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Antioxidant activity, total phenolic, flavonoid, and caffeine contents of robusta coffee (*Coffea canephora*) fermented with lactic acid bacteria

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Abstract

Robusta coffee is widely cultivated in Indonesia, but it struggles to dominate the global market due to its bitterness, slightly sour taste, and higher caffeine content. Coffee bean fermentation can be done to reduce undesirable characteristics in robusta coffee. This study aimed to evaluate the effects of lactic acid bacteria fermentation on the quality of coffee and its brewing characteristics, comparing it to spontaneous fermentation. The goal was to explore the potential of fermented coffee beans as functional beverages with health benefits. Three lactic acid bacteria (LAB) isolates were tested, and *Lactiplantibacillus plantarum* H 2.34 was identified as the most effective starter culture for coffee fermentation. After 12 hours of fermentation using coffee mucilage, LAB growth was significantly enhanced. A significant increase in flavonoid content was observed in coffee fermented with the starter culture. All brewed coffee samples demonstrated strong antioxidant activity, and LAB fermentation successfully reduced caffeine content by 4.85%. These results suggest that fermented robusta coffee could offer functional health benefits while improving its sensory profile.

[Keywords: antioxidant, flavonoid, *L. plantarum*, phenolic compounds]

Introduction

Coffee is an important plantation commodity in Indonesia with high economic value (Falahudin and Harmeni 2016). According to the Ministry of Agriculture, Indonesia's coffee production increased from 171.96 thousand tons in 2017 to 786.19 thousand tons in 2021 (KEMENTAN RI 2022). There are four types of coffee cultivated in Indonesia, namely arabica coffee (*Coffea arabica*), robusta coffee (*Coffea canephora*), liberica coffee (*Coffea liberica*), and excelsa coffee (*Coffea excelsa*). Robusta coffee is the most widely produced type of coffee in Indonesia, reaching 87.1% of total coffee production in Indonesia (Hartatie & Kholilullah, 2018). Although robusta coffee is the most widely cultivated variety, it struggles to dominate the global market due to its bitter and slightly sour taste and higher caffeine content (Darmanto et al., 2013).

Improving coffee quality requires careful attention to the post-harvest process. One of the stages of the post-harvest process is fermentation. Previous studies have analyzed the fermentation of coffee pulp using xylanolytic and cellulolytic bacteria but did not characterize the resulting coffee beans (Kurniawati et al., 2016; Putri et al., 2023). Typically, coffee fermentation focuses on improving bean processing efficiency and flavor, rather than enhancing health-related properties such as reducing caffeine levels or increasing flavonoid content and antioxidant activity (Kinyua et al., 2017; Haile & Kang, 2019).

Fermentation can take place immediately after harvest, but spontaneous fermentation can result in low quality coffee. In contrast, several fermentation studies have proven that fermentation using starter cultures improve coffee quality (Martinez et al., 2017; Bressani et al., 2018; da Mota et al., 2020). Elhalis et al. (2020) reported that the dominant microbial communities that play a role in coffee fermentation are yeasts, mesophilic aerobic bacteria, and lactic acid bacteria (LAB). The addition of yeast as a starter culture during coffee fermentation has been shown to increase flavonoid content and antioxidant activity compared to unfermented coffee beans (Kwak et al., 2018; Haile & Kang, 2019). Adrianto et al. (2020) have used lactic acid bacteria in robusta coffee fermentation but only presents data

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on the decrease in caffeine levels. The use of Indonesian LAB isolates as starter cultures in coffee bean fermentation, particularly for analyzing their effects on phenolic content, flavonoid content, caffeine levels, and antioxidant activity, has not been widely reported. This study aims to evaluate the effect of LAB fermentation on robusta coffee, focusing on improvements in health-related properties such as antioxidant activity and caffeine reduction.

Materials and Methods

Coffee sample preparation

Robusta coffee was obtained from the Cibulao coffee plantation in Tugu Utara Village, Cisarua District, Bogor Regency, West Java. The red coffee cherries from freshly picked robusta coffee were sorted by soaking them in water to separate ripe cherries and unripe ones (Huch & Franz, 2015). The ripe cherries were de-pulped to obtain the de-pulped coffee beans. These de-pulped beans were then subjected to wet fermentation using a selected LAB starter culture, which was compared to spontaneous fermentation.

