Agro Bali : Agricultural Journal
Vol. 8 No. 1: 242-253, March 2025
e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

Changes in Chemical Profile and Bioactive Potential of Cascara Water Kefir Probiotic Beverage During Fermentation

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Article history: submitted: December 8, 2024; accepted: February 25, 2025; available online: March 28, 2025

Abstract. The global demand for functional food products is on the rise, with probiotic beverages, including water kefir and kombucha, emerging as the preferred options among consumers. Water kefir is produced through the fermentation of sugar-based solutions by water kefir grains and contains an extensive variety of probiotic microorganisms. Cascara, also known as coffee skin, has been recognized as a viable raw material for functional beverages that possess high antioxidant content. Nonetheless, a comprehensive understanding of the alterations in chemical compounds within cascara during the fermentation process remains insufficient, especially regarding the development of water kefir probiotic beverages. This study aims to identify changes in chemical profiles that occur during fermentation of cascara-based water kefir. The research was conducted using a completely randomized design (CRD) method with fermentation times of 0, 24, 48, and 72 hours. The results showed significant increases in total acidity, with lactic acid rising from 2.25% to 8.10% and acetic acid from 1.50% to 5.40%, which correlated with a decrease in pH. Total phenolic content increased from 366.70 mg/L to 514.91 mg/L, while flavonoid content decreased from 703.12 mg/L to 265.62 mg/L. Additionally, tannin and caffeine contents increased significantly during fermentation, on the other hand the sugar content decreased over time. These findings suggest that fermentation enhances the bioactive potential of cascara-based water kefir, improving its antioxidant capacity while altering its flavor and chemical composition. This research contributes to the understanding of cascara's potential for probiotic beverages, providing valuable insights for the development of functional beverages. The suggestion for further research is the need to identify the bioactive effect of cascara water kefir beverages products on health benefit.

Keywords: beverage; bioactive potential; cascara; chemical profile; water kefir

INTRODUCTION

The global demand for functional food products is escalating swiftly, with consumers increasingly favoring alternative probiotic beverages like water kefir and kombucha. Water kefir is generated by the fermentation of carbohydrate-rich liquids using water kefir grains, which harbor numerous probiotic microbes. Diverse carbohydrate sources, including sucrose, coconut water, honey, tea, fresh or dried fruits, fruit juices, and organic sugar water, can be utilized to produce water kefir (Limbad et al., 2023). The microbiota present in water kefir exhibits significant diversity based on the substrate utilized; typically, water kefir comprises various microorganisms such as *Lactobacillus ssp*, *Streptococcus ssp*, *Leuconostoc ssp* bacteria, *Bifidobacterium psychraerophilum/crudilactis*, *Dekkera bruxellensis*, yeasts, along with *Acetobacter* which are embedded within a white, translucent matrix of dextran polysaccharide (Egea et al., 2022; Laureys & De Vuyst, 2014; Moretti et al., 2022). Water kefir is regarded as a potential source of organics, probiotics, and antioxidants for vegans and those with dairy intolerances or allergies (Limbad et al., 2023).

Cascara, or coffee cherry tea, is a raw resource that has been cultivated as a beneficial beverage. Cascara is known to be able to be made as a refreshing beverage characterized by high antioxidant activity and low caffeine concentration. Cascara, as a beverage, is recognized for its elevated total phenol content and antioxidant activity (Nalurita, et al., 2023). Geremu et al. (2016) reported that the pulp of red coffee cherries comprises several polyphenols and other antioxidant compounds, chlorogenic acid being the most common phenolic component. Cascara is a potential raw ingredient for the development of water kefir beverages.

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Cascara is a compelling choice for research and product innovation because of its high antioxidant content, potential health benefits, role in sustainable by-product utilization, innovation in the development of functional beverages, and alignment with growing consumer trends towards eco-friendly and health-conscious products.

