used up for growth, the probiotic growth would be decelerated and eventually remained constant. This phenomenon is known as the stationary phase. Usually, the death phase would happen following to stationary phase owing to the depletion of nutrients and accumulation of waste products. However, a fluctuation was observed instead. The fluctuation in growth observed after 48 hr was attributed to the competition among the individual isolates for the uptake of nutrients and acidification that influence probiotics growth (Ram *et al*., 2019). In this case, it is hard to predict the growth characteristics of each probiotic strains since they are grown in a complex co-culture matrix. As far as we understood, the dominant species continued to grow while the weaker species would be terminated. The acidic environment favored yeast growth while reducing the LAB and AAB growth. The continuous fermentation caused an increase in acidity, thus resulting in the dominance of yeast after 48 hr. This result was in close conformity with the findings of Stadie *et al*. (2013) and Tzavaras *et al*. (2022), who reported yeast are the dominant species in sugar substrate fermenting water kefir, as opposed to what has been discovered in the case of milk kefir, where LAB dominated.

In terms of optimal harvesting point, the probiotics are often harvested during the late exponential or stationary phase of growth to secure high cell numbers. Longer fermentation time did not guarantee high cell numbers, in contrast, resulted in a non-optimal fermentation process (Rickett *et al*., 2015). According to Figure 1, it is considered to harvest the probiotics at 30 to 42 hr for downstream processing. This is because the probiotics are highly stable and less metabolically active in their stationary phase. Although at 48 hr, the total cell count was at maximum number, however, they are metabolically active and less stable as compared to 30-42 hr. Broeckx *et al*. (2020) also documented that probiotics in the stationary phase (17 & 24 hr) have better stress tolerance which keeps them surviving during processing, as compared to the mid-log phase (6-7 hr).

pH and lactic acid content

Figure 2 depicts the acidity (pH and lactic acid content) of water kefir over 72 hr of fermentation. Generally, the pH of water kefir dropped while lactic acid content rose consistently as the fermentation

time extended. Initially, the pH of the sugar solution before inoculum was 5.16 ± 0.01. This value dropped to 4.43 ± 0.08 after inoculation of water kefir (0 hr). The pH reached 3.79 ± 0.00 at the first 24 hr and gradually dropped after 48 hr. The final pH of water kefir (72 hr) obtained was 3.35 ± 0.01. Meanwhile, the lactic acid content increased from 0.44 ± 0.07 to 2.16 ± 0.09 g/L. From 24 to 48 hr, the lactic acid content was 0.87 ± 0.05 g/L and 1.49 ± 0.12 g/L, respectively.

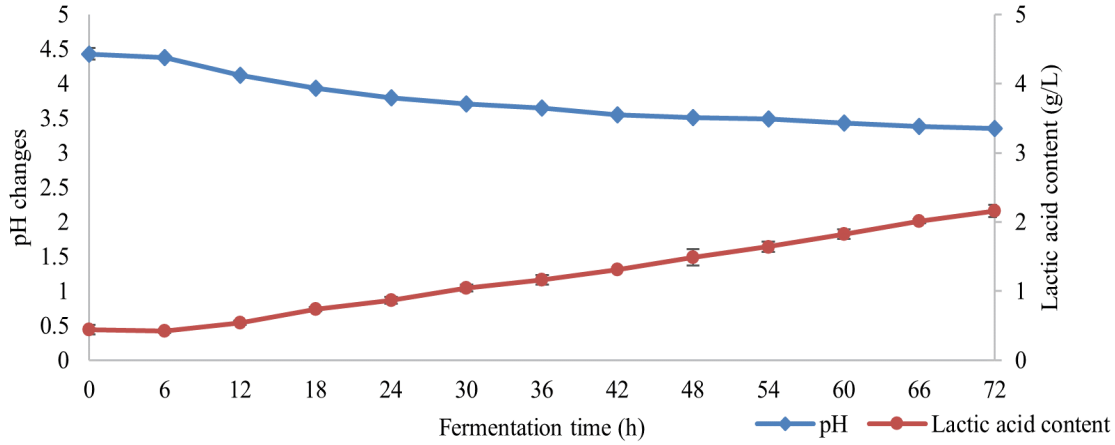

Fig. 2. pH changes and lactic acid content (g/L) of water kefir observed at 6 hr intervals, up to 72 hr of fermentation at room temperature.

