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Microbiological, Phytochemical Constituents, and Antioxidant Properties of Fermented Green Robusta Coffee Beans

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ABSTRACT

Robusta coffee is one of Malaysia's most planted species due to its ability to adapt to the local climate. Nonetheless, the coffee species was perceived as having lower quality and economic value due to bitterness and astringency. It is widely believed that higher caffeine and chlorogenic acid contents in Robusta coffee beans contributed to the unfavourable bitter and astringent flavour. Hence, the present study intends to evaluate the effect of spontaneous wet fermentation (SWF) of locally grown Robusta (*Coffea canephora* L.) coffee towards the microbiological properties, phytochemical constituents, in particular caffeine and chlorogenic acids (CGA), total phenolic content (TPC), and antioxidant properties. The SWF of green Robusta coffee beans from University Agricultural Park (UAP), Universiti Putra Malaysia, Serdang, Selangor, took place at ambient temperatures between 25 to 28°C, and the pH decreased from 5.2 to 3.64 over five days of fermentation. The total plate count, lactic acid bacteria (LAB) and yeasts were significantly increased to approximately 7 Log_{10} CFU/g. The SWF has reduced caffeine content by 35%, while the CGA has decreased by roughly 20%. The SWF also led to an increase in TPC of approximately 31.5% and an increase in antioxidant activity of approximately 60%.

Keywords: Antioxidant activities, caffeine, chlorogenic acid, Robusta coffee, spontaneous wet fermentation

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INTRODUCTION

The diversity in chemical compositions, such as chlorogenic acids (CGA) in the green Robusta coffee beans, notably influences the coffee brew's sensory characteristics, ultimately affecting its economic value in the trading market (Fioresi et al., 2021). Based on studies, it has been observed that green Robusta coffee beans contain higher concentrations of caffeine and CGA, both of which exhibit bitterness and astringency taste in the coffee brew and are regarded as lower quality compared with green Arabica coffee beans (Bicho et al., 2011; de Melo Pereira, de Carvalho Neto, Júnior, et al., 2019; Seninde & Chambers IV, 2020). Nonetheless, the phytochemical constituents of green Robusta coffee beans can be significantly influenced by various factors, including the agronomic environment and the methods used during post-harvest processing (Maxiselly et al., 2023). For instance, caffeine and CGA concentrations in green coffee beans negatively correlated with altitude (Girma et al., 2020). Likewise, the environment of coffee cultivation has a significant impact on the microbial communities. The bacteria communities interaction was reported to predominate at higher altitudes due to larger levels of soil organic matter and nutrients, whereas fungi-bacteria communities interaction predominates at lower altitudes (Veloso et al., 2020).

Post-harvest spontaneous wet fermentation (SWF) involves immersing the pulped coffee cherries in water at ambient temperature to degrade the inner mucilage of Robusta coffee. The SWF process was greatly affected by the variation of naturally occurring microbial communities (Velmourougane, 2013). For example, the presence of yeast in the SWF might improve the levels of isoamyl alcohol, acetaldehyde, and acetate, consequently resulting in improved roasted coffee brew sensory attributes (Elhalis, Cox, Frank, et al., 2020; Hadj Salem et al., 2020; Pereira et al., 2020). While the presence of bacteria capable of producing pectin-related enzymes is favourable in the SWF (Silva et al., 2013). Steer fermentation is undisputedly appropriate because viable microbial strains such as *Pseudomonas* sp. and *Aspergillus* sp. can be inoculated to achieve the desired result to target the degradation of caffeine and CGA in green Robusta coffee beans (Gokulakrishnan et al., 2005; Tai et al., 2014). In stark contrast, the results of SWF may not be as favourable as those of steer fermentation since microbial profiles are highly dependent on the coffee-growing environment. Also, to our knowledge, there has been no report of the efficiency of spontaneous wet fermentation in reducing caffeine and CGA in locally planted Robusta coffee. Therefore, this research aims to evaluate the SWF microbiological properties as well as the impacts of SWF on the phytochemical constituents of green Robusta coffee beans, specifically caffeine and CGA, along with TPC and antioxidant properties.

METHODS

Preliminary Screening of Washed Green Robusta Coffee Beans Samples Collected from Various Locations

Washed green Robusta bean samples were acquired from four different regions of Malaysia: (1) Sik, Kedah in the north, (2) University Agricultural Park (UAP), Universiti Putra Malaysia, Serdang, Selangor in the centre, (3) Kluang, Johor in the south, and (4) Tenom, Sabah in the east. The washed green Robusta beans were ground to a powder that passed through 600–710 microns (Liu et al., 2018).

Extraction of Washed Green Robusta Coffee Beans Samples Collected from Various Locations. The coffee extraction was conducted according to Rukayadi et al. (2008). Approximately 4 g of fermented green Robusta coffee powder was mixed with 100 ml ethanol (Merck, Germany) and shaken (170 rpm) for 1 hr at ambient room temperature (27°C) by orbital shaker-incubator (Biosan, Latvia). The mixture was placed in the dark for 48 hr at room temperature. The mixture was then sonicated using an ultrasonicator (Fisherbrand, USA) at 70% amplitude for 10 min in an ice bath before filtering using a Buchner funnel and Whatman No. 1 filter paper (Whatman, USA). Then, ethanol was removed using a rotary evaporator (Eyela, Japan) at 60°C under a vacuum. The coffee extracts were used for subsequent phytochemical constituent quantification, TPC, and antioxidant assays.

Quantifying Phytochemical Constituents in Green Robusta Coffee Beans using High-performance Liquid Chromatography (HPLC). The quantification of phytochemical constituents was performed in accordance with Abrahão et al. (2019). HPLC (Shimadzu, Japan) was used to quantify seven phytochemical compounds: (1) caffeine, (2) CGA, (3) caffeic acids, (4) 3,4 dihydrobenzoic acids, (5) 4 dihydroxybenzoic acid, (6) *p*-coumaric, (7) quercetin, and (8) kaempferol. The reverse phase column C-18 was used with a flow rate of 1 ml/min, mobile phase methanol, water, and acetic acid (Merck, Germany) (70:28:2 v/v/v), and a wavelength of 280 nm for caffeine with mobile phase methanol (Merck, Germany) and 0.1% formic acid (Merck, Germany), a wavelength of 320 nm for CGA, caffeic acids, and *p*-coumaric, a wavelength of 280 nm for 3,4 dihydrobenzoic acids and 4 dihydroxybenzoic acids, and a wavelength of 320 nm for quercetin and kaempferol. A five-point standard curve (R^2 = 0.99) was developed for each compound for phytochemical quantification in the sample. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard error (SE) of the intercept and the standard deviation (SD) of the intercept from the standard curve. The sample with the highest level of caffeine and CGA was chosen to proceed with the subsequent SWF experiments because a sample with a low level of caffeine and CGA may not be able to be identified and quantified using HPLC after SWF and roasting.

