

Effect of *Lactobacillus plantarum* Fermentation on Caffeine and Chlorogenic Acid in Robusta Coffee (*Coffea canephora*)

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Abstract: Robusta coffee (*Coffea canephora*) contains bioactive compounds such as caffeine and chlorogenic acid that contribute to antioxidant and metabolic health benefits. Microbial fermentation has emerged as a strategy to modulate phytochemical profiles and enhance functional properties in food products. This study investigated the effect of *Lactobacillus plantarum* fermentation (0, 6, and 24 h) on caffeine content, secondary metabolite composition, total phenolics, and antioxidant activity of Robusta coffee. LC-MS/MS and spectrophotometric assays revealed dynamic shifts in phytochemicals during fermentation. Fermentation at 6 hours resulted in the greatest reduction in caffeine and enhanced antioxidant capacity, suggesting increased bioactive potential, whereas prolonged fermentation partially restored initial levels of caffeine and chlorogenic acid. Overall, controlled short-term fermentation with *L. plantarum* demonstrated a promising approach for modulating coffee bioactives and improving antioxidant functionality. These findings provide new insights into microbial biotransformation pathways in fermented coffee and support the development of functional coffee-based beverages.

Keywords: antioxidant activity; *Lactobacillus plantarum*; LC-MS/MS; Robusta coffee; fermentation.

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1. Introduction

Robusta coffee (*Coffea canephora*) is a high-value agricultural commodity known for its characteristic flavor and aroma. Its economic importance is closely linked to bean quality [1–3]. Originating from the lowland tropical rainforests of the Congo River basin extending to Lake Victoria, Uganda, robusta coffee thrives in regions with average temperatures of 23–26°C and annual rainfall of approximately 2,000 mm over 9–10 months. It grows optimally at altitudes of 0–800 m above sea level and adapts well to similar climatic conditions in other regions, particularly where annual temperatures range from 22–26°C [4–6]. In Indonesia, three major coffee species are cultivated: Arabica, Robusta, and Liberica. Coffee beans contain diverse chemical constituents that influence their sensory and functional properties. These compounds are classified into volatile and non-volatile groups. Volatile compounds, such as aldehydes, ketones, hydrocarbons, alcohols, carboxylic acids, esters, pyrazines, pyrroles, pyridines, sulfur compounds, furans, phenols, and osazones, contribute significantly to coffee

aroma. Meanwhile, non-volatile components include caffeine, chlorogenic acids, and nutritional constituents such as carbohydrates, proteins, lipids, and minerals [7–9]. Robusta coffee typically contains higher caffeine levels than other species. Because excessive caffeine intake may pose health risks, appropriate processing is needed to reduce caffeine content while maintaining product quality.

Daily consumption of decaffeinated coffee rich in chlorogenic acids has been shown to reduce the risk of type 2 diabetes and lower blood pressure in hypertensive individuals [10]. Additionally, bioactive compounds in coffee, including caffeine, chlorogenic acids, trigonelline, and minerals, may offer various health benefits [11]. Acidity is a key determinant of coffee quality, contributing to its distinctive flavor profile. Chlorogenic acids are the predominant acidic components of coffee and are phenolic compounds. They are water-soluble esters formed from quinic acid and trans-cinnamic acids such as caffeic, ferulic, and p-coumaric acids [12–14]. Coffee beans contain approximately 3.6–6.0% chlorogenic acids (dry basis), with robusta beans generally exhibiting higher levels than arabica [15–17]. Beyond contributing to flavor, chlorogenic acids play a protective role in coffee plants against microbial attack, insect damage, and UV radiation [18–20].

Fermentation plays a crucial role in enhancing coffee quality by promoting biochemical reactions that generate key flavor precursors, including organic acids, amino acids, and reducing sugars. Wet processing is widely recognized for producing superior flavor profiles compared to dry processing, as enzymatic activity during fermentation converts sugars within the mucilage layer located between the exocarp and endocarp into alcohols through the action of microorganisms and atmospheric oxygen [21–25]. The progression and outcome of coffee fermentation largely depend on the composition and dynamics of the microbial community involved [26–28].

Among beneficial microbes, *Lactobacillus plantarum* has been shown to significantly enhance phenolic content during wet fermentation [25,27]. This bacterium can decarboxylate protocatechuic acid in green coffee to catechol, a compound with antioxidant properties [25,26,29], and its activity has been linked to increased antioxidant capacity in robusta coffee, which naturally contains higher levels of chlorogenic acids, 4,5-dicaffeoylquinic acid, and caffeine than arabica [30]. However, prolonged fermentation may lead to excessive breakdown of antioxidant molecules. *L. plantarum* also contributes to quality improvement by reducing furfuryl alcohol levels [31,32].

In addition to biochemical transformations, *L. plantarum* influences the aroma profile of coffee. It produces dominant aroma compounds compared to *Leuconostoc mesenteroides*, including grape-like (ethyl propionate) and pineapple-like (ethyl hexanoate) esters via esterification reactions between alcohols and free fatty acids [33]. Other metabolites include honey and floral notes (phenylacetaldehyde) derived from phenylalanine, and apple-like aromas (1-hexanal) formed from 3-keto-hexanoyl-CoA conversion [34,35]. *L. plantarum* is associated with chocolate and nutty notes, whereas *L. mesenteroides* tends to produce darker chocolate and caramel characteristics [10,36]. Importantly, *L. plantarum* exhibits demethylase activity capable of reducing caffeine during fermentation, while protease production in wet fermentation further supports caffeine degradation [37]. Other proteolytic microbes, such as *Pseudomonas putida*, can decrease caffeine content by up to 80% through the N-demethylation pathway [38]. Given that robusta beans contain higher caffeine levels (1.5–2.6%) than arabica beans (0.9–1.4%) [39,40], microbial fermentation offers a promising approach to reduce

caffeine content. Additionally, *L. plantarum* effectively removes mucilage by metabolizing sugars in the mucilage layer, thereby accelerating depulping efficiency [41,42].

Despite extensive studies on flavor development during coffee fermentation, limited research has focused on the targeted biotransformation of caffeine and chlorogenic acid by *L. plantarum* in robusta coffee, particularly on fermentation-time optimization. Understanding these biochemical changes is crucial to improving coffee quality while reducing caffeine content to better align with consumer health preferences. Therefore, this study aims to evaluate the effect of *L. plantarum* fermentation on caffeine concentration, chlorogenic acid levels, total phenolic content, and antioxidant activity in robusta coffee. We hypothesize that controlled *L. plantarum* fermentation will decrease caffeine while maintaining or enhancing phenolic-based antioxidant activity and chlorogenic acid content. Fermentation durations were selected based on preliminary reports indicating significant biochemical shifts in short- to mid-term lactic fermentations.