The increment in lactic acid content was concomitant with a decrease in pH values. The lactic acid content was discovered inversely proportional to the pH changes (P<0.01). The increment of lactic acid content might be owing to the residual substrates and production of organic acids by probiotics in water kefir. The organic acids were metabolized by lactic acid bacteria *via* either Embden Meyerhof Parnas (EMP) or the phosphoketolose pathway, depending on their metabolic characteristics (Verce *et al*., 2019). Overall, the range of lactic acid in water kefir was between 0.44 ± 0.07 g/L (0.044%) and 2.16 ± 0.09 g/L (0.216%). A similar result was found in the study of Pendón *et al*. (2021), where lactic acid in water kefir was between 0.17 to 0.25%.

The longer the fermentation time, the greater the acidity and the lower the pH values. In addition, the acidic nature of brown sugar and palm sugar also contributed to the acidity, therefore producing an acidic sugar solution (Kongkaew *et al*., 2014). An acidic growth condition can favor the growth of acid-tolerant microorganisms, but excessive acid might deteriorate the growth of microorganisms (Laureys *et al*., 2019; Laureys *et al*., 2022). Anyhow, the results reported were relatively lower than the study by Laureys and De Vuyst (2014) who reported a pH of 3.45 obtained at 72 hr of fermentation. The pH values can be varied, depending on the fermentation conditions, amount of substrate used, type of substrate, and amount of kefir grains used (Lengkey *et al*., 2014; Egea *et al*., 2022).

Reducing sugar content (RSC)

Figure 3 illustrates the RSC in water kefir over 72 hr of fermentation. In this study, kefir microflora was assumed to utilize some structural sugars. Indeed, there is a significant increase was observed in reducing sugar content after fermentation. The initial concentration of reducing sugar (0 hr) in the water kefir sample was 3.329 ± 0.38 mg/mL. At 6 hr, a reduction was observed where the RSC decreased to 2.051 ± 0.05 mg/mL. After 12 rh, the reducing sugar content showed a steady increase until the end of fermentation (72 hr), from 3.806 ±0.41 mg/mL to 13.660 ± 0.14 mg/mL, recording a 72.13% increment. The increment was due to the sugar metabolism by yeasts, where sucrose was hydrolyzed into monosaccharides, namely glucose, and fructose, *via* the action of invertase enzymes, leading to a higher reduced sugar production (Lynch *et al*., 2021).

Meanwhile, a high reducing sugar content observed at 0 hr might be due to the deposition of residual sugar on the water kefir grain. For better accuracy, the water kefir grains should be rinsed well before inoculation. In addition, the result obtained was found inconsistent with the findings of Destro *et al*. (2019), Limbad *et al*. (2023), and Łopusiewicz *et al*. (2020). Normally, a decreasing trend of reducing sugar would be observed, however, the trend was not shown in our findings. The reason might be due to excessive sugar amounts used in the fermentation, causing a prolonged hydrolysis process. A similar phenomenon was reported by Laureys *et al*. (2017), where a high concentration of sugars might lead

to substrate inhibition or excessive osmotic pressure. If the fermentation is inhibited or slowed down due to excessively high sugar concentrations, it might result in incomplete sugar utilization, leading to residual sweetness or unbalanced flavors in the water kefir.