Various advancements have been implemented to enhance the added value of cascara products as functional beverages, specifically the incorporation of cascara in kombucha production. The findings indicated an elevation in antioxidant activity and phenolic compound concentration, alongside alterations in chemical constituents resulting from the liberation of bioactive compounds during fermentation. In total, 53 organic chemicals and volatile compounds were identified in the cascara raw material, including aldehydes, acids, alcohols, esters, and ketones (DePaula et al., 2022). Nalurita et al. (2024) identified that the components in cascara comprise acidic compounds, pyrazines, furans, flavonoids, and alcohols.

The further development of cascara-based water kefir probiotic beverages has been limited so far. Putri et al., (2024) produced an optimized formulation for a cascara tea-based probiotic beverage, demonstrating variations in antioxidant activity due to alterations in grain concentration and fermentation duration. During the fermentation phase, variations were observed in antioxidant activity, total phenolic content, color, pH, total soluble solids, viscosity, total lactic acid bacteria, total mold, and yeast levels. This research is novel in its use of cascara as a raw material for water kefir probiotic beverages, examining chemical profiles to assess alterations in the characteristics of cascara tea and the fermentation products of cascara water kefir throughout the fermentation process. The objective of this study is to ascertain the alterations in the chemical profiles during the fermentation of cascara-based water kefir. The determined chemical profile will contribute to and correlate with sensory perception, elucidating the phenomena associated with variations in antioxidant activity and consumer acceptance of created food products.

METHODS

Materials and tools

The tools used are glass bottles, non-metal filters, plastic spoons, analytical scales, beaker glass, erlenmeyer, measuring pipettes, dropper pipettes, statives, burettes, spatulas, Spectrophotometer UV-Vis (Shimadzu UV-1900i), Reflux. The material used are water kefir grain, sugar, mineral water, Cascara arabica from the Mount Ijen farm area from Bondowoso, Folin ciocalteu reagent (1:10), aquadest, aquabidest, Quercetin standard, tannic acid standard, caffeine standard, $NaNO_2$ 5%, $AlCl_3$ 10%, NaOH 1 M, NaOH 0,1 N, Na_2CO_3 35%, Na_2CO_3, chloroform, Fenolftalein indicator.

Kefir grain adaptation

Water kefir grains were rinsed with mineral water twice. A 500 ml solution of water and 10% sugar is produced and pasteurized at 72°C for 15 minutes. Upon chilling, water kefir grains are included into this concoction. Every 48 hours, the grains are filtered, and a new mixture of water and sugar is incorporated using the same method as before. This procedure is reiterated for seven days prior to utilization.

Preparation of cascara tea

Cascara tea is prepared by placing 2 grams of cascara tea flakes into a tea bag, which is then used to brew tea with 150 milliliters of hot water. The tea bags with cascara flakes were infused in water at 90°C for 12 minutes. The tea bags were thereafter destroyed, and the cascara tea extract was incorporated with 10% sugar. The tea concoction was subsequently cooled to ambient temperature.

Preparation of cascara water kefir

The preparation of water kefir involved the introduction of kefir grains at an optimal concentration of 8.58%, followed by fermentation for durations of 0, 24, 48, and 72 hours. Upon completion of fermentation,

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

samples from various fermentation durations were collected for the analysis of the chemical constituent profiles generated throughout the process.

Total phenolic content, tanin, and flavonoid

The Folin-Ciocalteu technique was utilized to determine the concentration of total phenolic compounds. Milligrams of gallic acid equivalents per 100 millilitres of the sample were reported by (Araújo et al., 2023). The tannin content was quantified with the AOAC International method by spectrophotometry using folin-ciocalteu reagent. The content of tannin was ascertained by calculating the standard equation of tannic acid used (AOAC International, 2016). The assessment of flavonoid concentration was quantified using a spectrophotometer. The content of flavonoids was ascertained by calculating the standard equation of quercetin used (Nurlinda et al., 2021).