2. Materials and Methods

2.1. Materials.

The materials used were a lactic acid bacteria isolate, *Lactobacillus plantarum* InaCC-B1028, obtained from the Indonesian Culture Collection (InaCC) at BRIN, with accession/reference number B1028. Stock cultures were maintained at -80°C in MRS broth supplemented with 20% glycerol. The other materials are DeMan Rogosa Sharpe Broth (MRS Broth) growth media, distilled water, CaCO_3 , chloroform, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, and robusta coffee beans from the Patani Bogor coffee plantation.

2.2. Fermentation of robusta coffee with *L. plantarum*.

The robusta coffee beans to be fermented were first sorted, peeled, and washed. Then, 200 grams of coffee beans were weighed for each test sample, placed in a measuring cup, and sealed. All fermentations were performed in triplicate under aseptic conditions. Fermentation was carried out using wet coffee beans to which *L. plantarum* was added with a concentration of 1.89×10^9 CFU/g. The pH of the fermentation medium was monitored at 0, 24, 48, and 72 h using a calibrated digital pH meter. Each treatment consisted of 500 grams of coffee soaked in water containing LAB. Sterile, uninoculated coffee samples were used as negative fermentation controls. After that, all the beakers were closed and incubated for 0, 6, and 24 hours. The control incubation temperature was set at room temperature (25°C). The treatment temperature for the addition of *L. plantarum* culture was set at 37°C . The fermented coffee beans were air-dried at room temperature and ground into powder using a coffee grinder. The fermentation process followed the underwater tank method described by da Silva *et al.* [12].

2.3. Analysis of caffeine content in fermented Robusta coffee beans.

Two grams of coffee bean samples were weighed, and 150 mL of boiling water was added. The solution was stirred until completely dissolved, then filtered through Whatman No. 41 filter paper. The filtrate was transferred to a separating funnel, and 1.5 grams of CaCO_3 and 25 mL of chloroform were added. The mixture was shaken until no gas was formed. The two phases were then separated using a separating funnel, with the chloroform phase collected at the bottom. The separated chloroform phase was concentrated using a rotary evaporator, and

then distilled water was added to 100 mL. The solution was diluted 10-fold with distilled water, and the absorbance was measured at 275 nm using a spectrophotometer [33]. Caffeine quantification was performed using a caffeine analytical standard ($\geq 99\%$ purity; Sigma-Aldrich). A calibration curve (0–200 $\mu\text{g/mL}$) was constructed with five concentration points, and linearity ($R^2 \geq 0.99$) was verified before sample analysis.

2.4. LC-MS/MS analysis of fermented *Robusta* coffee.

Coffee bean extraction was carried out using a solvent-soaking method without stirring. The maceration time was 3 x 24 hours. The macerated material was separated from the simple dregs using Whatman filter paper, then evaporated in a rotary evaporator at 45–50 °C and 65–90 rpm to obtain a concentrated extract. LC-MS/MS analysis was conducted using an ESI system in negative ionization mode. Separation was achieved using a C18 reverse-phase column (2.1 × 100 mm, 1.7 μm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile) with a gradient elution program. Flow rate was 0.3 mL/min, injection volume 5 μL , and column temperature 40°C. Metabolite identification refers to the method described by Lemos *et al.* [37]. The compound discovery software processes LC-MS/MS data in RAW format. The workflow was selected in the processing section for untargeted metabolomics with unknown detected statistics using an ID from a local database. Then, add the RAW file to the workflow, and options appear, including selecting the spectrum, aligning retention times, detecting unknown compounds, grouping unknown compounds, predicting composition, searching the mass list, filling gaps, normalizing areas, and marking the compound background. The unknown compound section detected uses a tolerance of m/z 5 ppm and a minimum peak intensity of 2,000,000. In the search mass list, a manual database is used to identify compounds in the extract. Masslynx software version 4.1 was used for data acquisition and analysis as well as instrument control [36,37,39].

2.5. Analysis of antioxidant activity of fermented *Robusta* coffee.

Coffee bean extract was analyzed using the DPPH method. To prepare the extract solution, an 80% methanol solution was added to the coffee ethanol extract. The test mixture consisted of 0.4 mM DPPH solution, 1 M Tris-HCl buffer at pH 7.4, and 20% methanol in the same ratio. The mixture was homogenized and incubated in the dark for 20 minutes. Absorbance was measured using a visible light spectrophotometer at 517 nm [33]. DPPH absorbance was measured by mixing 2 ml of 40 ppm DPPH solution with 2 ml of 96% ethanol, vortexing, incubating for 30 minutes, placing in a cuvette, and measuring absorbance at 517 nm using a UV-Vis spectrophotometer. Free radical scavenging activity was measured by mixing 2 ml of 40 ppm DPPH solution with 2 ml of the test solution at concentrations of 20, 40, 60, 80, and 100 ppm, vortexing, and leaving for 30 minutes. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. DPPH radical scavenging activity was expressed relative to ascorbic acid standard curves. Blank controls were measured for background correction. All assays were performed in triplicate. The absorbance of each concentration was recorded and used to calculate the attenuation percentage.

$$\% \text{ Yield} = \frac{\text{DPPH absorbance} - \text{test sample absorbance}}{\text{DPPH absorbance}} \times 100 \% \dots\dots\dots(1)$$

From the percentage attenuation values at each concentration, a regression curve is fit, yielding the equation $y = bx + a$. The IC_{50} value will be obtained by linear regression, with the sample concentration (ppm) on the abscissa (x-axis) and the percentage of attenuation on the ordinate (y-axis). The IC_{50} value is determined by calculating the Y attenuation percentage at 50%.