TPC Comparison between Washed Green Robusta Coffee Beans from Various Locations. The TPC analysis was performed in accordance with Acidri et al. (2020), with minor modifications to the serial dilutions and three replications. The coffee extracts were diluted in five serial dilutions (0.2–1 mg/ml), and 1 ml was transferred to a universal container. The samples/gallic acid standard (0.02–0.1 mg/ml) were incubated with 5 ml of 10% Follin-Ciocalteu's reagent (Merck, Germany) for 1 min and then reacted with 4 ml of 20% (w/v) sodium carbonate (Friendemann Schmidt, Australia) solution in a dark ambient condition for 30 min. The absorbance was read at 765 nm using a spectrophotometer (Biomate 3). A five-point standard calibration curve ($y = 3.47x + 0.1446$) with $R^2 = 0.9934$ was used to calculate the TPC content.

Antioxidant Properties Comparison between Washed Green Robusta Coffee Beans from Various Locations. The Trolox equivalent antioxidant capacity (TEAC) was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay in accordance with Acidri et al. (2020), with minor modifications to the serial dilutions and three replications. The green coffee extracts were diluted with methanol in 10-fold serial dilutions (10–0.001 mg/ml), with 1 ml transferred into a new universal bottle. The green coffee extracts and freshly prepared Trolox (6–hydroxy–2,5,7,8–tetramethyl–chroman–2–carboxylic acid) standards (Aldrich, USA) were incubated with 9 ml of freshly prepared 0.1 mmol/l DPPH solution (Sigma-Aldich, Germany) for 10 min in a dark ambient environment. The absorbance was measured at 519 nm using a spectrophotometer (Biomate 3) with methanol (Merck, Germany) solvent as blank and DPPH (Sigma-Aldich, Germany) working solution as a control. The percentage inhibition of the DPPH radical was calculated through $\% \text{ inhibition} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) \times 100\%$. The concentrations that caused a 50% decrease in the initial concentrations of the DPPH radical (half maximal inhibitory concentration [IC_{50}]) and antioxidant capacity of the samples, i.e., TEAC, were determined from the absorbance curve ($y = 9.4141x + 0.0233$) with $R^2 = 0.9910$.

The antioxidant-reducing power was performed using a ferric-reducing antioxidant power (FRAP) assay performed in accordance with Tran et al. (2020) with three replications. The FRAP working solution was freshly prepared by mixing 300 mM acetate buffer (Merck, Germany, pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (Tokyo Chemical Industry, Japan), 40 mM hydrochloric acid (HCl, Fisher Scientific, Malaysia) and 20 mM iron(III) chloride ($FeCl_3$, R and M Chemicals, Malaysia) in the ratio 10:1:1. The mixture was incubated for 30 min at 37°C. The extracts were diluted in five serial dilutions (0.1–0.5 mg/ml), from which 0.5 ml was transferred into the new universal bottle. The samples/freshly prepared Trolox (Aldrich, USA) were incubated with 9.5 ml of FRAP working solution and allowed to stand for 30 min in a dark ambient environment. The

absorbance was measured at 593 nm using a spectrophotometer (Biomate 3, USA) with FRAP working solution as blank. The antioxidant capacity of the samples was calculated.

Physical Characteristics Comparison between Green Robusta Coffee Beans from Various Locations. The dimension of washed green Robusta coffee beans was performed in accordance with Bote and Vos (2017) with minor modifications of the sample size and three replications. Ten washed green Robusta coffee beans were randomly selected to measure their dimension using a calliper. The surface with the longest length was measured as length, the surface with the greatest width was measured as width, and the thickness was measured with the flat surface facing downwards.

The hardness of washed green Robusta coffee beans was measured in accordance with Pittia et al. (2007), with minor modifications of beans used and working temperature, and with three replications. The hardness of washed green Robusta coffee beans (10 beans) was measured using a TA.XTplusC texture analyser (Stable Micro Systems, England) equipped with a 1,000 N load cell at a rate of 0.83 cm/s and the working temperature was 25°C. The coffee beans were placed on the analyser plate with the flat side up.

The colour analysis was performed in accordance with Wongsa et al. (2019) with three replications. The colour of washed green Robusta coffee beans (10 g) was measured using a colourimeter (Konika Minolta CR 400 Chroma Meter, Japan) to determine the colour parameter values, i.e., L^* ($L^* = 100$ means white, $L^* = 0$ means black), a^* [redness (+) and greenness (−)], and b^* [yellowness (+) and blueness (−)].

Spontaneous Wet Fermentation (SWF) of Robusta Coffee from UAP

After a preliminary screening, Robusta coffee from UAP, UPM, was chosen for SWF. The ripe Robusta coffee cherries were harvested by hand using a sterile glove and container [(sprayed with 70% alcohol (Merck, Germany)]. The cherries' fruit skin (exocarp) and part of the mucilage (mesocarp) were manually pulped using a sterile [(spray with 70% alcohol (Merck, Germany)] mortar and pestle upon arrival at the UPM laboratory. The pulped cherries were spontaneously fermented using ultrapure water (Arium 611 UV, Germany) at ambient temperature for 0 to 5 days. Zero-hour fermentation (day 0) was used as a negative control. Fermentation temperature and pH (Delta 320 pH meter, USA) readings were taken for 0 (day 0), 24 hr (day 1), 48 hr (day 2), 72 hr (day 3), 96 hr (day 4), and 120 hr of fermentation (day 5).

Temperature and pH Evaluation during Fermentation. The internal fermentation temperature and pH were measured daily throughout the SWF periods, i.e., from day 0 (0 hr) to day 5 (120 hr), using a glass-electrode pH meter (Delta 320 pH meter, USA), with three replications. Prior to the measurement, the pH meter was calibrated with pH 4.1 and pH 7.0 buffer solutions.