Caffeine content

Samples of cascara water kefir are heated and boiled for ten minutes to ascertain the caffeine level. Samples were left to come up to room temperature. Each sample was collected in ten milliliters and put into the separatory funnel. Each sample was given one millilitre (20% w/v) of sodium carbonate solution and five millilitres of chloroform. The funnel was shaken for a few minutes to remove the caffeine, and then the bottom layer separated. UV-Vis spectrophotometry was used to measure the caffeine level at a wavelength of 273 nm (Ramanda et al., 2024). The caffeine content can be represented according to the following:

$$Caffeine\ content\ \left({}^{mg}/_{g}\right) = \frac{concentration\ \left({}^{mg}/_{L}\right) \times volume\ of\ dilution \times FP}{weight\ of\ sample\ (g)}$$

Titrable organic acid and pH

The measurement procedure involves placing 10 mL of cascara water kefir into a 100 mL volumetric flask and diluting it with distilled water to the calibration mark. Subsequently, two drops of 1% pp indicator are added, followed by titration with 0.1 N NaOH, the titration will be stopped until a pink color develops (Nugrahani et al., 2021; Rohman et al., 2019).

The pH measurement procedure refers to the study from Putri et al. (2024). As much as 30 ml of the sample is taken and then placed in a beaker glass, and then the pH meter that has been calibrated and washed with distilled water solution is inserted into the beaker glass. pH value will appear on the monitor and then be recorded. For every sample change, the probe must always be washed with distilled water.

Sugar content

The Luff Schoorl method is employed to determine the sucrose content. Introduce 10 ml of lead acetate, followed by homogenization, and subsequently add 15 ml of ammonium hydrogen phosphate. The mixture was homogenized and supplemented with distilled water to the designated limit mark. The mixture was permitted to remain undisturbed until all precipitate settled at the bottom of the flask. Subsequently, 25 ml of filtrate was utilized as the mother liquor, to which 25 ml of Luff Schoorl solution was added. This mixture was then heated with a condenser for 5 minutes. Following cooling, 10 ml of 20% KI solution and 15 ml of 25% H_2SO_4 were introduced. The liberated iodine is titrated using a 0.1 N $Na_2S_2O_3$ standard solution with an amylum indicator until a milky white endpoint is achieved. When adjusted according to the Luff method, the volume of Na2S2O3 solution utilized for titration can be determined as the quantity of glucose present in the sample (Pine et al., 2013).

Statistical data analysis

The data represent an analysis conducted using ANOVA (analysis of variance) with Minitab software to identify significant differences among the factors employed. If a significant difference is observed in the fermentation time factor, the analysis will proceed with the Tukey test.

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

RESULTS AND DISCUSSION

pH value

Based on the data shown in Figure 1, the pH of cascara water kefir decreased from 6.05 to 2.75 with the length of fermentation time. This is due to the formation of organic acids resulting from secondary metabolites produced by microbiota contained in water kefir grain. This study aligns with the results that show the formation of acid, which states that the fermentation process of cascara with water kefir grain leads to the production of organic acids, such as lactic acid and acetic acid, as fermentation time increases to 72 hours. The formation of these organic acids can increase the concentration of $H+$ ions, thereby lowering the pH (Siggaard-Andersen, 2006). Water kefir grain contains lactic acid bacteria (LAB) in algae crystals that break down glucose into lactic acid. Lactic acid produced during fermentation is strongly linked with a product's pH value or acidity level.