Selection of LAB isolates

The LAB isolates used were *Lactiplantibacillus plantarum* H 2.34, LAB H 3.1, and LAB H 0.17, which were selected for their ability to produce total acid (Tsaaqifah et al., 2023). The isolates were rejuvenated by inoculating 10 mL of de Man, Rogosa, and Sharpe Broth (MRSB) medium and incubating at 37°C for 24 hours. The grown bacteria were streaked onto de Man, Rogosa, and Sharpe Agar (MRSA) medium and incubated for 24 hours at 37°C to check for purity. Next, 10 mL of rejuvenated LAB cultures were grown in 100 mL of minimal medium (glucose, K_2HPO_4, KH_2PO_4, NaCl, $CaCl_2$, NH_4Cl, $MgCl_2.6H_2O$, KCl, yeast extract, and $NaHCO_3$) containing 10% de-pulped coffee beans, and incubated for 24 hours at 37°C (David et al., 2018). The parameters measured included bacterial cell count using the total plate count (TPC) method on MRSA medium, pH using a pH meter, and total acid content in the fermentation liquid using the titration method. The LAB isolate showing the highest growth, lowest pH, and highest total acid was used as the starter culture in the coffee fermentation process.

Preparation of growth curve

The inoculum was prepared by inoculating one loop of the selected LAB isolate into 10 mL of MRSB medium and incubating at 37°C for 24 hours until the optical density (OD) reached 0.6-0.8. Subsequently, 1 mL of this culture was added to 100 mL of fresh MRSB medium. Every 3 hours during the 24-hour incubation, OD was measured using a spectrophotometer at 600 nm, and bacterial colonies were counted using the TPC method. The petridishes were then incubated at 37°C for 24 hours.

Coffee fermentation process

Fermentation was carried out in a 500 mL Erlenmeyer flask containing 300 g of de-pulped coffee beans, 300 mL of distilled water, and 10% starter culture at the exponential growth phase (maximum specific growth rate value). Samples were observed at 0, 12, and 24 hours under anaerobic conditions (Pereira et al., 2016), as modified for this study. Spontaneous fermentation was performed by adding 300 mL of distilled water without starter culture. After fermentation, the coffee beans and fermentation liquid were separated. The coffee beans were dried in an oven at 35 ± 1°C for 5 days until the moisture content reached 10-12%. The fermentation liquid was heated to 80°C to halt fermentation activity, then analyzed for fermentation performance.

Fermentation performance analysis

Fermentation parameters, including bacterial growth, pH, total acid, soluble solid content, total sugar content, and reducing sugar content, were analyzed. Bacterial growth was determined using the TPC method on nutrient agar (NA) medium for total bacterial colonies, and MRSA + 1% $CaCO_3$ medium for LAB.

pH was measured using a pH meter, and total acidity was determined with titration. A 10 mL sample of fermentation liquid was treated with 3 drops of 1% phenolphthalein and titrated with 0.1 N NaOH until a stable pink color persisted for 30 seconds (Hadiwiyoto, 1994). Soluble solid content was measured using a SCM-1000 refractometer (HM Digital, Korea). For total sugar content, 0.5 mL of 5% phenol was added to 1 mL of supernatant, followed by 5 mL of concentrated H_2SO_4. After cooling for 20 minutes, the absorbance was measured at 490 nm using a UV-Vis spectrometer (DuBois et al., 1956). Reducing sugar content was measured by adding 1 mL of DNS solution to 1 mL of supernatant, boiling for 15 minutes, cooling, and measuring absorbance at 540 nm (Miller, 1959).

Roasting and grinding of coffee beans

Green coffee beans were roasted to a medium roast to ensure uniform roasting. A total of 150 g of green coffee beans were roasted using a William Edison x Suji roaster at 207-210°C for 7-11 minutes (Wang et al., 2019). After roasting, the beans were cooled for 30 minutes and stored at room temperature. The roasted beans were ground using a FOMAC COG-HS600 grinder to a size of 4.0. The ground coffee was then sieved using a multistage sieve with mesh sizes of 24, 40, 60, and 80 to separate the grounds by particle size.

Analysis of coffee brewing performance

For brewing, 5 g of coffee powder was mixed with 100 mL of hot water at 95°C. The coffee was stirred for 1 minute using a magnetic stirrer, then cooled in an ice water bath while stirring for 2 minutes. Filtration was done using Whatman No. 1 filter paper (Herawati et al., 2019).