Microbiological Analysis. The United States Food and Drug Administration (FDA) (n.d.) performed the microbe enumeration and direct plating with minor solution volume adjustments. The analysis was performed with three replications. Fermented pulped cherries were transferred into a stomacher bag containing 100 ml of 1% peptone water (Oxoid CM 0509, United Kingdom) to determine the microbial population throughout 5 days of SWF. The bags were gently shaken for 5 min. Up to 10^{-8} serial dilutions in 1% nutrient broth (Oxoid CM0001, United Kingdom) were performed. For the total plate count enumeration, an aliquot (100 µl) of the three most diluted solutions was pipetted in duplicate onto plate count agar (Oxoid CM0325, United Kingdom) and incubated at 28°C for 48 hr. While De Man-Rogosa-Sharpe (MRS) agar (Oxoid CM0361, United Kingdom) was used for LAB enumeration at 37°C for 48 hr, potato dextrose agar (BD Difco, USA) was used for yeast and mould enumeration at 28°C for 48 hr. The visible colonies formed in the agar were calculated in logarithmic numbers of colony-forming units per gram ($\log_{10}$ CFU/g).

Extraction, Quantification of Phytochemical Constituents, TPC, and Antioxidant Activity Analysis of SWF Green Robusta Coffee Beans. The green Robusta coffee beans were dried to 10–12% moisture, and the parchments were manually removed. Following this, the extraction was conducted according to the methods outlined in the preceding extraction procedures. The phytochemical constituent quantification, TPC analysis, DPPH radical assay, and FRAP assay were conducted according to the methods outlined in the preceding analysis procedures.

Hardness SWF Green Robusta Coffee Beans. The hardness of SWF green Robusta coffee beans was conducted according to the methods outlined in the preceding hardness testing procedures.

Statistical Analysis

All results were reported as mean ± standard deviation (SD), and statistical analyses were performed using one-way analysis of variance (ANOVA). Tukey's multiple range tests were used with a probability of $p < 0.05$ to identify significant differences between the results. General linear model (GLM) regression analysis was used to explain the variance of the dependent variables, i.e., (1) pH, (2) LAB, (3) caffeine, (4) CGA, (5) caffeic acid, (6) TPC, and (7) antioxidant capacity influenced by the independent variables, i.e., fermentation periods through adjusted R^2. The direction and strength of correlation between the variables were evaluated using Pearson correlation. The software for statistical analysis was Minitab V.19 (Minitab Inc., USA).

RESULTS AND DISCUSSION

Phytochemical Constituents of Washed Green Robusta Coffee Beans from Various Locations

In the current study, seven phytochemicals were attempted to be measured using

HPLC; nonetheless, only four were detected (> LOD) statistically, as shown in Table 1. Because 4 dihydroxybenzoic acids, *p*-coumaric acid, quercetin, and kaempferol were either not detected or lower than LOQ throughout the analysis, the results were reported as undetected (ND). Among the four phytochemicals, caffeine had the highest concentration in all instances, ranging from 1 to 6%, followed by CGA, ranging from 0.6 to 2.1% per gram of dry weight (DW). Washed green Robusta coffee beans from UAP have the highest concentration of all locations. This observation could be attributable to the fact that the site of the plantation has the highest average temperature and lowest average yearly precipitation compared to other locations (Table 2). Such agroclimatic conditions enhance the propensity for smaller bean size, significantly impacting phytochemical constituents (Kath et al., 2021).

Table 1
Quantification of washed green coffee Robusta beans phytochemical constituents using high-performance liquid chromatography

Phytochemicals	Sik	UAP	Kluang	Tenom	LOD	LOQ
Chlorogenic acid (mg/g DW)	16.40 ± 1.10 ^{b}	21.05 ± 1.44 ^{a}	15.00 ± 0.76 ^{b}	6.69 ± 0.40 ^{c}	2.25 ± 0.00	6.92 ± 0.00
Caffeine (mg/g DW)	24.24 ± 0.08 ^{b}	60.26 ± 0.30 ^{a}	22.44 ± 0.23 ^{b}	9.19 ± 0.08 ^{c}	5.80 ± 0.00	17.55 ± 0.00
Caffeic acid (mg/g DW)	2.29 ± 0.11 ^{b}	3.53 ± 0.19 ^{a}	2.48 ± 0.00 ^{b}	1.52 ± 0.01 ^{c}	1.13 ± 0.00	3.54 ± 0.00
3,4-dihydroxybenzoic acid (mg/g DW)	6.45 ± 0.04 ^{a}	4.66 ± 0.08 ^{b}	4.43 ± 0.02 ^{b}	3.50 ± 0.08 ^{c}	1.29 ± 0.00	4.03 ± 0.00
4 dihydroxybenzoic acid	ND	ND	ND	ND	1.29 ± 0.00	4.03 ± 0.00
p-coumaric acid	ND	ND	ND	ND	5.15 ± 0.00	15.62 ± 0.00
Quercetin	ND	ND	ND	ND	1.45 ± 0.00	4.67 ± 0.00
Kaempferol	ND	ND	ND	ND	1.43 ± 0.00	4.34 ± 0.00

Note. DW = Dry wieght; UAP = University Agricultural Park; LOD = Limit of detection; LOQ = Limit of quantification; ND = Not detected; $^{a\text{-}c}$ Mean values ± standard deviations within the same row without a common superscript are significantly different ($p < 0.05$)

Table 2
Climate conditions in different sampling areas

	Sik	UAP	Kluang	Tenom
Average temperature (°C)	24.99 – 33.21	25.57 – 34.56	25.56 – 31.7	25.6 – 28.56
Average annual precipitation (mm)	84.3	26.77	54.14	92.73
Average rainy days per year	191.2	57.13	142.09	207.34
Average days without rain per year	173.8	307.87	222.91	157.66
Elevation (m)	3.33	44.67	18.79	281.51

Note. Data were obtained from https://weatherandclimate.com/; UAP = University Agricultural Park

In contrast, the lowest caffeine and CGA concentrations were observed in the washed green beans from Tenom, where the plantation takes place at a higher altitude. This observation is consistent with that of Girma et al. (2020), who made a similar observation; nevertheless, the study did not clarify the causes behind this occurrence. Other researchers believe that one of the major causes was the influence of altitude's environmental temperature (both air and soil temperature) during coffee cherries' development on a number of metabolic pathways that directly affect the CGA, lipids, and soluble sugar levels (Joët et al., 2010). Caffeic acids, one of the CGA precursors that exhibit a striking similarity trend in Table 1, could support the claim that altitude and environmental temperature influence CGA metabolism. Additionally, the higher the altitude and cooler the temperature at which coffee beans are harvested, the higher their sensory score (Barbosa et al., 2012). Such a discovery has raised the alarm about global warming, which could substantially impact coffee's sensory quality.

