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Effect of Fermentation Time on the Antioxidant and Antibacterial Activity of Fermented Cascara Beverage

Monayanti Simanjuntak, Diva Yolanda Hutabarat, Ditha Klarissa Sutan, Eunike Harjanto, Dwi Aditiyarini*

Biology Study Program, Faculty of Biotechnology, Universitas Kristen Duta Wacana, Yogyakarta, Indonesia

*Corresponding author (dwi.aditiyarini@staff.ukdw.ac.id)

Abstract

Indonesia is a country with a high consumption of coffee, which is in direct comparison with the increased production of coffee waste. Arabica coffee husk (cascara) is coffee industry waste that has not been utilized optimally, which is only 0.76% of the existing potential. Cascara is known to have a secondary metabolite compound that can be utilized as a good product for human health. The effects of Arabica cascara fermentation time on the antioxidant and antibacterial activity of cascara beverage were studied in this study. This research is using a completely randomized non-factorial design. Cascara was obtained from Puntang Mountain, West Java, and was fermented with *Lactiplantibacillus plantarum* (previously known as *Lactobacillus plantarum*) for 10, 14, and 18 days. pH value was measured with a pH meter, total sugar content with phenol sulfuric acid method, caffeine content with high performance liquid chromatography (HPLC) method, total lactic acid bacteria (LAB) with drop plate method, total flavonoid and phenolic content with UV-Vis spectrophotometry method, antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, and antibacterial activity against *Helicobacter pylori* with disc diffusion method. Data were analyzed using One-Way ANOVA and DMRT test at the significance level of 0.05. The results showed that prolonged fermentation times reduce the quality of Arabica cascara beverage. The optimal fermentation times were found to be 10 days producing the best balance of pH (3.66), total sugar content (1.29%), caffeine content (0.043%), total LAB (1 x 10^7), total phenols (12.74 ppm), total flavonoids (3.40 ppm) and highest antioxidant IC_{50} activity (215 ppm) while for antibacterial activity has not been able to inhibit the growth of *Helicobacter pylori*.

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Introduction

Indonesia is one of the leading coffee producers in the world. According to the data of the International Coffee Organization (ICO), consumption of coffee in Indonesia reached 5 million bags of 60 kg from 2021 to 2022 and increased in the subsequent period (ICO, 2023). According to the Central Bureau of Statistics (BPS), coffee production in Indonesia in 2021 has increased by 3.12% with a total production of 786.19 thousand tons (BPS, 2022). The increase in coffee production is directly proportional to the increase in coffee production waste, especially coffee husk (cascara). The extraction process of 100 kg coffee produces 56.8 kg of seeds and 43.2 kg waste of pulp and skin. Coffee production waste, especially cascara, can be reprocessed into derivative products with economic value (Garis et al., 2019). According to Sholichah et al. (2019), cascara contains bioactive compounds such as phenolic, caffeine, flavonoids, chlorogenic acid, caffeic acid, anthocyanins, tannins, and pectins. The compounds in cascara are almost the same as in coffee. Cascara has not been utilized optimally in Indonesia, with only 0.76% of its potential being used (Ramon et al., 2019). According to Aryadi et al. (2020), the caffeine content in coffee was 1.77% of the total weight of 1 gram sample, whereas the caffeine content in cascara was 1.3% lower than coffee in general (Garis et al., 2019).

Secondary metabolites in cascara have been beneficial in some aspects of health, including preventing free radicals and protecting the stomach layer (Sari et al., 2021). One of the health disorders in the stomach is gastritis, which is caused by an infection of the bacterium *Helicobacter pylori* (Malfertheiner et al., 2023). Fermented beverages are known to inhibit the growth of disease-causing pathogenic bacteria (Le et al., 2024). Fermentation is one of the processing methods that can increase the bioactive content of cascara through enzymatic reactions. There are several factors that can affect the fermentation capacity, namely the type of starter, fermentation temperature, pH, and fermentation time. Generally, the starter used for cascara fermentation is Symbiotic Culture of Bacteria and Yeast (SCOBY), and Lactic Acid Bacteria (LAB) in cascara is still rarely used. Research by Nguyen et al. (2015), revealed that the use of LAB in fermentation can produce organic acids, bioactive compounds such as vitamin B, polyphenols that can increase antioxidant activity, and increase antimicrobial effectiveness against pathogens. Another factor that determines the quality of the cascara is the long fermentation time. Puspaningrum et al. (2022) stated that 14-day long fermentation is the optimal time for cascara kombucha fermenting with SCOBY starter, but the time for LAB starter is still rarely explored. In this study, *Lactiplantibacillus plantarum* ATCC8014 (previously known as *Lactobacillus plantarum*) was used as a starter with fermentation time for 10, 14, and 18 days. The study aims to determine the optimal fermentation time in the fermented Arabica (*Coffea arabica*) cascara beverage production and to explore the potential of antioxidant and antibacterial activity in the fermented Arabica (*Coffea arabica*) cascara beverage.