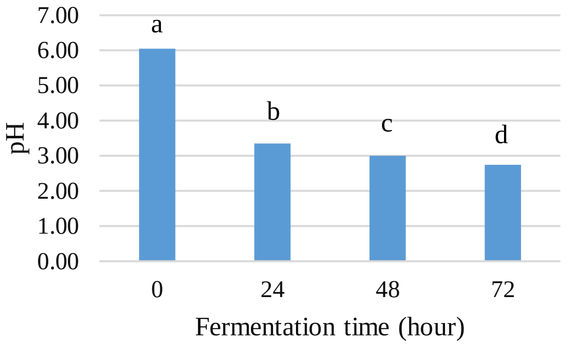

Figure 1. pH value of cascara water kefir at different fermentation times

In addition to lactic acid content, water kefir grain contains acetic acid bacterial yeast that can convert sugar into alcohol into acetic acid. The sour taste in green coconut water kefir results from the metabolic process of glucose and fructose from green coconut water into lactic acid by lactic acid bacteria. The breakdown of lactose into lactic acid by microbiota in water kefir grain occurs in water kefir grain through the process of glycolysis, which converts lactose into pyruvate and then breaks down into lactic acid by lactic acid bacteria (Moretti et al., 2022; Rohman et al., 2019). The presence of acetobacter also contributes to acetic acid production in the solution (Gomes et al., 2018; Moretti et al., 2022). The decrease in pH of water kefir products with increasing fermentation time also aligns with research conducted by Firdaus & Rizqiati (2019), who conducted a fermentation process for Green Coconut-based Water Kefir products. A decreased pH value was also found in whey kefir in fermentation for 12 to 48 hours.

Titrable organic acid

The alterations in total acidity in cascara water kefir influence the duration of fermentation; this result shows the levels of lactic acid and acetic acid of the sample tested (Figure 2). A significant decrease ($p < 0.05$) in the total acidity of the cascara water kefir sample was observed with the length of fermentation time. The initial lactic acid (Figure 2a) concentration in the water kefir was 2.25% to 8.10% over the 72-hour fermentation period. The acetic acid content (Figure 2b), initially measured at 1.50%, subsequently increased to 5.40%. The total acidity content values of cascara water kefir consistently increased, whereas the pH values decreased as fermentation time progressed from 0 to 72 hours.

According to the findings of this study, Araújo et al. (2023) also found that fermented products had lower pH values and acidity levels. The growth of lactic acid bacteria, a kind of microbiota found in both homofermentative and heterofermentative

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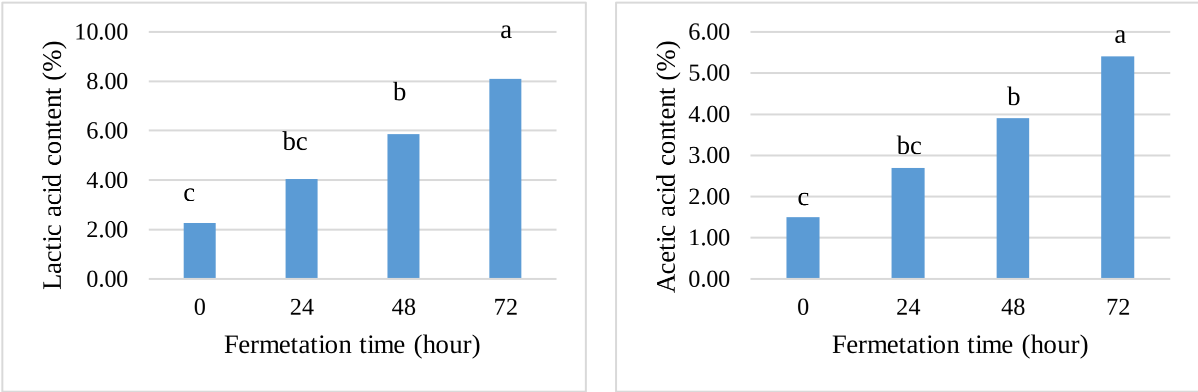
water kefir, influences the increase in overall acidity. These LAB's metabolic process creates organic acids. According to Putri et al. (2024), the increase in organic acid content created during fermentation causes an increase in H+ ion concentration, which lowers the pH of the fermented beverage product.



(a) (b)

Figure 2. Lactic acid and acetic acid content of cascara water kefir at different fermentation times

Total phenolic content (TPC)

Polyphenols constitute the most prevalent category linked to the health advantages of tea products. The TPC of the cascara water kefir samples during fermentation was assessed using the Folin–Ciocalteu method. Figure 3 shows a significant increase in TPC of the unfermented cascara tea from 366.70 mg/L to 514.91 mg/L as the fermentation time increased. The augmentation of total phenolic content (TPC) during fermentation can be attributed to the capacity of microbes in kefir grains to alter the concentration of beneficial substances, including phenolic compounds.