Caffeine, CGA, and caffeic acid were not significantly different between Sik and Kluang washed green Robusta coffee beans, most probably due to the comparable growing conditions at both plantations, including low altitude and average temperature. Irrigation, on the other hand, was a crucial component for the survival and development of coffee plants, but it also had no major effect on the phytochemical composition of green coffee beans (da Silva et al., 2005).

The soil composition influenced by the application of fertilisers exerts a significant influence on the phytochemicals of green beans; however, the present study did not report on the type and schedule of fertiliser and pesticide application because farmers were unwilling to disclose commercially sensitive information. Nevertheless, it has been reported that the availability of soil nutrients such as nitrogen, magnesium, potassium, calcium, and phosphate has a substantial positive correlation with caffeine and CGA (Getachew et al., 2022).

TPC and Antioxidant Activity of Washed Green Robusta Coffee Beans from Various Locations

The total phenolic contents of washed green Robusta coffee beans from various locations were illustrated in Figure 1, and it showed that the TPC of washed green Robusta coffee beans varies on their geographical origins. Likewise, green coffee beans from UAP had the highest TPC concentration compared to other regions, but it was insignificant compared to green coffee beans from Sik. Again, green coffee beans from Tenom had the lowest TPC by almost 50% compared to green coffee beans from UAP and Sik. Although CGA is a phenolic compound, it would be too facile to conclude that lower CGA levels in coffee beans from Tenom, as outlined in Table 1, result in low TPC because not all TPC is CGA. A trace amount of 3,4-dihydroxybenzoic acid detected in all cases can also be classified as phenolic compounds. The present TPC result is consistent with the findings of Martinez et

al. (2021), who demonstrated that plantation altitude may not significantly affect TPC in green coffee beans and that other factors, such as maturity phases, may influence it.

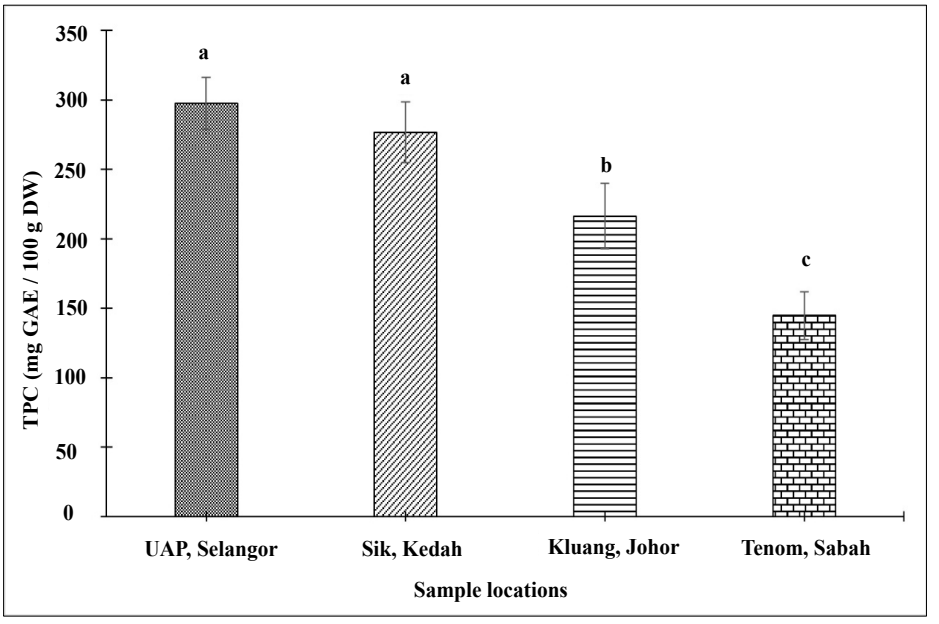

Figure 1. TPC of washed green Robusta coffee beans from various locations

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Concerning antioxidant activity, Figure 2 illustrates the antioxidant-reducing power and TEAC of washed green Robusta coffee beans in various regions from the FRAP and DPPH assays, along with the IC_{50}. The antioxidant-reducing power of washed green Robusta coffee beans from UAP was approximately 2.5 to 5 times greater than that of other regions, as measured by the FRAP assay. It may suggest that phytochemical compounds that stimulate antioxidant activity are particularly prone to the single-electron transfer (SET) mechanism. UAP still contained significantly higher TEAC than other regions for the TEAC, but there was not as substantial a difference as in the reducing power. The TEAC of washed green beans from UAP was roughly 10% to 50% higher compared with other regions. In line with the antioxidant-reducing power and TEAC from FRAP and DPPH assays, UAP beans had the lowest antioxidant concentration required to scavenge 50% of the initial DPPH radicals (IC_{50}). It suggests that the antioxidant activity of UAP beans is more potent than beans from other regions. The reducing power and TEAC result indicated that Tenom's beans have the lowest antioxidant activity.