2.6. Total phenolic content of fermented Robusta coffee.

The analysis of total phenol content was carried out using a UV-Vis spectrophotometer. Total phenol testing was carried out according to the modified procedure of Jeszka-Skowron *et al.* [34]. Preparation of test solution (sample) by weighing 0.1 g of dry extract sample and dissolving it in 10 mL of Aquadest. 0.5 ml of the solution was taken and put into a dark test tube. The solution was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent in aquadest. The mixture of extract and Folin reagent was homogenized and incubated for 5 minutes in the dark at room temperature. 2 ml of 7.5% (w/v) sodium carbonate solution in Aquadest was added. The solution was homogenized and incubated for 30 minutes in the dark at room temperature. The blank used was 0.5 ml of distilled water plus 2.5 ml of 10% folin reagent and 2 ml of 7.5% sodium carbonate solution. The maximum absorbance at 765 nm was measured using a UV-Vis spectrophotometer. The amount of phenolic compounds was measured using a gallic acid standard curve and expressed as mg gallic acid equivalent (GAE)/g extract. Blank controls were measured for background correction. All assays were performed in triplicate. The levels of phenolic compounds in the extract were calculated using the following equation:

$$C = \frac{c \times fp \times V}{m} \dots\dots\dots(2)$$

Where C = TPC concentration (mg GAE/g extract); fp = dilution factor; c = gallic acid concentration (μ g GAE/mL); V = Volume of extract solution taken for testing (ml); m = weight of extract used for testing (g)

2.7. Data analysis.

All research data were analyzed using IBM SPSS Statistics version 29. A one-way analysis of variance (ANOVA) was conducted at $\alpha = 0.05$ to determine differences among treatments. Each analysis was performed with 3 experimental replicates, and results are presented as mean \pm standard deviation (SD). When the ANOVA indicated a significant difference ($p < 0.05$), post-hoc comparisons were conducted using Duncan's multiple range test.

3. Results and Discussion

3.1. Caffeine content in Robusta coffee beans.

Figure 1 shows that the lowest caffeine content is found in *Lactobacillus plantarum* fermentation for 6 hours in robusta coffee (*Coffea canephora*). This result is supported by ANOVA, which shows a significant difference ($p < 0.05$) with the addition of *L. plantarum*. The exponential phase in fermentation occurs over 6 to 24 hours, as part of the growth curve of microorganism bacteria. Bacterial cells divide rapidly and regularly, reaching a maximum rate. The results of the study, shown in Figure 1, show that coffee without *L. plantarum* treatment

contains 0.12% caffeine. In comparison, a 6-hour treatment with *L. plantarum* yields a caffeine content of 0.08%, and a 24-hour treatment yields 0.13%. Caffeine levels decreased at 6 hours and increased again at 24 hours.

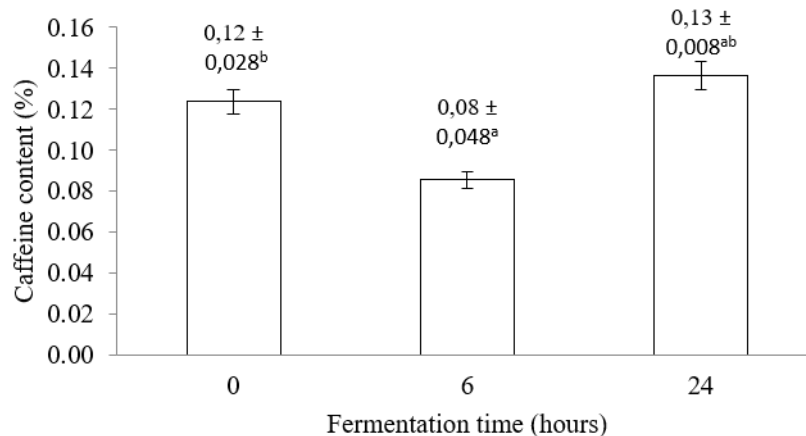


Figure 1. The caffeine content in *Lactobacillus plantarum* fermented robusta coffee varies over time. Note that different lowercase letters indicate statistically significant differences ($\alpha=0.05$).

3.2. Profiling of chlorogenic acid compounds in Robusta coffee with LC-MS/MS.

LC-MS/MS analysis identified chlorogenic acid in robusta coffee beans, providing initial information on the secondary metabolite composition of robusta coffee extracts. Table 1 presents the scanning results of four Cladonia extracts, including the abundance (%) of precursor ion mass (positive mode, m/z), fragment ions produced, and the resulting formulas. Robusta coffee extracts contained two groups of depsides, chlorogenic acid with an abundance (%) of 4.70 for unfermented robusta coffee, 3.37 for robusta coffee fermented with *L. plantarum* for 6 hours, and 4.73 for robusta coffee fermented with *L. plantarum* for 24 hours (Figure 2).

Table 1. LC/MS analysis results for robusta coffee fermentation with *L. plantarum*

m/z (PubChem)	Formula	Compound	Yield (%)		
			0 hour	6 hour	24 hour
147.0531	C5H9NO4	Glutamic acid	8.26	7.41	8.22
343.1240	C12H22O11	Sucrose	3.90	2.87	3.87
355.1029	C16H18O9	Chlorogenic acid	4.73	3.87	4.70
181.0712	C6H12O6	Glucose	4.47	3.57	4.42
999.8381	C66H110O6	Triglycerides	14.77	17.14	14.97

The target compound is chlorogenic acid with an ion mass (m/z) of 355.1029. For coffee without *L. plantarum* bacteria, the abundance (%) is 4.73. For coffee samples fermented for 6 hours with *L. plantarum* bacteria, the abundance (%) is 3.87, and for coffee samples fermented for 24 hours with *L. plantarum*, the abundance (%) is 4.70. Other compounds identified in robusta coffee include several compounds without *L. plantarum* bacteria 36. For coffee samples processed for 6 hours with *L. plantarum* bacteria, 26 were identified, and for coffee samples fermented for 24 hours with *L. plantarum*, 28 compounds were identified. The LC-MS/MS analysis showed that the fermentation process with the addition of *L. plantarum* decreased chlorogenic acid content.