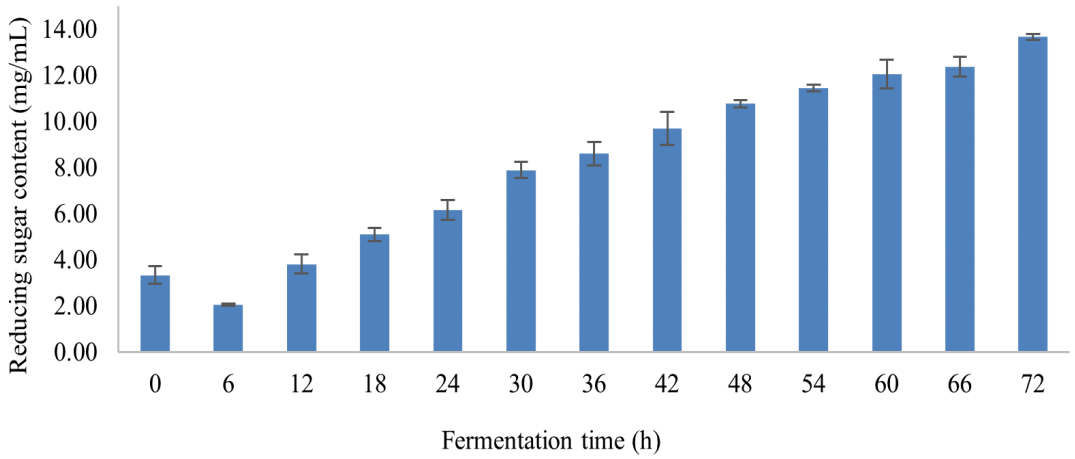

Fig. 3. The concentration of reducing sugar content (mg/mL) observed at 6 hr intervals, up to 72 hr of fermentation at room temperature.

Total soluble solids (°Brix)

Figure 4 presents the °Brix values of water kefir during fermentation of 72 hr. Brown sugar and palm sugar were the main substrates in the water kefir fermentation, which comprised nearly 70−80% of sucrose content. The reduction in sucrose concentration correlated with the reduction in total soluble solids (ºBrix). Overall, a reduction of TSS was observed with increasing fermentation time. °Brix values reduced from 7.37 ± 0.12 (0 hr) to 5.83 ± 0.06 over 72 hr of fermentation, recorded 22.26% of reduction. The decrease in °Brix values during fermentation owing to the catalytic metabolism of sucrose into glucose and fructose, which is catalyzed by the enzyme invertase of the yeast. Besides, the reduction of °Brix values also correlated with the degradation of monosaccharides to organic acids by lactic acid bacteria as reported by Destro *et al*. (2019). Gökırmaklı *et al*. (2023) reported that the °Brix values of water kefir beverages can be varied, and depending on the substrate used for fermentation. The °Brix values of water kefir beverages can be varied between 5.87 and 9.97.

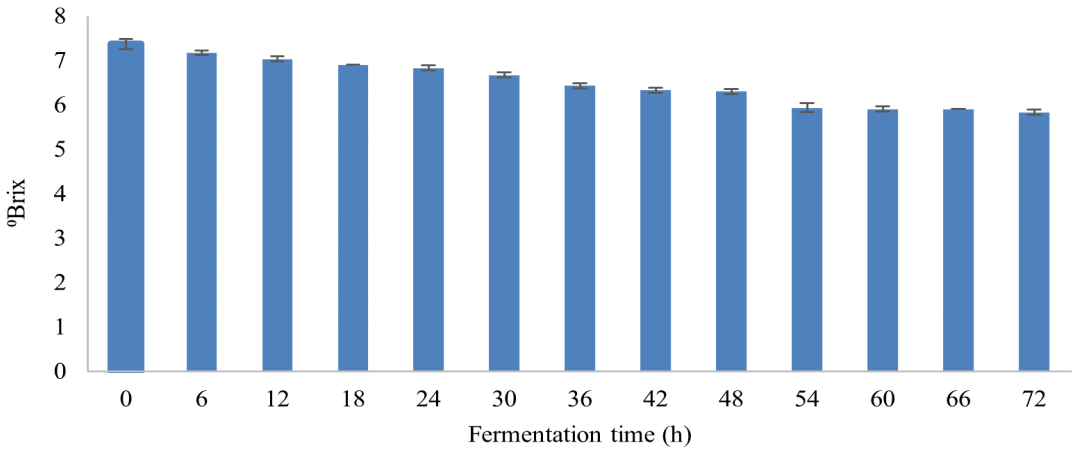

Fig. 4. The TSS (Brix°) of water kefir observed at 6 hr intervals, up to 72 hr of fermentation at room temperature.