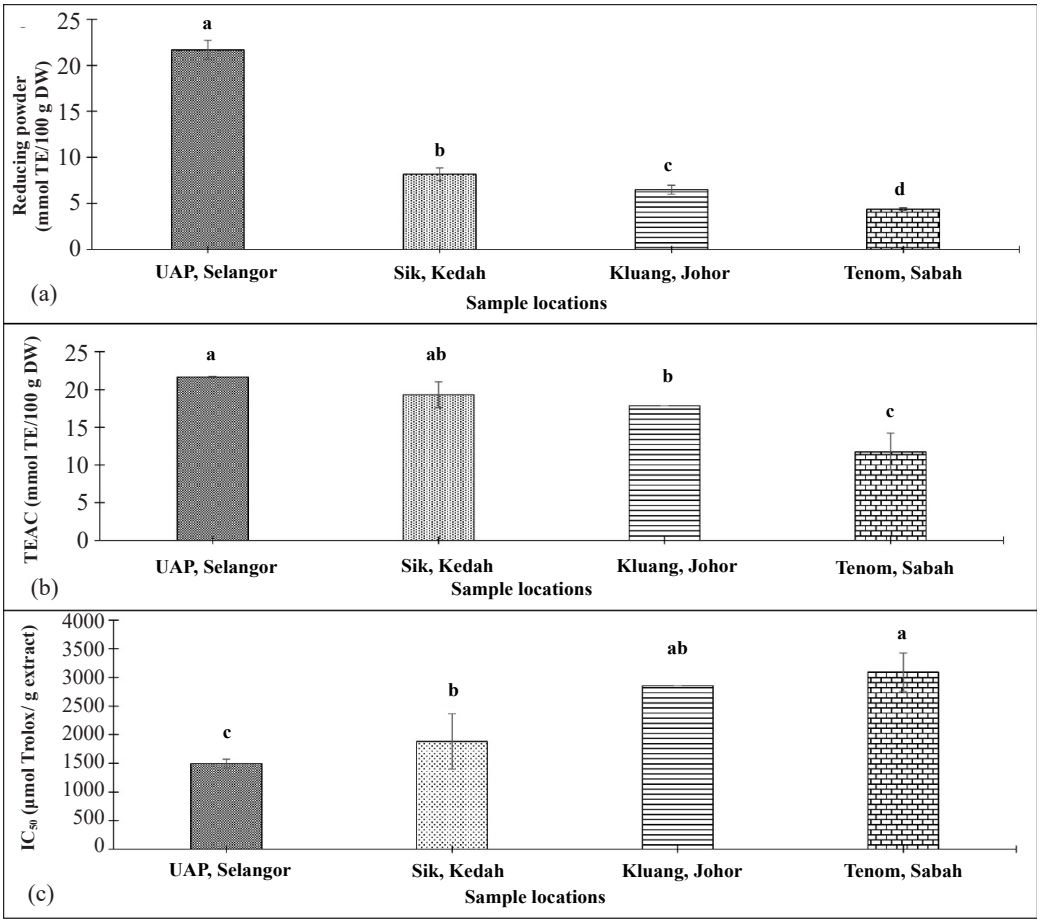

Figure 2. Antioxidant properties of washed green Robusta coffee beans from various locations. (a) Reducing power, (b) Trolox equivalent antioxidant capacity (TEAC), and (c) Half maximal inhibitory concentration (IC_{50})

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Physical Properties of Washed Green Robusta Coffee Beans from Various Locations

As washed coffee Robusta beans from Tenom were compared to other locations, the physical dimensions of length, width, and thickness were significantly larger, as illustrated in Figure 3, possibly due to higher altitude (Bote & Vos, 2017). Bean size has often been one of the primary physical attributes used to determine the worth of coffee in commerce; larger beans tend to have a higher economic value. Also, larger beans (from higher altitudes) are perceived to give superior cup quality, possibly due to lower caffeine and CGA concentrations, as shown in Table 1. Green beans from UAP were considerably smaller in length, width, and thickness compared to the other samples, yet they contained the most caffeine, CGA, TPC, and antioxidant properties (Table 1, Figures 1 and 2). The

preliminary screening results suggested that larger beans with greater economic worth may not always possess superior bioactive characteristics. Similar reports showed that coffee beans rated as having the best quality do not always exhibit the highest antioxidant activity and that Robusta coffee, which is perceived to be of poorer quality, has higher antioxidant activity than Arabica coffee beans (Jeszka-Skowron et al., 2016; Ramalakshmi et al., 2008). The "lower quality" label ascribed to Robusta coffee may result from a poor sensory experience from greater caffeine and CGA concentrations. The measurement and identification of sensory attributes were not conducted in this research. This observation has been noted and will be considered for future studies.