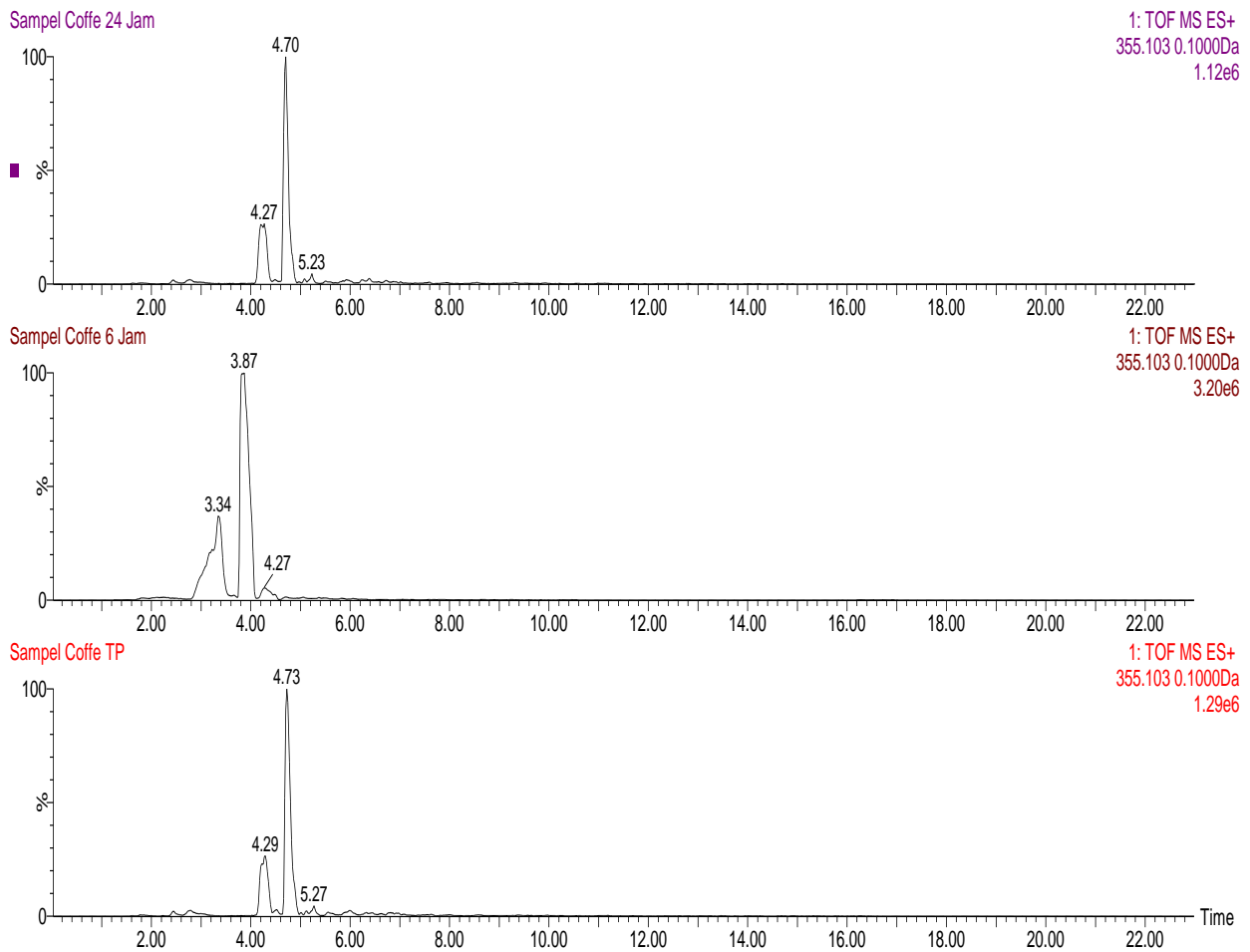


Figure 2. Compounds identified by LC-MS/MS in fermented robusta coffee extract from *L. plantarum*.

3.3. Antioxidant activity in fermented Robusta coffee with *L. plantarum*.

The antioxidant activity of fermented coffee decreases over time, reaching a minimum at 24 hours of fermentation (Figure 3). Based on the three robusta coffee samples, the IC₅₀ obtained in coffee that was not given fermentation treatment using *Lactobacillus plantarum* was 51.435 ppm, robusta coffee that was given fermentation treatment using *L. plantarum* for 6 hours produced an IC₅₀ of 49.737 ppm, and robusta coffee that was given fermentation treatment using *L. plantarum* for 24 hours had an IC₅₀ of 50.808 ppm. The antioxidant activity of the filtrate from the water extract of robusta coffee beans was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The DPPH antioxidant test is one of the methods used to quantify the antioxidant activity of robusta coffee. The DPPH method was chosen because it is simple, fast, and sensitive, and requires only a very small sample to evaluate the antioxidant activity of natural substances [33].

A lower IC₅₀ value indicates greater antioxidant activity. The maximum DPPH wavelength was determined using a 40 ppm DPPH solution, with absorbance measured over 400-800 nm. The maximum absorbance was observed at 518 nm. This wavelength provides the highest absorption and greatest sensitivity of the test solution [34]. The IC₅₀ value of the robusta coffee bean filtrate was obtained from the linear regression curve in Figure 3. Robusta coffee filtrate, untreated, is classified as having strong antioxidant activity. In contrast, robusta coffee fermented with *Lactobacillus plantarum* for 6 hours and 24 hours exhibits very strong

antioxidant activity. The low antioxidant activity of robusta coffee beans is thought to be due to variations in the number of *Lactobacillus plantarum* bacteria during fermentation.

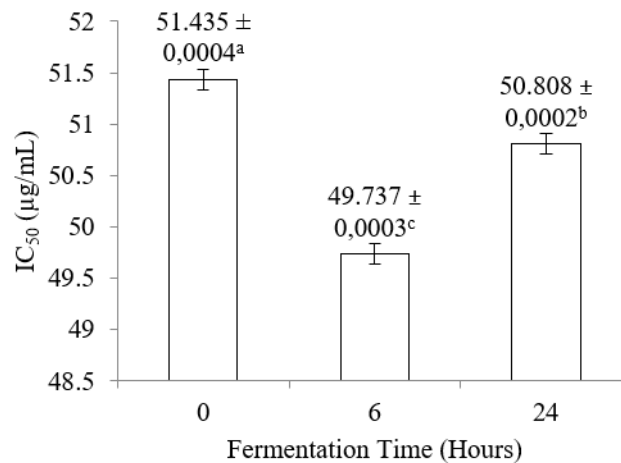


Figure 3. Analysis of the antioxidant activity of fermented coffee with different fermentation time variations.

3.4. Total phenolic content of fermented Robusta coffee.

The robusta coffee samples were conducted using three robusta coffee samples with different fermentation times. The robusta coffee results shown in Figure 4 were obtained from coffee that did not receive *L. plantarum* during fermentation, at 99.95 mg GAE/mL. Robusta coffee treated with *L. plantarum* for 6 hours contained a total of 89.65 mgGAE/mL of phenols, while coffee treated for 24 hours contained 93.44 mgGAE/mL.