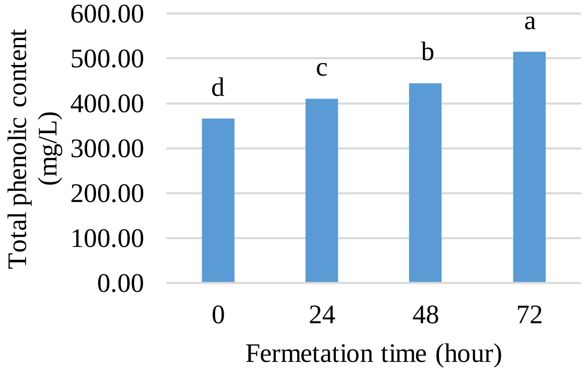


Figure 3. Total phenolic content of cascara water kefir at different fermentation times

According to Mousavi et al. (2011) the bioprocess involving fermentative microorganisms produce enzymes such as tannase, phenolic acid decarboxylase, benzyl alcohol dehydrogenase, decomposing complex phenolic compounds into simpler forms during fermentation, thereby increasing total phenolic content (Leonard et al., 2021). The fermentation process of tea leads to differences in the levels of phenolic acids, such as chlorogenic acid, gallic acid, salicylic acid, and vanillic acid, which are influenced by the duration of fermentation

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

(Somsong et al., 2020). Tannase contributes to the metabolic processes of gallic acid throughout the fermentation of pu-erh tea, resulting in an enhanced concentration of gallic acid throughout the fermentation process (Liu et al., 2020).

However, certain studies indicate trends that diverge from those identified in this research. The research by Gülhan (2024) indicated significant changes in total phenol compounds in fermented beverages, which were established from black teas, utilizing various infusion methods. Both infusion methods exhibited comparable decreasing trends in total phenol content as fermentation time progressed, which was also found in research by Araújo et al. (2023), who observed a tendency for a reduction in total phenolic compounds as fermentation progressed during the fermentation of green tea.

Flavonoid content

Recent investigations into flavonoid polyphenols in tea have predominantly focused on the categories of substances known as flavonols, flavanols, and flavones. The total flavonoid content obtained from 0 to 72 hours of fermentation of cascara water kefir is presented in Figure 4. The duration of fermentation influenced the flavonoid compounds present in cascara water kefir. The fermentation duration significantly impacted the total flavonoid content ($p<0.05$), resulting in a reduction of the flavonoid content in the cascara water kefir from 703.13 to 265.63 mg/L.

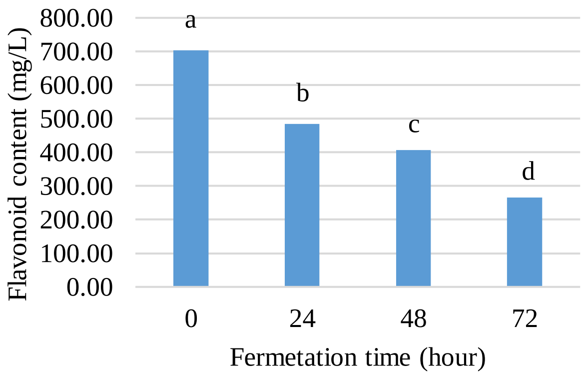


Figure 4. Flavonoid content of cascara water kefir at different fermentation times

The reduction in flavonoid content in water kefir may result from the degradation of compounds associated with the flavonoid group by microorganisms. The microbiota present in water kefir grain contributes to the degradation of flavonoid compounds in cascara tea. Sholichah et al. (2021) indicated that the fermentation of cascara using *Saccharomyces cerevisiae* results in a reduction of cascara flavonoid levels when compared to fermentation processes involving *Lactobacillus plantarum* and spontaneous fermentation.

In previous research conducted by Putri et al. (2024) cascara water kefir contains lactic acid bacteria as much as 8.54±0.10 Cfu/ml and total yeast & molds of 10.93±0.04 Cfu/ml. This result is in line with the study conducted by Moretti et al. (2022) and Laureys & De Vuyst (2014) who found that water kefir starter contains a variety of microorganisms, including LAB, AAB, yeast, *Bifidobacterium*, and *Dekkera*. Microorganisms in the fermentation process typically metabolize sucrose, producing a variety of acid, ethanol, and polysaccharide that might contribute to the decrease in flavonoid levels. The inclusion of yeast is thought to lower flavonoid levels in cascara water kefir. The content of flavonoid compounds is influenced by factors like fermentation degree, duration, temperature, and other variables (Li et al., 2021). Wang et al. (2022) reported a reduction in flavonoid

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

glycosides in green tea extracts fermented by lactic acid bacteria, possibly due to flavonoids' absorption and utilization.