Correlation analysis

Pearson's correlation matrix revealed the dependence among the studied variables at $p<0.01$ as presented in Table 1. The pH changes over 72 hr of fermentation were positively correlated with the

TSS (ρ=0.938), but negatively correlated with lactic acid content (ρ=-0.863) and reducing sugar content (ρ=-0.896). A negative correlation was also observed between reducing sugar content and total soluble solids (ρ=-0.816). During fermentation, kefir microorganisms metabolize the reducing sugars, leading to their conversion into various metabolites and organic acids like lactic acid. The pH values tend to decrease due to the accumulation of acids, resulting in a negative correlation between pH changes and lactic acid content (Abedi *et al*., 2020).

Table 1. Pearson's coefficients of the correlation between the probiotic growth and physicochemical parameters of water kefir

	pH		TSS		Lactic acid content		RSC	
	ρ	p-Value	ρ	p-Value	ρ	p-Value	ρ	p-Value
Physicochemical								
pH	/	/	0.938*	0.000	-0.863*	0.000	-0.896*	0.000
RSC	/	/	-0.816*	0.001	0.975*	0.000	/	/
TSS	/	/	/	/	-0.838*	0.000	-0.816*	0.001
Lactic acid content	/	/	/	/	/	/	0.975*	0.000
Microbiological								
Total cell count	-0.886*	0.000	-0.738*	0.004	0.681**	0.010	0.803*	0.001
Lactic acid bacteria	-0.861*	0.000	-0.729*	0.005	0.632**	0.020	0.746*	0.003
Yeast	-0.869*	0.000	-0.746*	0.013	0.694*	0.008	0.814*	0.001
Acetic acid bacteria	-0.872*	0.000	-0.690*	0.009	0.660**	0.014	0.784*	0.002

*P<(0.01); **P<(0.05); "/": without value, n=13 fermentation times; Total soluble solids (TSS); Reducing sugar content (RSC).

Besides, the correlation analysis between the total cells (LAB, yeast & AAB) and physicochemical properties was analyzed. A highly significant negative correlation (p<0.01) between the total cell count, pH, and total soluble solids. This suggested that the greater the total cell count, resulting in the lower pH values and TSS. Next, a significant positive correlation was detected between the total cells, lactic acid content, and reducing sugars, suggesting that the probiotics possess the capability to produce reducing sugars and organic acids. The positive correlation with pH and TSS suggests the uptake of sucrose as a carbon source. Meanwhile, a positive correlation between RSC and lactic acid content suggests the occurrence of sugar metabolism which caused metabolite production. In brief, the growth of probiotics is associated with a change in physicochemical properties, suggesting the uptake of sugars as a carbon source for metabolic activity and the production of organic acids as metabolites. A higher total cell count also correlates with increased metabolic activity, including the breakdown of sugars and other soluble compounds. As microorganisms consume sugars and other nutrients, they produce byproducts such as organic acids, alcohols, and gases (Yang *et al*., 2024). These byproducts contribute to the total soluble solids (TSS) in the fermentation medium.

CONCLUSION

This study showed four bacterial growth phases, with the stationary phase occurring at 30-42 hr, and was recommended as the optimal harvesting point. The optimal harvesting point can be useful to determine the optimal concentration of beneficial compounds such as probiotics, organic acids, vitamins, and enzymes which is useful for processing water kefir into powder with enhanced nutritional benefits. The fermentation involved the growth of probiotics with the uptake of sugars as a carbon source resulting in the production of organic acids as the final product. Overall, longer fermentation time produced lower pH values and lower total soluble solids while higher production of lactic acid and reducing sugars. As a recommendation, a nitrogen source can be added to boost probiotic growth aside from the primary carbon source (sucrose) in water kefir, since different substrates can have different effects in terms of fermentation stability, metabolite production, and grain growth. Nevertheless, future research should also focus on the growth of multi-strain probiotics in co-culture fermentation of water kefir as the standard plate count method has its limitations in predicting the accurate probiotic growth.

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ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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