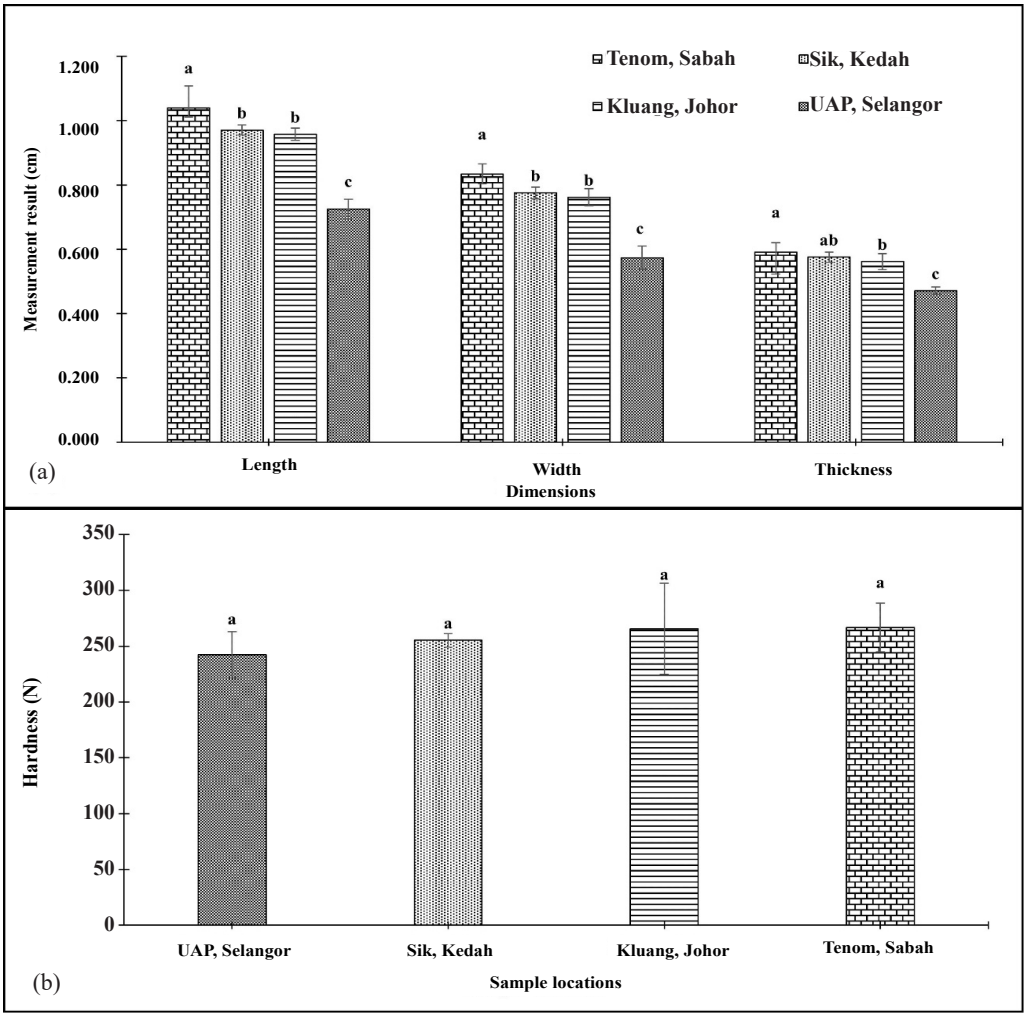

Figure 3. (a) Physical dimension and (b) hardness of washed green coffee beans from various locations

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

Regarding the hardness of green Robusta coffee beans, there were no statistically significant differences among sampling locations, as illustrated in Figure 3. It is likely because all beans were subjected to a similar post-harvest wash process. It was believed that linear galactomannans were the primary component of the cell wall responsible for the hardness of green Robusta coffee beans (Li et al., 2021). Nonetheless, the hardness of fermented Robusta coffee beans will be compared in the subsequent SWF sections.

Table 3 depicts the colour of washed green Robusta coffee beans from various locations; all beans exhibited a greenish-brown hue. There was no significant variation in lightness (L^*) in all situations. At the same time, UAP coffee beans exhibited a darker brown pigmentation with a lower red/green value (a^*) and blue/yellow value (b^*).

Table 3
Colour properties of washed green Robusta coffee beans from various locations

Colour	Sik	UAP	Kluang	Tenom
L^*	50.68 ± 2.45 [a]	52.07 ± 2.60 [a]	54.17 ± 0.54 [a]	55.56 ± 0.11 [a]
a^*	4.20 ± 0.05 [a]	3.68 ± 0.03 [c]	4.83 ± 0.11 [b]	3.57 ± 0.11 [c]
b^*	19.08 ± 1.67 [a]	15.46 ± 1.10 [b]	21.56 ± 0.39 [a]	20.96 ± 0.11 [a]

Note. UAP = University Agricultural Park; [a-c] Means values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

SWF Temperature and pH

Robusta coffee from UAP was chosen for the SWF study because it contains the highest levels of caffeine and CGA. Such a large quantity will provide assurance that both compounds can still be detected and measured by HPLC even if their concentrations fall over SWF time. Over the SWF periods, the fermentation temperature (Figure 4) varied between 26 and 28°C, with the lowest temperature being on day five at 26.38°C. Although some food fermentations, such as cocoa and vinegar, resulted in exothermic reactions that could reach as high as 50°C, the temperature fluctuation in the current study was mostly influenced by the open environment because SWF was not conducted in the bioreactor model. Its purpose is to imitate real-world SWFs that lack particular environmental control. So, at the end of the study, it can be suggested that SWF of green Robusta coffee beans is still possible without a complicated fermentation model that is economically advantageous and practical for small businesses and farmers.

Regarding fermentation pH, the initial pH at day 0 was 5.20, decreasing significantly throughout the five days of SWF. As depicted in Figure 4, the pH reached its lowest value between 3.64 and 3.70 on days 3 and 4, but it slightly rebounded to 4.05 on day 5. This pH rise may be attributed to a decreased LAB load, which declined

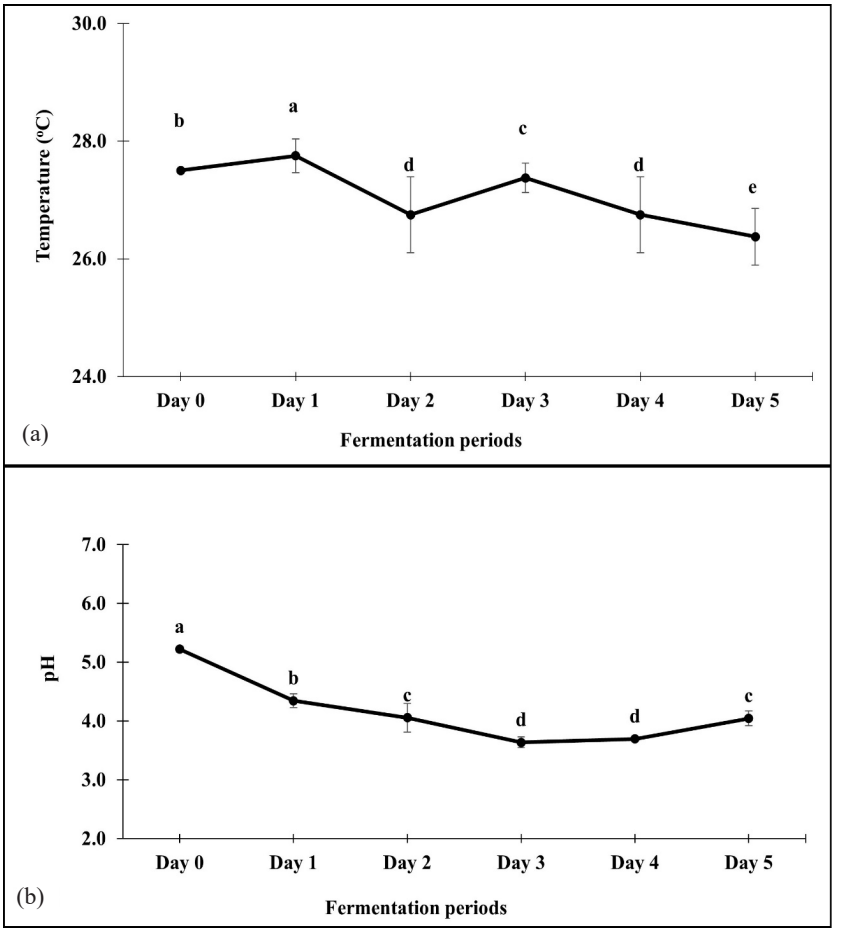

Figure 4. (a) Spontaneous wet fermentation temperature and (b) pH along the fermentation periods from day 0 to day 5

Note. [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

from 7.32 $\log_{10}$ CFU/g on day 4 to 7.12 $\log_{10}$ CFU/g on day 5. Furthermore, it is noticeable that the total plate count exhibited a similar reduction trend from 7.65 to 7.44 $\log_{10}$ CFU/g. This observation suggests that bacteria growth may have entered the death phase during an extended wet fermentation. The following microbiological properties discussion comprehensively analysed the correlation between LAB loads and pH. The presence of lactic acids from LAB could cause the pH to drop because the low oxygen SWF environment (facultative anaerobe) provides a favourable environment for their growth (the following discussion in the microbiological properties section elucidates the correlation between pH and LAB).