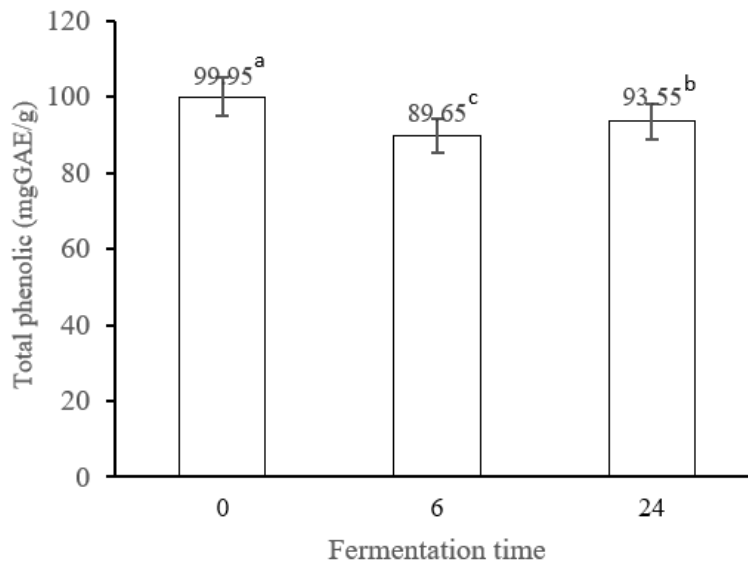


Figure 4. Total phenolics in fermented coffee with *Lactobacillus plantarum*.

The results showed that the longer the fermentation time, the greater the increase in the total phenol content of robusta coffee. Total phenols increased in robusta coffee without treatment and decreased during *L. plantarum* fermentation increased for 6 hours and then again during the 24 hours of fermentation. The increase and decrease in total phenols in robusta coffee are thought to be due to the formation of phenolic compounds due to enzymatic activity by *L. plantarum* microbes in degrading other matrix components [32]. Phenolic compounds can be formed from the degradation of the matrix of other material components due to the enzymatic activity of bacteria, and chlorogenic acid is a phenolic compound from the phenolic acid group [34,39].

3.5. Discussion.

3.5.1. Caffeine content in Robusta coffee beans.

The mechanism of action of caffeine in the body is inversely proportional to the function of adenosine. Caffeine does not inhibit the movement of body cells [33, 34]. However, caffeine inhibits adenosine activity, thereby relieving drowsiness and inducing feelings of freshness and joy [39]. It also speeds up the heart, increases blood pressure, enhances muscle contractions, and increases sugar output [37]. Coffee with lower caffeine content is produced by the breakdown of proteins, which increases the levels of free amino acids. Cellulolytic and xylanolytic bacteria degrade cellulose and hemicellulose in coffee, so organic acids are formed due to the activity of bacteria breaking down sugar. The breakdown of sugar produces lactic acid and other acids such as ethanol, butyric acid, and propionate [32-33]. Coffee beans have an outer layer of mucus consisting of 80% pectin and 20% sugar. This layer functions as a substrate for the inoculum. The inoculum reduces the substrate, allowing water to quickly enter the coffee beans through the horn-like skin, which has pores. Water that penetrates the coffee beans dissolves the caffeine due to its water-solubility [39].

The solubility of caffeine in water is due to its ability to bind to one water molecule. In addition, the decrease in caffeine content is caused by the esterification process, which breaks down the complex caffeine compound into chlorogenic acid. The caffeine compound becomes free, with reduced size and molecular weight, making it easier to move and diffuse through cell walls and to dissolve in water [33,39]. During fermentation, glucose, a substrate for *L. plantarum*, is broken down into organic acids, thereby degrading the mucilage layer on coffee beans [21,22]. Degradation of the mucilage layer makes it easier for water to penetrate the coffee beans through the horny skin layer. Water that penetrates the coffee's pores dissolves the caffeine [23]. This relates to caffeine's solubility in water and its ability to bind to water molecules. The amount of dissolved caffeine decreases caffeine levels [30]. Research by Jeszka-Skowron *et al.* [33] reported that during fermentation, microorganisms degrade caffeine as a carbon source via caffeine demethylation. Caffeine demethylation breaks the bonds of complex caffeine compounds into smaller, free caffeine compounds, making them easier to move [34]. Caffeine compounds are broken down into dimethyl xanthine, such as theobromine, paraxanthine, and theophylline [33]. The decrease in caffeine levels is influenced by fermentation time and the activity of proteolytic bacteria that produce protease enzymes [33,39].

Cruz-O'Byrne *et al.* [11] reported that coffee beans on the outside have a mucus layer consisting of 80% pectin and 20% sugar. This layer is a substrate for the inoculum. Then the substrate will be reduced by the inoculum, allowing water to easily enter the beans through the horny skin via pores [14,16]. Water that seeps into coffee beans dissolves the caffeine. This is due to caffeine's water solubility. Lopes *et al.* [39] reported that the solubility of caffeine in water is due to the binding of one water molecule. In addition, the decrease in caffeine content is due to esterification, which breaks down the complex caffeine compound into chlorogenic acid. The caffeine compound is small in size and low in molecular weight, making it easy to move and diffuse through cell walls and to dissolve in water. Coffee contains more caffeine than tea leaves and yerba mate leaves, around 1.6% - 2.5%, so the effect of this xanthine alkaloid compound is more pronounced when consuming coffee than other foods [32,37]. The activity of lactic acid bacteria decreases caffeine levels [33,34]. The decrease in caffeine levels

is also influenced by fermentation time [39]. The presence of proteolytic bacterial activity, which produces relatively high protease levels, reduces caffeine levels in coffee beans as fermentation lengthens [33]. The activity of the lactic acid bacterium *L. plantarum* also reduces caffeine levels. The activity of proteolytic bacteria producing protease enzymes is high, leading to decreased caffeine levels in coffee beans as fermentation lengthens [18,19].

The reduction of caffeine content during fermentation with *L. plantarum* is closely linked to the organism's metabolic capacity to utilize alkaloids as nitrogen and carbon sources [21]. *L. plantarum* expresses enzymes associated with demethylation and nitrogen assimilation pathways, enabling partial degradation of caffeine molecules. During fermentation, caffeine can be transformed into lower-molecular-weight derivatives via microbial demethylation, in which methyl groups are removed and incorporated into cellular metabolism [22]. This bioconversion process reduces the caffeine concentration while generating metabolites that may be further catabolized or incorporated into microbial biomass. The acidic fermentation environment resulting from lactic acid accumulation may also contribute to caffeine solubility and subsequent enzymatic access, thereby enhancing breakdown efficiency over time [23].