The following parameters were measured: pH, total phenolic content, total flavonoid content, antioxidant activity (IC_{50}), and caffeine content. Total phenolic content was determined by mixing 0.1 mL of brewed coffee with 95% ethanol (1.9 mL), 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was vortexed and allowed to stand for 5 minutes. Then, 1 mL of 5% Na_2CO_3 solution was added and the mixture was incubated in the dark for 1 hour. The absorbance was measured at 725 nm using a UV-Vis spectrometer. Standard solutions of gallic acid were prepared to generate the standard curve. The results were expressed as mg GAE g^{-1} of coffee extract (Vignoli et al., 2014).

Total flavonoid content was measured by mixing 250 µL of brewed coffee with 1 mL of distilled water and 75 µL of 5% $NaNO_2$. After 5 minutes, 150 µL of 10% $AlCl_3$ solution was added, followed by incubation for 6 minutes. Then, 500 µL of 1 N NaOH solution was added, and the mixture was incubated for 11 minutes. The absorbance was measured at 510 nm using a UV-Vis spectrometer. Standard solutions of quercetin were prepared to generate the standard curve. Results were expressed as mg QE g^{-1} of coffee extract (Haile & Kang, 2019).

Antioxidant activity (IC_{50}) was analyzed using the DPPH radical scavenging assay. Coffee brew solutions were prepared at concentrations of 2,000-20,000 ppm in methanol. A test sample (0.2 mL) was mixed with 0.5 mL of 100 ppm DPPH solution and 1 mL of methanol, and incubated at room temperature for 15 minutes in the dark. Absorbance was measured at 517 nm, and the percentage of inhibition was calculated. The IC_{50} value, which indicates the concentration that causes 50% scavenging of DPPH radicals, was derived from a linear regression of the inhibition percentage (Priftis et al., 2015).

Caffeine content was measured using high-performance liquid chromatography (HPLC) according to SNI ISO 20481-2008. Caffeine was extracted from the coffee brew, washed, evaporated, dissolved in methanol and water, and injected into the HPLC system. The analysis was done at a wavelength of 272 nm using a mobile phase of distilled water and methanol at a flow rate of 1 mL min^{-1}.

Data analysis

Data for each parameter based on treatment and fermentation time were summarized using Microsoft Excel 2013. Statistical analysis was performed using ANOVA at the 5% significance level, followed by Duncan's multiple range test using IBM SPSS 30.0 software.

Results and Discussion

Selection of LAB isolates

The ability of the three isolates to grow on minimal medium containing 10% de-pulped coffee beans was observed by counting the number of bacterial colonies, pH and total acid (Table 1). The *L. plantarum* H 2.34 isolate demonstrated high growth capacity, reaching 8.02 log CFU mL^{-1} in the medium-containing coffee. Additionally, it produced a lower pH of 3.09 and the highest total acid concentration of 10.81 mg mL^{-1}. The ability of *L. plantarum* to produce acid enables the inhibition of pathogen growth (Arena et al., 2016) and limits the growth of other microorganisms (Pothakos et al., 2020). Thus, *L. plantarum* H 2.34 is a promising candidate as a starter culture for coffee fermentation.

Growth curve of L. plantarum H 2.34 isolate

The growth curve of the *L. plantarum* H 2.34 isolate was determined to identify the appropriate growth phase and optimal harvesting time for cells to be used as a starter culture in the coffee fermentation process. A typical bacterial growth curve consists of four phases: lag phase, log (exponential) phase, stationary phase and death phase (Maier & Pepper, 2015). Based on observations over 24 hours, the OD of *L. plantarum* H 2.34 indicated a lag phase from 0 to 3 hours. The log phase, characterized by rapid cell growth, occurred between 3 and 21 hours.

Table 1. LAB growth count, pH, and total acid on minimal medium + 10% de-pulped coffee beans for 24 hours at 37°C

Isolate	Total microbes (log CFU mL^{-1})	pH	Total acid (mg mL^{-1})
LAB H.0.17	8.08	3.17	9.23
L. plantarum H 2.34	8.02	3.09	10.81
LAB H.3.1	7.75	3.13	9.91

In contrast, based on the TPC, the log phase was observed from 0 to 9 hours. Therefore, to maximize cell viability and activity, the cells were harvested at 6 hours, during the exponential growth phase (Figure 1). The growth pattern observed was consistent with previous findings by Hidayatulloh et al. (2019).