Also, an acidic environment is necessary for the dissociation of green coffee Robusta beans mucilage and the formation of aromatic compounds in roasted coffee

beans such as (1) aldehydes, (2) long chain alcohols, (3) carboxylic acids, and (4) ketones (de Melo Pereira, de Carvalho Neto, de O. Junqueira, et al., 2019; Schwan & Fleet, 2014; Velmourougane, 2013). Hence, there exists a mutually advantageous interaction in which the dissociation of mucilage provides vital nutrients to the microflora population. On the other hand, pulped coffee beans exposed to a low oxygen and pH environment could experience abiotic stress, resulting in the synthesis of γ-aminobutyric acid (GABA), which is renowned for its calming and relaxing effects. Additionally, the germination of coffee beans by isocitrate lyase in the initial two days of SWF could significantly increase GABA levels as well as alter several phytochemical concentrations, including caffeine (Kim Kim et al., 2018; Zhang et al., 2019).

SWF Microbiological Properties

Present SWF postulates the participation of complicated naturally occurring microorganisms such as yeasts and bacteria (Figure 5). From day 0 to day 5, the total plate count increased significantly from 5.82 $\log_{10}$ CFU/g to 7.44 $\log_{10}$ CFU/g. However, the bacteria loads did not significantly differ after reaching 7.3 $\log_{10}$ CFU/g. According to Pearson correlation, the total plate count was highly positively correlated (p = 0.000, r = 0.950) with LAB loads as well as the pH (p = 0.000, r = -0.899). The subsequent LAB discussion further discussed the correlation and effects between total plate count, LAB and pH variable. This increment of total plate count after 24 hr of soaking pulped beans to between 7 to 8 $\log_{10}$ CFU/g was comparable with another study indicating fermentation (Nasanit & Satayawut, 2015). It indicates that alteration in phytochemical contents,

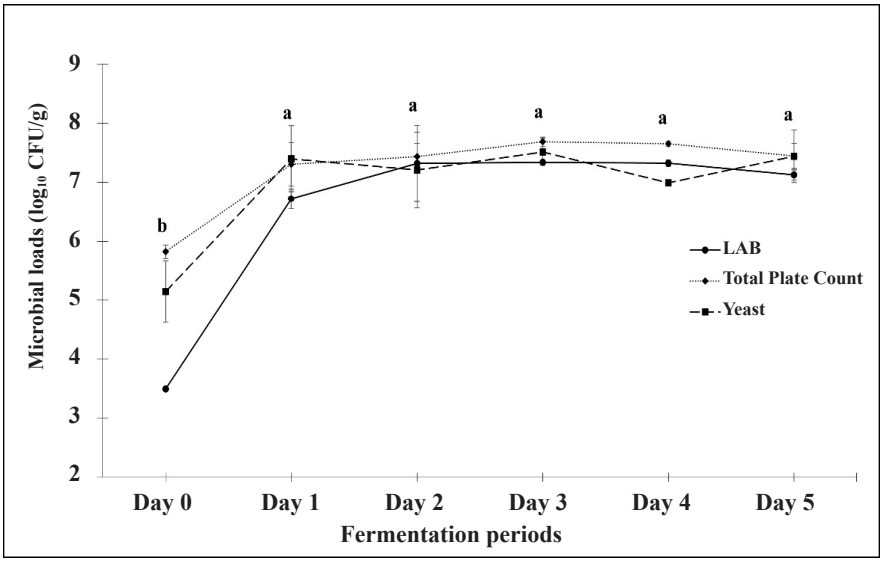

Figure 5. Spontaneous wet fermentation temperature microbial load from day 0 to day 5

Note. [a-b] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

antioxidant properties, and physicochemical properties could be observed, and the details will be discussed in subsequent phytochemical, antioxidants, and physical properties sections.

With regard to bacteria proliferation trends throughout SWF periods, different authors reported different proliferation trends. Nasanit and Satayawut (2015) reported that the initial total plate count loads were between 6 to 7 $\log_{10}$ CFU/g, and the trend decreased gradually after 24 hr to the end of 48 hr of fermentation. However, Elhalis, Cox, and Zhao (2020) and Evangelista et al. (2015), in line with the present study, reported that the initial loads of total plate count were between 3.8 to 5 $\log_{10}$ CFU/g, then increased significantly to between 7 and 8 $\log_{10}$ CFU/g and remained steady until the end of 48 hr fermentation periods. The initial rapid rise in the number of bacteria could be attributable to the rapid multiplication of microbes following adaptation to the fermentation medium. In contrast, the bacteria load remains constant (stationary phase) after that, which may be due to nutrient depletion and ribosome hibernation to conserve energy. The variability of microbial trends between studies is a peculiarity of SWF because of the diversity of microbial profiles intimately linked to the plantation environment; therefore, SWF remains the greatest barrier to the consistency of the final product when compared to steer fermentation (Bertranda, 2019).

Lactic acid fermentation is considered the most important trait of SWF, and its function has been described in the preceding pH section. In the present study, convincing evidence of LAB fermentation was observed, with a roughly 3-fold increase in LAB in merely 24 hr of SWF but a constant range of 7 $\log_{10}$ CFU/g throughout the fermentation periods. When the total plate count and LAB loads were compared, it was discovered that approximately 60% of the bacteria from the total plate count could be LAB at the initial SWF. Similarly, a study reported that LAB loads were up to 60% prevalent in the microflora community of SWF (de Oliveira Junqueira et al., 2019). After 24 hr of SWF, bacteria began to be displaced by LAB, plausible due to intolerance for the hostile acidic environment, and from 48 hr of SWF onwards, more than 90% of the bacteria in the total plate count could be LAB. Researchers have effectively isolated *Lactobacillus* sp., such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus casei*, and *Lactobacillus mesenteroides* from SWF and recognised its significant role in the dissociation of mucilage, altering phytochemical constituents such as caffeine and enhancement of the coffee brew's sensory profile (de Carvalho Neto et al., 2018; Djossou et al., 2011; Nasanit & Satayawut, 2015; Purwoko et al., 2022; Ribeiro et al., 2020).