During fermentation, the loss of the mucilage layer makes it easier for proteolytic enzymes from *L. plantarum* to enter the cytoplasm and break down caffeine in coffee beans [21]. Coffee with low caffeine levels is produced from proteins that have been broken down so that free amino acids increase. Cellulolytic and xylanolytic bacteria degrade cellulose and hemicellulose in coffee so that organic acids are formed due to the activity of these bacteria in breaking down sugar [22]. Caffeine, a non-volatile component in green coffee beans, is a nitrogen-containing metabolite widely known to have an arousal effect. Although debate continues over whether consuming caffeine is beneficial or detrimental to human health, several studies have suggested that caffeine has positive effects, such as increased alertness, improved mood and focus, and the ability to stay awake [23]. In addition, it can improve memory consolidation. Caffeine is thought to affect the bitterness, body, and strength of coffee drinks [33]. Elhali *et al.* [25] reported that the highest caffeine levels were obtained after coffee was fermented with *Pediococcus pentosaceus* isolates, followed by *L. plantarum*, *Saccharomyces cerevisiae*, *Leuconostoc mesenteroides*, and the lowest in *Candida parapsilosis*. These results indicate that the caffeine content of green coffee beans can be controlled differently during fermentation using different starter microbes [14,23,24].

Coffee with low caffeine, in addition to producing a delicious taste and aroma, is also better because it stimulates the nervous system, improves mood, and eliminates fatigue [6,15]. Lactic acid bacteria (LAB) can be used to reduce caffeine levels in coffee through fermentation. Several LAB species, such as *Lactobacillus* and *Pediococcus*, can modify components of coffee, including caffeine, via various enzymatic mechanisms [8]. Lactic acid bacteria can reduce caffeine levels through several main mechanisms, including enzymatic biodegradation, adsorption to cell walls, co-metabolism, changes in the composition of fermentation media, production of organic acids, and interactions with other microbes [26,27].

One mechanism that plays an important role in the biodegradation of caffeine is the demethylase enzyme, which is carried out by lactic acid bacteria, which remove methyl groups (-CH₃) from caffeine molecules, producing metabolites such as paroxetine, theobromine, and theophylline [32]. Some microbes, including lactic acid bacteria, can produce this enzyme under certain fermentation conditions, as demonstrated in tea fermentation using *Lactobacillus* strains [33]. In addition to enzymatic degradation, adsorption of caffeine to the bacterial cell

wall also contributes to reducing caffeine levels, where caffeine interacts with polysaccharides, proteins, and lipids in the cell wall of lactic acid bacteria [39].

Lactobacillus plantarum is known to have the capacity to adsorb various phenolic compounds and alkaloids, including caffeine, the effectiveness of which depends on pH, temperature, and composition of the fermentation medium [26]. In addition, under certain conditions, lactic acid bacteria can co-metabolize carbohydrates, using caffeine as a secondary carbon source [30]. *Lactobacillus fermentum* can convert caffeine into other compounds via co-metabolism under long-term fermentation or limited nutritional conditions [31]. Furthermore, fermentation by lactic acid bacteria can alter the chemical composition of the fermentation medium, thereby decreasing caffeine levels [26,33]. In coffee fermentation, lactic acid bacteria can interact with other microbes, such as *Saccharomyces cerevisiae* and *Acetobacter* sp., which accelerate caffeine degradation through a combination of enzymatic activity and adsorption [19,20]. Natural fermentation in coffee beans can reduce caffeine levels by around 15-20% through a combination of enzymatic activity and adsorption [21].

The role of organic acid production in fermentation is also very important in accelerating caffeine degradation. Lactic acid bacteria produce lactic acid, acetic acid, and citric acid, which lower the pH of the fermentation medium and affect the stabilization of caffeine, thereby accelerating its degradation into simpler compounds [26,30]. Lactic acid bacteria fermentation in tea-based drinks could reduce caffeine by up to 30% through a combination of acidification and enzymatic activity [31]. In addition, in natural fermentation, lactic acid often interacts with other microbes, such as yeast (*Saccharomyces*) and acetic acid bacteria (*Acetobacter*), thereby enhancing the degradation of caffeine [8,9]. The combination of *Lactobacillus* and *Saccharomyces* in kombucha fermentation resulted in a greater reduction in caffeine levels than fermentation with lactic acid bacteria alone [8,10]. Other studies also show that spontaneous fermentation in coffee beans involves various microbes that contribute to caffeine degradation, including lactic acid bacteria that produce demethylase enzymes and enzymes that degrade caffeine structures [11].

3.5.2. Profiling of chlorogenic acid compounds in Robusta coffee with LC-MS/MS.

Jeszka-Skowron *et al.* [33] reported that the CGA content in raw coffee beans is 7.73%, while the chlorogenic acid content in roasted coffee beans at temperatures of 75°C, 150°C, and 225°C is 6.93%, 9.33%, and 7.12%, respectively. Green coffee beans (*Coffea arabica* L.) obtained from East Gojjam, West Gojjam, Awi, Bahir Dar, and South Gondar were analyzed using HPLC-VWD, with caffeine content ranging from 0.78% to 1.55% and chlorogenic acid content ranging from 3.29% to 7.73% [34]. Chlorogenic acid (*5-caffeoylquinic*) is an ester of quinic acid and caffeic acid. This compound is a natural phenolic antioxidant widely distributed across the plant kingdom and is well-represented in green coffee beans (raw coffee beans) [33,34]. Depending on the species, green coffee beans contain around 3.6-6.0% chlorogenic acid in dry form, with higher levels of chlorogenic acid in robusta coffee beans than in arabica coffee beans [33,34]. Coffee extracts have many beneficial effects on human health, such as lowering blood pressure, inhibiting lipid accumulation, increasing body weight, and controlling blood glucose levels [5]. The presence of chlorogenic acid in robusta coffee extracts has several potential biological properties, including antioxidant, anti-inflammatory, anticancer, anti-obesity, antihypertensive, and anticonvulsant [33]. Chlorogenic acid can protect coffee growth from microorganisms, insects, and UV radiation [34]. Meanwhile, the benefits of chlorogenic

acid for human health include its antioxidant, antiviral, and hepatoprotective properties, as well as its antispasmodic activities [33,34].

The esterification process breaks down complex caffeine compounds into chlorogenic acid. Caffeine compounds become free with a small molecular size and weight, and become easy to move to diffuse through cell walls and dissolve in water [39]. Chlorogenic acid released from caffeine is then decomposed into other organic compounds, becoming soluble in the fermentation medium [33,39]. The longer the dissolution process, the more chlorogenic acid becomes soluble in the fermentation medium [34]. In coffee, glucose can be degraded into lactic acid by *L. plantarum* during the coffee fermentation process. In addition, *L. plantarum* produces an amylolytic enzyme that degrades starch into glucose, which is then converted to lactic acid. Therefore, the longer *L. plantarum* ferments coffee beans, the more lactic acid is produced, so that the aroma of fermented coffee will smell increasingly sour along with the length of the fermentation process [8].