Tanin content

The total tannin content of cascara water kefir, measured over a period from 0 to 72 hours, is presented in Figure 5. Tannin levels generally increase with extended fermentation durations. In cascara water kefir, the tannin content increased to 326.02 mg/L after 72 hours of fermentation, suggesting that fermentation may facilitate the breakdown of tannins into simpler molecules, potentially contributing leads to the production of phenolic chemicals. The results comply with the study carried out by Dartora et al. (2023); results indicated that the concentrations of total polyphenols and condensed tannins were improved until the 7th day of fermentation, after which they reached a state of stabilization.

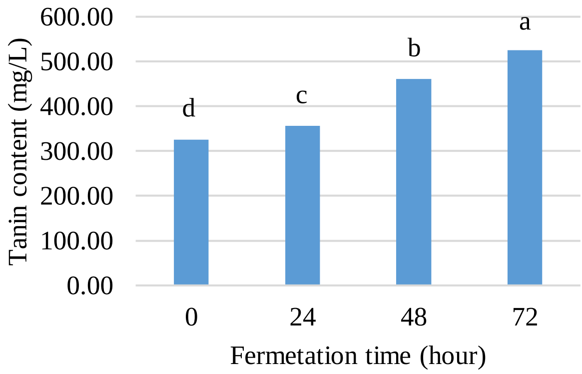


Figure 5. Tanin content of cascara water kefir at different fermentation times

Lactobacilli in fermented foods produce enzymes that degrade tannins, potentially disrupting the molecular bonds in phenol compounds throughout fermentation and preservation. Studies have shown increased extractable phenolic content during fermentation, as seen in pearl millet and oatmeal. Nishino et al. (2003) reported alterations in major polyphenol molecules in green tea leaves during fermentation and storage, showing a modest rise in total extractable phenolic content. However, the concentrations of epicatechin gallate and epigallocatechin gallate declined, suggesting gallic acid may be emitted. The Folin-Ciocalteu methods quantify total extractable phenolics and tannins, but the reaction to Folin solutions does not directly correlate with phenolic compounds. The capacity of hydroxyl groups in phenolic compounds to reduce molybdic acid is the basis for the Folin-Ciocalteu methods, which quantify the total extractable phenolics and tannins. As a result, the reactivity to Folin solutions is not directly proportional to the amount of phenolic chemicals present.

Caffeine content

Caffeine is an alkaloid compound and a natural chemical known for its stimulant effects. Several factors influence caffeine content, such as processing methods, the variety of coffee cherries, and plantations. The results indicate that fermentation time significantly affects ($p<0.05$) the cascara water kefir caffeine content, as illustrated in Figure 6, increases during the fermentation process.

The increase in caffeine content in this study aligns with previous studies by (X. Wang et al., 2008), who found that the concentration of caffeine in tea increases following treatment with mixed microorganisms over a specified duration. This study also stated the possibility of caffeine precursors present in raw materials that can be synthesized into caffeine so that

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

the amount increases after fermentation. Hlahla et al. (2010) mentioned that fermentation contributes to tea's caffeine content. The tea's caffeine content increases with fermentation time. Kato et al. (1996), demonstrated that microorganisms can establish a novel biosynthetic pathway for caffeine synthesis, distinct from tea plants. They can also break down, destroying coffee cherries' caffeine content during fermentation. Caffeine is ultimately synthesized through the methylation of N-7 in theophylline. Moreover, the microorganism could hydrolyze and eliminate caffeine content present in coffee cherries during the fermentation process. Caffeine can be converted into chlorogenic acid, which subsequently degrades into smaller compounds that can be released or dissolved in water (Tawali et al., 2018).