Fermentation performance analysis

Fermentation performance was assessed based on the ability of both treatments to remove coffee mucilage. In both spontaneous fermentation and fermentation using the *L. plantarum* H 2.34 starter culture, the total bacterial count and total LAB count peaked at 12 hours of incubation (Figure 2). The number of LAB in the starter culture treatment was higher than in spontaneous fermentation, consistent with findings by de Carvalho Neto et al. (2018). This result indicates that *L. plantarum* H 2.34 was able to dominate the coffee fermentation process.

The pH of the fermentation liquid decreased over time in both treatments, reflecting increasing acidity (Figure 3A). Total acid content, measured by titration, was higher in the fermentation using the starter culture and showed a continuous increase, from 2.57 mg mL^{-1} at the beginning to 4.91 mg mL^{-1} after 24 hours (Figure 3B). The decrease in pH and the increase in total acid confirm the ability of LAB to degrade coffee mucilage and convert it into organic acids (de Carvalho Neto et al., 2018).

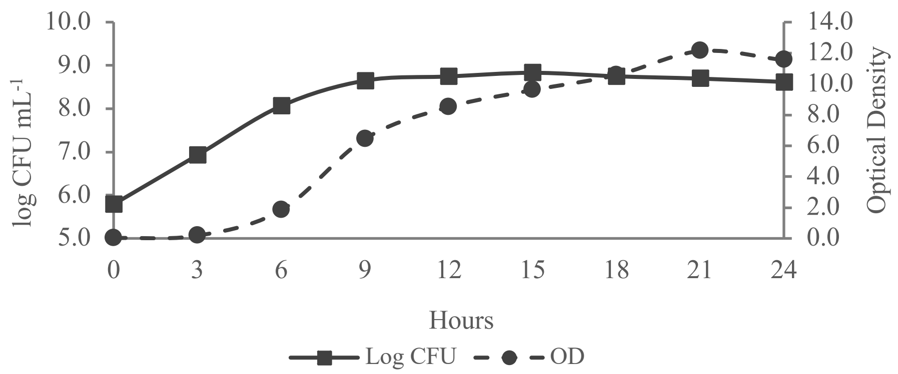

Figure 1. Growth curve of *L. plantarum* H 2.34 on MRSB medium for 24 hours

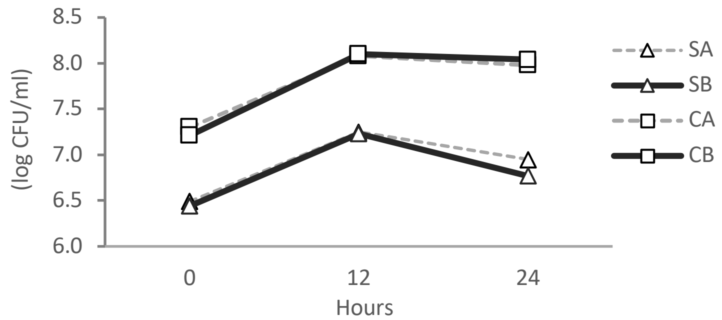

Figure 2. Total bacteria (SA) and total LAB (SB) growth in spontaneous fermentation; total bacteria (CA) and total LAB (CB) in fermentation using LAB starter culture

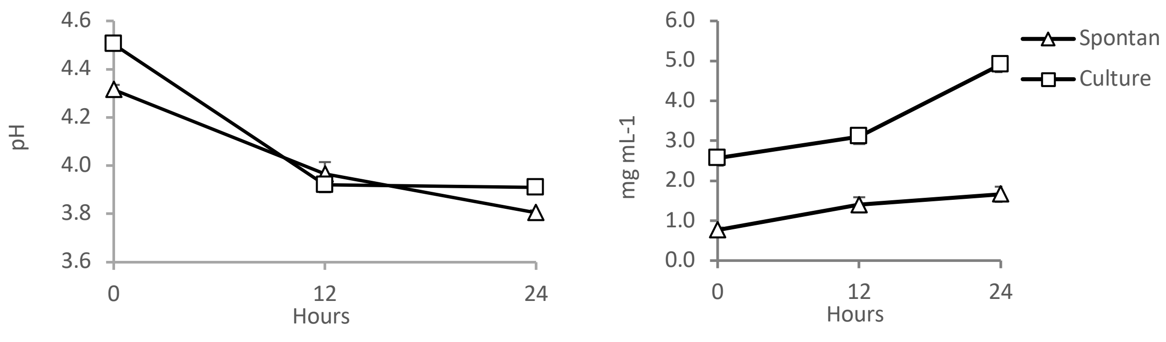

Figure 3. pH (A) and total acid contents (B) in spontaneous and LAB fermentation cultures