Materials and Methods

Materials

Arabica cascara was obtained from Puntang Mountain, West Java. *Lactiplantibacillus* ATCC8014 (previously known as *Lactobacillus plantarum*) was obtained from Agritama Synergy Innovation LLC (AGAVI) Bandung, Indonesia. *Helicobacter pylori* was obtained from Integrated Laboratory, Samarinda, East Kalimantan, Indonesia. The chemicals used in this research were of analytical reagent grade, including phenol 5%, sulfuric acid 96%, methanol PA, Folin-Ciocalteu reagent, ethanol PA 96%, Na_2CO_3 1 M, $AlCl_3$ 10%, acetic acid 5%, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid.

Methods

The study was conducted from April-June 2024. Arabica cascara was ground using a Philips HR 215 blender manufactured in the Netherlands and sieved through a 50 mesh filters. The preparation and fermentation of the Arabica cascara beverage followed these steps: The beverage was prepared with ratio of 30 g cascara powder to 1 L of boiling water and 100 g of sugar, then pasteurized at 5°C for 30 minutes using an autoclave (Hirayama) manufactured in the Netherlands. 5% (v/v) starter of *Lactobacillus plantaru*m ATCC8014 was inoculated into the fermenter. Incubation was conducted for 10,14, and 18 days at room temperature (27±1℃) under anaerobic conditions (Puspaningrum et al., 2022).

pH value was measured using a Bench pH meter (OHAUS AB33PH-F) manufactured in the United States (Puspaningrum et al., 2022). Total sugar content was analyzed using the phenolic acid sulfate method (Yue et al., 2022) by diluting the sample (1:100), adding 5% phenol and 96% sulfuric acid, and allowing the reaction to stand for 10 minutes. The sample was heated at 100℃ for 15 minutes and cooled, and its absorbance was measured at 490 nm. Caffeine levels were determined using the high-performance liquid chromatography (HPLC) method (Hidayah et al., 2024) with a UV-Vis detector, a C18 column with 26 °C temperature, and a mobile phase consisting of deionized water and methanol PA (50:50). The mixture was filtered with a 0,45 µm syringe filter and had a flow rate of 1 mL/min. Measurements were taken of 271 nm. Total lactic acid bacteria (LAB) count was determined using the de Man Rogosa Sharpe Agar (MRSA), manufactured in India, through the drop plate method and calculated using the Total Plate Count (TPC) method (Soesetyaningsih and Azizah, 2020). Samples were diluted to 10^{-8} and dropped into MRSA medium, followed by incubation for 48 hours at 37°C.

The total flavonoid and phenolic content were analyzed using UV-Vis spectrophotometry (Sholichah et al., 2021) with modifications. Gallic acid was used as a standard for total phenol content analysis. Samples were diluted (1:10) with 96% ethanol, reacted with 10% Folin-Ciocalteu reagent, left for 10 minutes, and then combined with Na_2CO_3 (1 M) and distilled water. The mixture was incubated for 2 hours in the dark before measuring absorbance at 758 nm. For total flavonoid content, quercetin was used as a standard in the analysis. Samples were diluted (1:10) with 96% ethanol and reacted with 10% $AlCl_3$ and 5% acetic acid, then the mixture was incubated for 15 minutes at room temperature before measuring the absorbance value at 435 nm.

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Lestari et al., 2023) with modifications. Ascorbic acid was used as the positive control. A 1000 ppm stock solution was prepared and diluted into a series of concentrations ranging from 100 to 500 ppm. For each concentration, 2 mL was mixed with 2 mL of 50 ppm DPPH solution, incubated for 30 minutes in the dark condition, and measured for absorbance at 517 nm. Antibacterial activity was tested using the disc diffusion method (Pradana et al., 2023) with modifications. Bacteria cultured to match the 0,5 McFarland standard were inoculated onto Mueller-Hinton Agar (MHA) medium using a sterile cotton swab twice. Then, 25 µL of cascara sample at different concentrations (6.25%, 12.5%, 25%, 50%, and 100%) was dropped onto sterile dics and placed on the MHA medium. Amoxicillin was used as a positive control, and distilled water served as a negative control. The results of these test were further used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) following Monteiro et al. (2019). All tests were carried out triplo except for caffeine test with only one repetition, the obtained data were statistically analyzed using one-way ANOVA and Duncan's Multiple Range Test using SPSS 25.0. The level of significance was set at $\alpha = 0.05$.