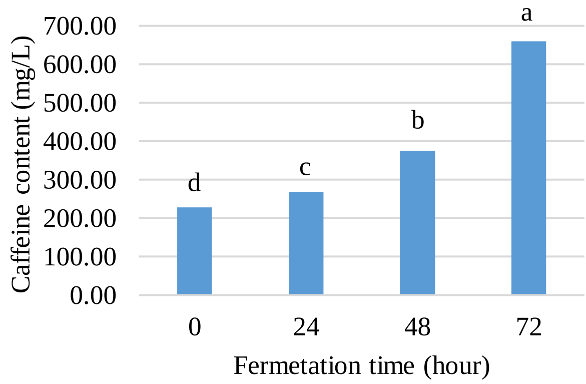


Figure 6. The caffeine content of cascara water kefir at different fermentation times

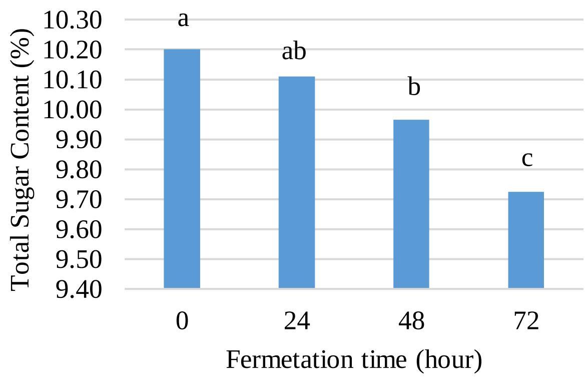


Figure 7. Total sugar content of cascara water kefir at different fermentation times

Total sugar content

Figure 7 illustrates the changes in the total sugar content of cascara water kefir prior to and throughout the fermentation process. A statistically significant reduction ($p < 0.05$) in the total sugar content of cascara water kefir samples was observed with increasing fermentation time. The initial total sugar content of the cascara tea samples was 10.2%, which decreased to 9.97% following a 72-hour fermentation period. Reduced total sugar content results from the fermentation of microorganisms found in water kefir grains. These microorganisms utilize sugars found in tea as a substrate, developing simpler compounds, including alcohol and organic acids such as acetic acid, formic acid, and citric acid. This study correlates with those stated by Ozcelik et al. (2021), who found that fruit juices have a range of Total Soluble Solids values from 9.85% to 12.80%. After fermentation in a water kefir medium, these

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v8i1.2178

values decrease, with pomegranate juice showing the most significant decrease. The reduction in sucrose was associated with decreased total soluble solids (ºBrix). This decrease indicates a decline in sugar content, as sugar acts as a solute in the juice's water. Longer fermentation time also leads to a reduction in Total Soluble Solids. The yeast enzyme invertase assists in catalytic sugar conversion, and Lactic acid bacteria convert monosaccharides to organic acids, which contributes to this decrease. Sugars comprise 80-100% of the sucrose content and decrease during fermentation (Sin et al., 2024). Furthermore, Lactic acid bacteria were shown to convert monosaccharides to organic acids, resulting in a drop in total sugar content (Destro et al., 2019).

CONCLUSION

The research findings on the fermentation of cascara-based water kefir reveal significant biochemical changes that align with the study's objectives. Both lactic acid and acetic acid contents increased notably during the fermentation process and contributed to the decrease in pH. The total phenolic, tannin and caffeine content also showed a significant rise. However, the flavonoid content exhibited a marked decline due to the degradation of these compounds by the microorganisms present in the kefir grains. Additionally, the total sugar content decreased steadily, reflecting the consumption of sugars by microorganisms, which in turn produced simpler compounds such as organic acids and alcohols. Overall, these findings highlight the complex changes in chemical composition during fermentation, which enhance the bioactive potential of cascara-based water kefir while also influencing its flavor profile and nutritional value, making it a promising functional beverage.

ACKNOWLEDGEMENTS

This research project was financially supported by the Directorate General of Higher Education, Research, and Technology Indonesia for the fiscal year 2024 under the *Penelitian Dosen Pemula Reguler* scheme.

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