Changes in total soluble solids were also observed during fermentation. In spontaneous fermentation, total soluble solids increased by 61.54% by the end of fermentation. In contrast, fermentation using the starter culture showed a 26% decrease in total soluble solids, consistent with the findings of Polanía Rivera et al. (2024) (Figure 4A). These results suggest that starter culture fermentation is more efficient at utilizing available substrates compared to spontaneous fermentation.

The total sugar content increased over the course of fermentation, reaching its highest level at 24 hours. In spontaneous fermentation, the total sugar content increased by 49.16%, while fermentation using the starter culture showed a smaller increase of 28.03% (Figure 4B). The reducing sugar content in spontaneous fermentation decreased by 32.97% from the start to 24 hours. In the starter culture treatment, reducing sugar content initially decreased at 12 hours and then increased again by 24 hours (Figure 4C). This pattern aligns with bacterial growth, which also peaked around 12 hours, suggesting that bacteria were actively utilizing reducing sugars as a substrate at that point.

Coffee fruit mucilage is an insoluble hydrogel rich in sugars that serves as a suitable substrate for microbial growth (Silva et al., 2013). The interaction between extracellular enzymes and organic acids during fermentation leads to the hydrolysis of macromolecules such as carbohydrates, proteins, and polyphenols, producing important aroma precursors, reducing sugars, proteins, amino acids, and phenolic compounds (Lee et al., 2015).

Particle size measurement

This step was carried out to determine the appropriate grinding scale for coffee processing. The sieving results of ground coffee using a grinding scale of 4.0 revealed the following size distribution: 6% passed through a 24-mesh sieve, 70% through a 40-mesh sieve, 14% through a 60-mesh sieve, and 10% through an 80-mesh sieve.

Coffee brewing performance analysis

The pH of the coffee brew resulting from fermentation with a starter culture was higher than that of spontaneous fermentation (Figure 5). ANOVA results indicated that fermentation treatment (spontaneous or starter culture) significantly affected the pH of the brewed coffee ($p<0.05$). The pH across both treatments and fermentation durations ranged from 5.15 to 5.26. This variation in pH is attributed to microbial activity, which produces organic acids such as malic, acetic, chlorogenic, and quinic acids (Fauzi et al., 2017).

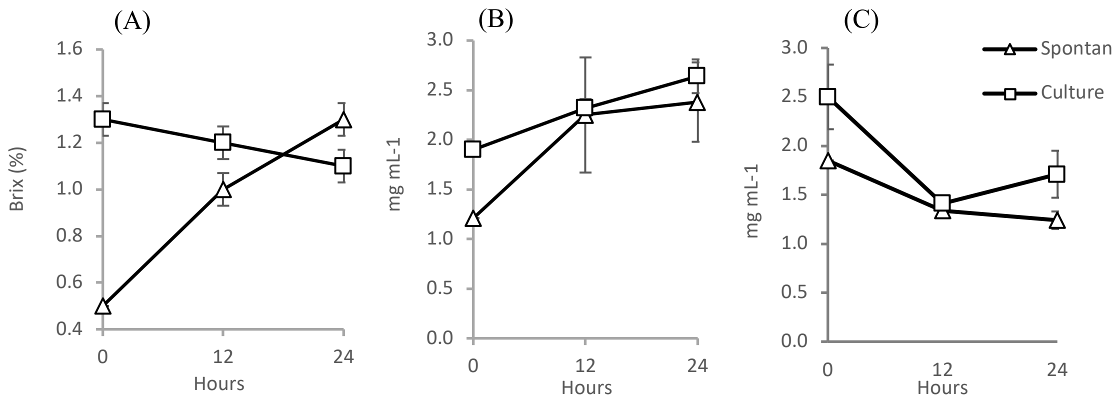

Figure 4. Total soluble solids (A), sugar content (B), and reducing sugar content (C) in spontaneous and LAB fermentation cultures.