Results and Discussion

pH Value

pH value of the unfermented cascara sample was 4.47, but after 10-18 days of fermentation, pH values decreased to 3.66, 3.62, and 3.58 sequentially in Table 1. The decrease in pH is caused by the accumulation of

Table 1. Chemical and Microbiological Characteristics of Fermented Arabica Cascara Beverage

Parameters	Fermentation Times (Days)			
	0	10	14	18
pH	4.47±0.1[b]	3.66 ±0.0[a]	3.62±0.1[a]	3.58±0.1[a]
Total Sugar Content (%)	6.62±0.1[c]	1.29 ±0.1[b]	1.08±0.2[a]	0.88±0.1[a]
Caffeine Content (%)	0.033	0.043	0.039	0.041
LAB (CFU/mL)	-	1 x 10^7±0.4[b]	6 x 10^6±0.5[a]	4.08 x 10^6±0.6[a]
Total Phenolic Content (mgGAE/g)	4.32±0.1[a]	12.74±0.1[d]	8.97±0.2[c]	7.41±0.1[b]
Total Flavonoid Content (mgQE/g)	2.45±0.1[a]	3.40±0.1[d]	2.91±0.0[c]	2.67±0.0[b]
IC_{50} (ppm)	403±0.2[a]	215±0.2[d]	300±0.2[a]	368±0.2[a]

Results are mean±standard deviation; Different superscript letters in the same row indicates the significant differences ($p < 0.05$) (except for caffeine content)

lactic acid and organic acid produced by lactic acid bacteria during the fermentation, thereby promoting a decreased pH value in Arabica cascara beverage (Rosidah et al., 2021). Based on Puspaningrum et al. (2022), the pH value of kombucha cascara is safe for consumption should not be less than 3. The results showed that the pH of the cascara beverage in each sample was at the safe boundary for consumption and was acceptable to the body. The decrease in pH value during the fermentation process is not only essential for food safety but also contributes to the development of a flavorful and potentially health-beneficial beverage (Puspaningrum et al., 2022).

Total Sugar Content

The total sugar content of the fermented cascara beverage obtained a value that indicates a decrease in the sugar levels during the time of fermentation. The unfermented beverages had a total sugar content of 6.62%, then decreased by 1.29%, 1.08%, and 0.88% on the 10, 14, and 18 days sequentially in Table 1. The fermentation time affects the sugar content of the fermented cascara beverage. The sugar content of Arabica cascara beverage decreased due to sugar as a source of carbon for the lactic acid bacteria found in the fermenter (Fatima et al., 2024). The lactic acid bacteria use sugar as an energy source for cell reproduction, carry out metabolic activity, and produce lactic acids, so the longer the time of fermentation makes the concentration of sugar in a fermenter will decrease (Cahyanti et al., 2021). The decrease in total sugar content of the fermented Arabica cascara beverage is directly related to the decreases in the fermenters's pH levels, as a result of lactic acid accumulation produced by bacteria.

Caffeine Content

Caffeine is an alkaloid compound commonly found in coffee or tea. From Table 1, the caffeine content of cascara beverage before fermented was 0.033% and after fermented for 10, 14 and 18 days is in the range of 0.039% - 0.043%. The low caffeine levels in unfermented Arabica cascara beverage can be attributed to the inherent properties of Arabica, which naturally contains 1-2% caffeine in its seeds. Additionally, the production of kombucha from cascara, the dried husk of coffee cherries, involves minimal extraction of caffeine during the fermentation process, especially when fermentation is not applied (Muzaifa et al., 2021). The caffeine content in the Arabica cascara beverage remains relatively constant during fermentation, with slight variations from 0.039% to 0.043%. In another study by Li et al. (2022), the caffeine content of tea beverage by-product fermented using *Lactobacillus plantarum* RLL68 remained relatively constant during the fermentation process with slight variations of 0.0158% to 0.0167%. This indicates that the use of *Lactobacillus plantarum* as a lactic acid bacteria starter does not significantly affect the caffeine content in the cascara beverage. When compared to other studies, the results of caffeine content in this study are close to the results from Sholichah et al. (2019), which shows that the caffeine content in cascara fermented using *Lactobacillus plantarum* starter was 0.0528%.