During fermentation, *L. plantarum* metabolizes carbohydrates via the glycolysis and pentose phosphate pathways, producing lactic acid as the main product [10]. However, fermentation by *L. plantarum* tends to reduce chlorogenic acid in robusta coffee beans, not increase it [18]. This decrease in chlorogenic acid levels occurs due to enzymatic activity that breaks down the compound during fermentation [41]. During coffee fermentation, chlorogenic acid levels decrease significantly. This decrease occurs due to various biochemical mechanisms influenced by enzymes, microorganisms, and environmental conditions during fermentation [34]. One of the main mechanisms for the decrease in chlorogenic acid in fermented coffee is hydrolysis catalyzed by esterase [33]. This enzyme breaks the ester bond in chlorogenic acid, producing caffeic acid and quinic acid. The activity of this enzyme increases during fermentation due to supportive environmental conditions, such as a more neutral pH and the presence of microorganisms that produce the enzyme [12]. Coffee fermentation involves various types of microorganisms, including lactic acid bacteria (*Lactobacillus spp.*), yeast (*Saccharomyces spp.*), and fungi [4,11]. These microorganisms secrete enzymes that can degrade phenolic compounds, such as chlorogenic acid. This process produces metabolites that contribute to changes in coffee taste, such as increased sweetness and reduced bitterness, as seen with chlorogenic acid. In addition to hydrolysis, chlorogenic acid can be oxidized by the enzyme polyphenol oxidase (PPO) [33,34]. This enzyme catalyzes the oxidation of phenols to quinones, which can then polymerize to form complex compounds. This oxidation process can cause color changes in coffee beans and contribute to the formation of compounds that affect the aroma and final taste of coffee [39,41].

Fermentation conditions, such as pH and temperature, also influence the degradation of chlorogenic acid. Higher temperatures can accelerate enzymatic reactions and increase oxidation rates, while more acidic or neutral pH supports the activity of hydrolytic enzymes. Therefore, controlling fermentation parameters is crucial for determining the final chlorogenic acid levels in coffee [33]. A decrease in chlorogenic acid levels during fermentation can affect coffee's taste profile [34]. Caffeic acid, formed from the breakdown of chlorogenic acid, has a bitter taste, while quinic acid contributes to the acidity of coffee. Therefore, LAB fermentation can alter the balance of coffee flavors, making them smoother or increasing their complexity [4]. In terms of health, although fermentation can reduce chlorogenic acid levels, the degradation products, such as caffeic acid, still have antioxidant and anti-inflammatory benefits [33,34]. Therefore, fermentation with LAB can improve coffee's health benefits in various

ways. Fermentation with lactic acid bacteria significantly affects chlorogenic acid levels in coffee [39]. Through enzymatic hydrolysis and the production of organic acids, this fermentation can reduce chlorogenic acid levels while also producing derivative compounds that contribute to coffee's taste and health benefits. With a better understanding of this process, the coffee industry can optimize fermentation to produce higher-quality coffee with greater health benefits [41,42].

Chlorogenic acid degradation by *L. plantarum* primarily involves the action of microbial esterases and phenolic-metabolizing enzymes. Chlorogenic acids are first hydrolyzed into caffeic acid and quinic acid, a reaction facilitated by cinnamoyl esterases, which are widely reported in lactic acid bacteria [39]. Following hydrolysis, caffeic acid undergoes further metabolic transformations, including decarboxylation and reduction, yielding derivatives such as dihydrocaffeic acid, which may exhibit enhanced antioxidant activity [40]. This bioconversion process reflects the ability of *L. plantarum* to detoxify phenolic substrates while simultaneously generating bioactive metabolites beneficial to fermented products. Collectively, these enzymatic pathways demonstrate how *L. plantarum* fermentation contributes to the breakdown of caffeine and chlorogenic acids, supporting the observed reductions in these compounds and potentially enhancing the functional quality of the final product [41,42].

3.5.3. Antioxidant activity in fermented Robusta coffee with *L. plantarum*.

Fermentation of robusta coffee beans can increase antioxidant activity by altering the levels of specific bioactive compounds [32]. The main compound that increases antioxidant activity is chlorogenic acid, the main phenolic compound in coffee, thereby increasing the antioxidant capacity of robusta coffee [33,34]. In addition, fermentation can increase the availability of caffeic acid and ferulic acid, which are derivatives of chlorogenic acid and are known to have strong antioxidant properties, thereby enhancing coffee's antioxidant activity [33,34]. In addition, selecting the right fermentation microorganisms, such as *Saccharomyces cerevisiae* or *Lactobacillus plantarum*, can maximize the increase in the content of these bioactive compounds, thereby enhancing antioxidant activity in fermented robusta coffee [34]. The LC-MS/MS technique identifies and quantifies bioactive compounds, such as chlorogenic acid, caffeic acid, ferulic acid, and flavonoids, that contribute to coffee's antioxidant activity [32-34].

Jeszka-Skowron *et al.* [33] reported that fermentation can modify the concentration of these compounds, thereby increasing antioxidant capacity. The study using LC-MS/MS to analyze the chlorogenic acid content in mossto-fermented robusta coffee beans found that chlorogenic acid levels increased after fermentation, thereby increasing the coffee's antioxidant activity [34]. The test results for robusta coffee indicated antioxidant activity. Robusta coffee fermented with *Lactobacillus plantarum* for 6 hours and 24 hours had strong and very strong antioxidant activity. Antioxidants are classified into 5 categories: <50 ppm (very strong), 50-100 ppm (strong), 100-150 ppm (moderate), 150-200 ppm (weak), and >200 ppm (very weak) [33,34].

The antioxidant results demonstrated an inverse correlation between total phenolic content and IC₅₀ values, indicating that samples with higher phenolic concentrations required lower extract volumes to achieve 50% radical inhibition [33,34]. This negative association suggests that phenolics, including chlorogenic acid and its bioconversion derivatives,

contributed significantly to the antioxidant activity of the fermented coffee. As fermentation progressed, enzymatic hydrolysis by *L. plantarum* increased the availability of free phenolic compounds, thereby enhancing radical-scavenging capacity and lowering IC₅₀ values [30,32]. These findings support the role of phenolic metabolites as key antioxidant components, reinforcing the mechanistic link between phenolic enrichment and improved antioxidant potency during fermentation.