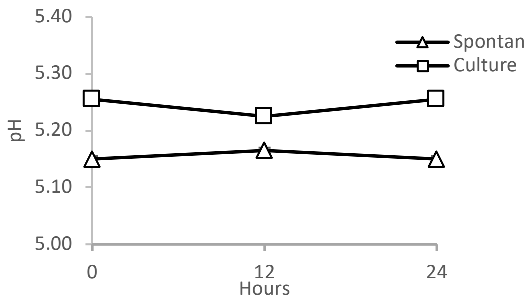

Figure 5. The pH of brewed coffee fermented in spontaneous and LAB cultures

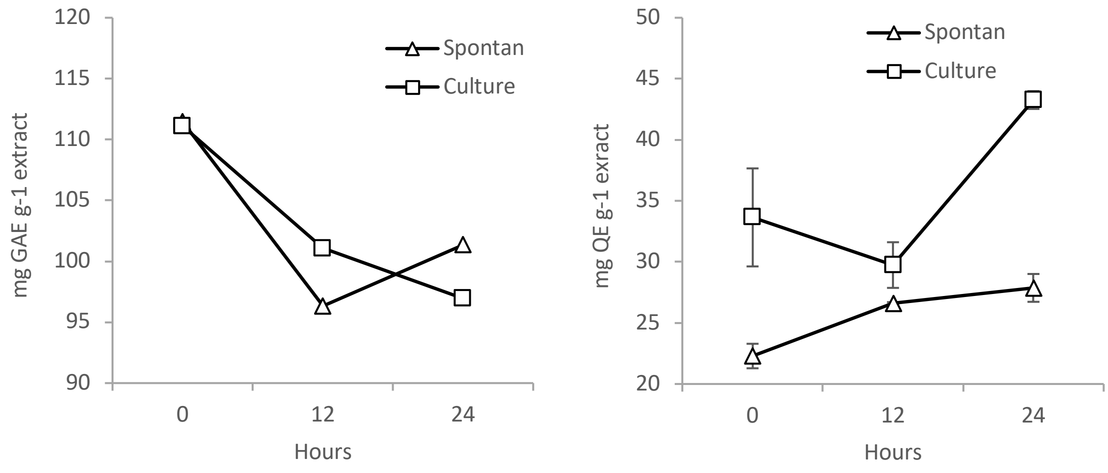

Figure 6. Analysis of coffee brewing. (A) Total phenolic (A) and flavonoid (B) contents of brewed coffee fermented in spontaneous or LAB cultures

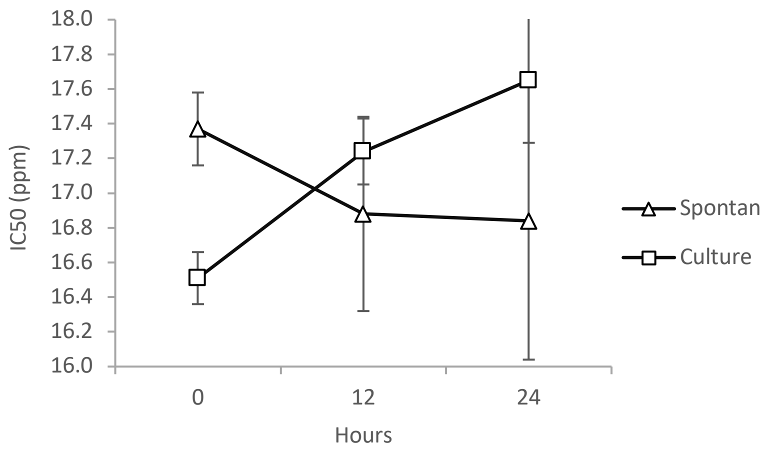

Figure 7. Antioxidant activity (IC_{50}) of coffee fermented in spontaneous or LAB cultures