Total Lactic Acid Bacteria (LAB)

A total of 5% LAB was inoculated into the unfermented cascara beverage sample. On day 0, the total LAB count showed no growth, remaining at 0 CFU/mL. In a sample with a 10-day fermentation period, the total LAB count reached 1 x 10^7 CFU/mL. After 10 days of fermentation, there was an increase LAB in the beverage, indicating that the inoculated LAB utilized the substrates in the cascara beverage, supporting LAB cell proliferation due to the glucose content in the beverage (Oktaviani et al., 2020). At 14 and 18 days, there was a significant decrease in the total LAB count to 6 x 10^6 CFU/mL and 4.08 x 10^6 CFU/mL, respectively. This decline is related to the metabolic activity of lactic acid bacteria, which use sugars as a nutrient source to support their growth. The reduction in bacterial growth indicates that bacteria died due to the depletion of nutrients in the fermenter, leading to a lower bacterial count in subsequent days (Mahjani and Putri, 2020).

Total Phenolic Content

The fermentation process is known to affect phenolic content, causing modification or the formation of monomers or polymers. Through the tests that have been carried out, showed that the total phenol content of non-fermented cascara beverage is 4.32 mg GAE/g, as seen in Table 1. The total levels of phenols after the process of fermenting increased by 12.74 mg GAE/g on the 10th day. The highest-rated cascara was on the 10th day of fermentation. During the fermentation process of cascara beverages, the activity of LAB will damage the cell wall structure, resulting in the synthesis of various bioactive compounds. *Lactobacillus plantarum* produces several key enzymes that play a crucial role in the metabolism of phenolic compounds during fermentation. These include phenolic acid decarboxylase, hydroxycinnamic acid reductase, esterases, and glycosidases (Gaur and Gänzle, 2023; Landete et al., 2021) which work together in the metabolic pathways leading to the formation of 4-vinyl phenol and 4-vinyl guaiacol as phenols (Nafisah et al., 2023). A decrease in the phenolic content along with a reduction in the sugar content during fermentation thus inhibits LAB metabolism (Nafisah et al., 2023).

Total Flavonoid Content

The total flavonoid content of unfermented cascara beverage was 2.45 mg QE/g, while cascara that had been fermented on the 10, 14, and 18 days were 3.40 mg QE/g, 2.91 mg QE/g and 2.67 mg QE/g sequentially. Table 1 shows that the highest flavonoid content of cascara fermentation drinks is found in the fermenting process for 10 days, which is 3.40 mg/g QE. The increase in the levels on the 10 days is influenced by the activity of lactic acid bacteria that produce enzymes to break down sugar, dissolve complex phenolic compounds, and remove phenol compounds from the substrate, thus adding a group of phenols to form the flavonoids, but it can be seen that there is a decrease in the level on the 14 and 18 days that may be affected by the length of the fermentable time, according to Aryantini et al. (2020) long fermented process will cause the occurrence of decreased levels of flavonoids contained in the sample. According to Liu et al. (2020), the increase and decrease in flavonoid content in samples can be related to the concentration of LAB in the fermenter. The decrease in total LAB count on the 14 and 18 days of fermentation leads to a reduction in the produced phytochemical compounds.

Antioxidant Activity

Inhibitory concentration (IC_{50}) is a value that indicates an oxidation inhibitory activity of 50%. The lower the IC_{50} indicates the higher of antioxidant activity. During the fermentation, Arabica cascara produced IC_{50} values ranging between 215-403 mg/L. The highest IC_{50} activity was found in samples with a 10-day fermentation time of 215 ppm that were included in the moderate activity of IC_{50}. The results are consistent with a significant increase in total phenol content (TPC) and total flavonoid content (TFC) content after fermentation. The increased content is associated with the aglycon release produced by the microbes' enzymes. Previous research suggests that the increased antioxidant capacity during fermentation is believed to be due to the oxidation mechanisms of *Lactiplantibacillus* ATCC8014 that are stimulated by enzymes and non-enzymes, thereby increasing the antioxidant capacity in the process (Rehman et al., 2016). The decrease in antioxidant capacity can be caused by an increased concentration of organic acids produced by bacterial activity. The increase in organic acid content can cause phenolic compounds to become stable and difficult to release protons that can bind to DPPH, leading to a decrease in antioxidant ability (Situmeang et al., 2022). It suggests that long fermentation times can affect the antioxidant ability of Arabica cascara beverage.