The correlation between total phenolic content and antioxidant activity in fermented coffee underscores the role of phenolic compounds as the primary contributors to antioxidant activity. During fermentation with *L. plantarum*, enzymatic hydrolysis of complex phenolic compounds increases the concentration of free phenolics, which are more reactive and readily donate hydrogen atoms to neutralize free radicals [32,34]. As a result, samples with higher total phenolic content generally show stronger antioxidant capacity. This trend aligns with previous findings demonstrating that phenolic compounds, particularly hydroxycinnamic acids and their derivatives, significantly enhance antioxidant potential in plant-based fermented products [30,33].

Chlorogenic acids showed a dynamic relationship with antioxidant activity during fermentation. Although fermentation reduced the native chlorogenic acid content through enzymatic hydrolysis, the resulting degradation produced caffeic acid and other lower-molecular-weight derivatives with stronger antioxidant potential [32,33]. The biotransformation process helped maintain, or even enhance, antioxidant levels at certain fermentation stages. This indicates that the antioxidant capacity of the fermented coffee system is not solely dependent on the original chlorogenic acid concentration, but also on the generation of metabolites with higher antioxidant reactivity [33,34]. Jeszka-Skowron *et al.* [34] reported that the degradation of chlorogenic acid by microbes converts chlorogenic acid into caffeic acid, and caffeic acid is broken down into cinnamic acid. Caffeic acid is a phenolic compound of the flavonoid group. *S. cerevisiae* in kombucha culture is able to produce phenol reductase enzymes and decarboxylate cinnamic acid [1]. Cinnamic acids such as trans-4-hydroxy methoxy cinnamic acid (phytic acid) and trans-4-hydroxycinnamic acid (p-coumaric acid) undergo decarboxylation to form phenolic compounds, namely 4-vinyl guaiacol (4-VG) and 4-vinyl phenol (4-VP) [1,8].

Overall, the observed correlations suggest a synergistic association between total phenolics, chlorogenic acid bioconversion, and antioxidant activity [33]. A decline in intact chlorogenic acids does not necessarily correspond to reduced antioxidant performance, as degradation products such as caffeic acid and dihydrocaffeic acid contribute substantially to radical-scavenging activity [32]. This biochemical interaction underscores the importance of phenolic metabolism during fermentation, where microbial transformations enhance the bioavailability and functional value of polyphenolic compounds [32].

3.5.4. Total phenolic content of fermented Robusta coffee.

The activity of enzymes produced by microbes used as starters in the fermentation process releases phenolic compounds bound to cell wall components, such as cellulose, lignin, and protein, via ester bonds, thereby increasing free phenolic compounds [33,36]. Some lactic acid bacteria possess specific enzymes (β -glucuronidase, β -galactosidase, and α -rhamnosidase) that allow them to metabolize phenolic compounds as carbon sources [34]. Some lactobacilli, thanks to their enzymes (gallate decarboxylase), can degrade gallic acid to pyrogallol. This

compound is degraded to cis-aconitate, which then enters the Krebs cycle, keeping these microorganisms metabolically active. It is important to consider that the extract used contains a mixture of compounds, with caffeine and chlorogenic acid as the predominant compounds [38].

The total phenolic content in fermented robusta coffee is closely correlated with its antioxidant activity. Phenolics in fermented robusta coffee are closely correlated with natural antioxidant activity, likely through free-radical capture [37]. The fermentation process in coffee can increase or decrease total phenolic levels, depending on factors such as the type of microorganisms involved, fermentation duration, and environmental conditions [32]. However, under certain conditions, fermentation can also cause phenolic degradation due to enzymatic or uncontrolled oxidation reactions [4]. Therefore, optimizing the fermentation process is a key factor in maximizing the antioxidant potential of fermented Robusta coffee.

Many studies have shown a positive correlation between total phenolic content and antioxidant activity. The higher the phenolic content of an extract, the greater its antioxidant capacity. This is because phenolics can be effective hydrogen and electron donors to neutralize free radicals. However, this relationship is not always linear. Not all phenolic compounds have the same antioxidant activity. Flavonoids with more hydroxyl groups tend to have higher activity [33]. The presence of other compounds, such as vitamin C, vitamin E, and carotenoids, can enhance or diminish the antioxidant activity of phenolics. Various testing methods have different sensitivities to certain types of phenolic compounds. Total phenolic content is closely related to antioxidant activity [34,39]. However, this relationship can be influenced by various factors such as phenolic structure, testing methods, and the presence of other compounds in the sample [32,34]. Therefore, although total phenolic content can serve as an early indicator of a material's antioxidant capacity, a more holistic approach is needed to assess the overall effectiveness of antioxidants [34]. Further studies using a combination of analytical methods can help deepen understanding of the mechanisms and potential of phenolic compounds as natural antioxidants.

4. Conclusions

This study demonstrates that fermentation of Robusta coffee with *Lactobacillus plantarum* for 6 hours yields the most favorable balance between caffeine reduction and antioxidant activity, achieving concentrations of 0.08% caffeine and 49.737 ppm antioxidant activity. Although chlorogenic acid and total phenolics decreased by 3.87% and 89.65 mg GAE/mL, respectively, this controlled reduction may contribute to a smoother flavor profile and improved consumer acceptability. Metabolite profiling revealed 36, 26, and 28 compounds across the three coffee samples, indicating significant biochemical transformations during fermentation. Collectively, these findings highlight the practical relevance of a 6-hour fermentation period as a feasible processing guideline for enhancing functional and sensory attributes in Robusta coffee. However, this study was limited to laboratory-scale fermentation and did not include sensory evaluation. Future research should therefore explore pilot-scale applications, flavor and aroma analysis, consumer acceptance testing, and metabolic pathway elucidation to optimize fermentation parameters further and enhance commercial potential.

Author Contributions

Conceptualization, K.N., D.A., and R.H.B.S.; methodology, D.A. and R.H.B.S.; formal analysis, K.N., D.A., and R.H.B.S.; data curation, K.N.; writing—original draft preparation, K.N., D.A., and R.H.B.S.; writing—review and editing, K.N., D.A., and R.H.B.S. All authors contributed equally to this work and have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Definition
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
DPPH	2,2-difenil-1-pikrilhidrazil
IC ₅₀	Inhibitory Concentration 50%
UV-Vis	Ultra Violet - Visible
LAB	Lactic Acid Bacteria
CGA	Chlorogenic Acids
ANOVA	Analysis of Variance

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