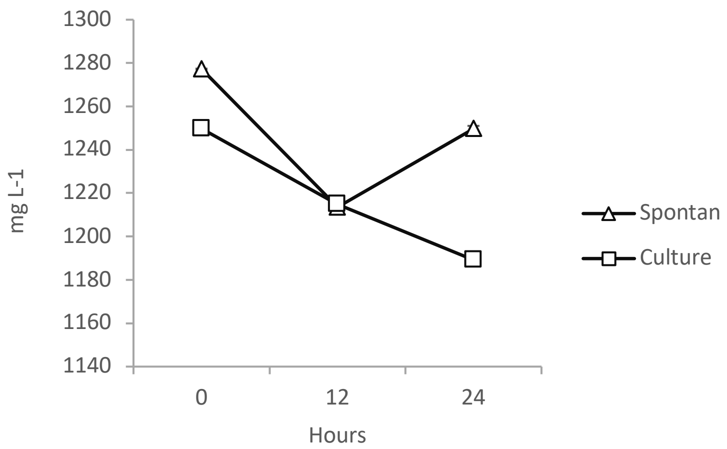

Figure 8. Caffeine content of coffee fermented in spontaneous or LAB cultures

ANOVA results also showed that both fermentation time and treatment significantly affected the total phenolic content ($p < 0.05$). A Duncan test revealed a decline in total phenolic content with increasing fermentation time for both spontaneous and starter culture fermentations (Figure 6A). This trend aligns with previous findings by Zofia et al. (2020), who also reported a reduction in phenolics during coffee extract fermentation. The decline may be due to the volatilization of phenolic compounds (Lee et al., 2017). Phenolic compounds are known for their antioxidant and anti-inflammatory properties (Farah et al., 2008; Alonso-Salces et al., 2009).

Similarly, ANOVA analysis of total flavonoid content showed a significant effect of fermentation time and treatment ($p<0.05$). According to Duncan's test, flavonoid levels increased during fermentation in both treatments. The highest flavonoid content was observed in the starter culture fermentation after 24 hours, reaching 46.91 ± 6.35 mg QE g^{-1} extract (Figure 6B). This result is consistent with the findings of Kwak et al. (2018), who reported enhanced flavonoid content in coffee fermented with a starter culture. The increase is attributed to enzymatic activity by *L. plantarum*, which degrades complex polyphenols into simpler molecules, thereby increasing flavonoid levels (Arwangga et al., 2016).The IC_{50} value of the coffee brew decreased over time in spontaneous fermentation, indicating increased antioxidant activity. In contrast, fermentation with the LAB starter culture showed a reduction in antioxidant activity with extended fermentation time (Figure 7). A lower IC_{50} value reflects stronger antioxidant activity against DPPH free radicals. Although ANOVA analysis found no significant differences in antioxidant activity between the two treatments, all samples exhibited very strong antioxidant potential, with IC_{50} values below 50 ppm (Winahyu et al., 2019). Antioxidants play a crucial role in neutralizing toxic free radicals produced during cellular metabolism. While the human body synthesizes endogenous antioxidants, it still relies on dietary sources (Fiedor & Burda, 2014). Coffee is a rich source of antioxidants, mainly contributed by phenolic compounds, alkaloids, caffeine, and diterpenes such as cafestol and kahweol (Sroka et al., 2015).

Caffeine content was analyzed to assess its reduction during fermentation. ANOVA revealed that both fermentation time and treatment significantly affected caffeine levels ($p < 0.05$). The lowest caffeine content was found in the coffee fermented with the starter culture for 24 hours (Figure 8). This finding is consistent with the study by Tawali et al. (2018), which also reported decreased caffeine levels over time. The reduction is likely due to protein breakdown and a corresponding increase in free amino acids (Marcone & Alrifai, 2019). While caffeine has recognized benefits such as enhanced psychomotor performance and stimulation of the nervous system and gastric secretions (Kartasasmita & Addyantina, 2012), excessive intake may lead to adverse effects like anxiety, insomnia, nausea, hypertension, and seizures (Dong et al., 2015). According to SNI 01-3542-2004, acceptable caffeine levels in post-roasted coffee range from 0.9% to 2%. The caffeine content in all treatments remained within this standard.

Conclusion

The *L. plantarum* H 2.34 isolate is a promising candidate for use as a starter culture in coffee fermentation due to its ability to grow and produce a high total acid content in media containing at least 10% coffee beans. Fermentation using the LAB starter culture demonstrated increased LAB growth within the first 12 hours, accompanied by a reduction in pH, an increase in total acid, and a decrease in both total soluble solids and total reducing sugars compared to spontaneous fermentation. These results indicate the effectiveness of the LAB starter culture in enhancing the degradation of coffee mucilage. Based on the coffee brew performance analysis, phenolic content decreased with prolonged fermentation time, while flavonoid content increased in coffee fermented with the starter culture. Although differences in antioxidant activity between spontaneous and starter culture fermentation were not statistically significant, all brewed coffee samples exhibited very strong antioxidant activity. Additionally, the LAB starter culture was able to reduce caffeine levels in the coffee brew by up to 4.85%.

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