Organoleptic Test

The organoleptic test was carried out with 30 untrained panels that gave a score according to the individual's level of preference or hedonic test for cascara beverage. The parameters of the organoleptic test include color, aroma, taste, aftertaste, and overall. In Figure 1 showed that panels prefer the color, aroma, taste, aftertaste, and overall of the cascara beverages with fermentation time at 10-days long. Cascara beverages with 10-days long fermentation time showed a high score of color at 3.53, this is due to the presence of alpha-diketone pigment, which gives a brown color to the beverage. The aroma result showed the highest score at 3.43. After fermentation, the presence of LAB also affects the acid smell that comes out of the cascara. The longer the fermentation time, the more organic acids will be formed, so that the sour scent that appears will be stronger. The flavor result showed the highest score at 3.37. This is due to the acidity and slight sweetness of the cascara, which results in a combination that fits and refreshes. During the fermentation process, LAB produces organic acids that add acidity to the beverage (Nurhayati et al., 2020). The length of fermentation time also affects the flavor of the Arabica cascara beverage, the longer it is fermented, the more acidic it will be, so the panelist prefers the taste of cascara with a fermenting time of 10 days. The aftertaste of the sampled cascara is rated to be not so concentrated and still light to consume. Lastly, the highest evaluation in the organoleptic test in terms of flavor, aroma, and aftertaste is obtained in the 10-day fermentation cascara sample treatment with an average score of 3.3 out of 5.

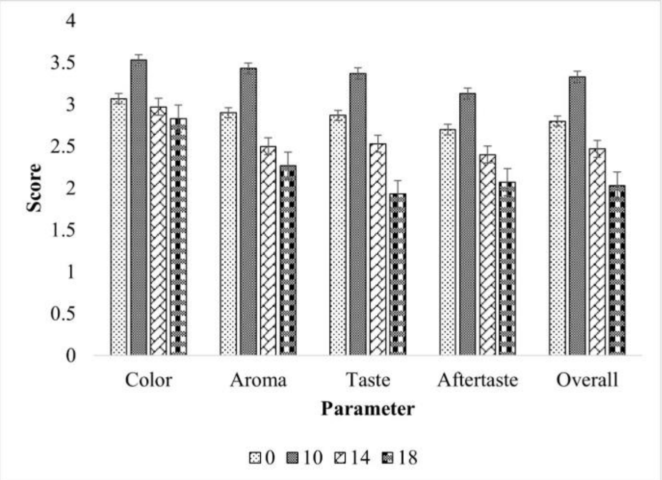

Figure 1. Average score of organoleptic ratings from fermented arabica cascara beverage

Antibacterial Activity

The antibacterial potential can be identified by observing the formation of an inhibition zone during antibacterial testing. Both the results of the antibacterial test using a sample of cascara beverage and amoxicillin as the positive control didn’t show any formation of an inhibition zone against the growth of *Helicobacter pylori.* Further testing of minimum inhibitory concentration (MIC) was conducted to determine the lowest concentration of an antimicrobial agent that can inhibit the growth of a microorganism, and minimum bactericidal concentration (MBC) was used to determine the lowest concentration of an antimicrobial agent that can kill a specific microorganism (Oktaviani et al., 2020). The same goes for the antibiotic amoxicillin, which did not show any inhibitory response or kill bacteria in the trial. This is supposed to be because *Helicobacter pylori* can adapt by producing urea and ammonia to help adapt bacteria in acidic environments and inhibit bacterial metabolism and growth. In addition, *Helicobacter pylori* has developed resistance due to a chromosome-encoded mutation that affects the growth inhibitory capacity of *Helicobacter pylori* (Boyanova et al., 2023). The sample of the bacteria used in this test was *Helicobacter pylori* strain CPY6081, isolated from a gastric biopsy homogenate from a stomach cancer patient in Yamaguchi Prefecture, Japan. Amoxicillin is a commonly used antibiotic to treat *Helicobacter pylori* with low levels of resistance around the world, but recent research suggests that high levels have occurred in some countries, including Japan. Other research reports that *Helicobacter pylori* bacteria have experienced increased resistance to antibiotics and have produced several strains that are resistant to many medicines causing significant challenges to treatment and inhibition (Hasanuzzaman et al., 2024). To further explore the antibacterial potential of cascara for treating gastritis, additional testing using other bacterial species associated with gastritis is necessary.

Conclusion

Sustained fermentation time shows a significant influence on antioxidants and the sensory of the Arabica cascara drink. The best treatment is found with a long 10-day fermenting time that produces Arabica cascara beverage with a good balance of total bioactive compounds, the activity of antioxidants, and consumer reception to cascara beverages. Although there is not yet an Arabica cascara capable of being an antibacterial agent against *Helicobacter pylori.*

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