Since lactic acid is the chief metabolite produced by LAB, the drop in pH in the current study was strongly believed to be attributable to lactic acid from LAB (S.-J. Lee et al., 2021). The GLM and correlation analysis presented in Tables 4 and 5 validated the significance and strength

of the association between fermentation times, pH, and LAB loads. The *p*-values for all the analysed dependent variables in GLM were less than 0.05, confirming a statistically significant relationship with the independent variables. The coefficient in the regression equation showed the distance between the factor levels and the overall mean. If the coefficient exhibits a positive value, an increase in the independent variable corresponds to an increase in the mean value of the dependent variable. Conversely, the negative coefficient exhibits an opposite relationship. For instance, the overall mean for the pH regression equation was 4.152. On day 0 of SWF, the pH increased by 1.068 units from the overall mean. Likewise, the first day of SWF had a positive coefficient with a slight rise of 0.203 units from the total pH mean, yet the pH was still lower than day 0. Continuing SWF from day 2 to day 5 resulted in lower pH values than the overall pH means. The comparison of the positive coefficient on day 1 and the negative coefficient on day 2 suggests that it is preferable to conduct SWF for a minimum of two consecutive days since the low pH of SWF appeared to be more prevalent from the second day of SWF forward. The adjusted R^2 of the fermentation period and pH equal 93.33%, indicating that if there is a variation in pH, 93.33% is due to the change in fermentation periods, and only 6.67% is due to error or unexplained factors. All following GLMs were interpreted in the same way. In terms of the intensity and direction of correlation, fermentation periods strongly correlated with LAB loads

Table 4
GLM of fermentation periods with pH, lactic acid bacteria (LAB), caffeine, chlorogenic acid (CGA), caffeic acid, total phenolic content (TPC), and antioxidant properties of fermented green Robusta coffee beans

Independent variable	Dependent variables	Model *p*-value	Regression equation
Fermentation periods	pH	0.000	pH = 4.152 + 1.068 fermentation day 0 + 0.203 fermentation day 1 - 0.087 fermentation day 2 - 0.517 fermentation day 3 - 0.462 fermentation day 4 - 0.207 fermentation day 5 Adjusted R^2 = 93.33%
	LAB	0.000	LAB = 6.677 - 3.183 fermentation day 0 + 0.037 fermentation day 1 + 0.638 fermentation day 2 + 0.858 fermentation day 3 + 0.958 fermentation day 4 + 0.692 fermentation day 5 Adjusted R^2 = 93.92%
	Caffeine	0.000	Caffeine = 63.007 + 15.265 fermentation day 0 - 2.751 fermentation day 3 - 12.514 fermentation day 5 Adjusted R^2 = 99.85%

Table 4 *(Continue)*

Independent variable	Dependent variables	Model *p*-value	Regression equation
	CGA	0.000	CGA = 22.071 + 2.675 fermentation day 0 - 1.020 fermentation day 3 - 1.655 fermentation day 5 Adjusted R^2 = 83.53%
	Caffeic acid	0.001	Caffeic acid = 3.765 - 1.041 fermentation day 0 - 0.237 fermentation day 3 + 1.279 fermentation day 5 Adjusted R^2 = 98.57%
	TPC	0.000	TPC = 279.490 - 76.800 fermentation day 0 + 24.000 fermentation day 1 + 18.000 fermentation day 2 + 20.400 fermentation day 3 - 0.100 fermentation day 4 + 14.400 fermentation day 5 Adjusted R^2 = 62.47%
	TEAC	0.000	TEAC = 20.184 - 5.375 fermentation day 0 + 1.410 fermentation day 1 + 1.458 fermentation day 2 + 0.801 fermentation day 3 + 0.824 fermentation day 4 + 0.883 fermentation day 5 Adjusted R^2 = 96.89%
	Reducing power	0.000	Reducing power = 20.367 - 6.498 fermentation day 0 + 1.237 fermentation day 1 + 1.286 fermentation day 2 + 0.273 fermentation day 3 + 1.734 fermentation day 4 + 1.969 fermentation day 5 Adjusted R^2 = 88.05%

Table 5
Pearson correlations of fermentation period with pH, lactic acid bacteria (LAB), caffeine, chlorogenic acid (CGA), caffeic acid, total phenolic content (TPC), and antioxidant properties of fermented green Robusta coffee beans

	Fermentation periods	LAB loads	Caffeine	CGA	TPC	Reducing power
Microbiological properties						
LAB loads	0.738					
pH	-0.789	-0.905				
Phytochemical constituents						
Caffeine	-0.998					
CGA	-0.914		0.925			
Caffeic acid	0.954		-0.938	-0.781		

Table 5 *(Continue)*

	TPC and antioxidant properties			
TPC	0.451			
Reducing power	0.683		0.776	
TEAC	0.573		0.882	0.927

Note. All correlations were significant ($p < 0.05$)

while strongly correlated negatively with pH. The LAB loads demonstrated a very significant negative correlation with pH; as LAB loads increased, SWF's pH decreased.

Yeast, likewise, was a participatory microbe in the SWF, with the load significantly increasing from 5.15 to 7.44 $\log_{10}$ CFU/g from day 0 to day 5. The load did not differ significantly after reaching 7.4 $\log_{10}$ CFU/g on the first day of SWF. Several yeast strains, including *Meyerozyma caribbica*, *Hanseniaspora uvarum*, *Torulaspora delbrueckii*, *Pichia kudriavzevii*, *Pichia fermentans*, *Pichia kluyveri*, and *Saccharomyces cerevisiae*, have been isolated from SWF and show remarkable results in modulating phytochemical contents and improving sensory quality (Elhalis, Cox, Frank, et al., 2020; Evangelista et al., 2014; Evangelista et al., 2015). Yeast-fermented green coffee beans contained significantly more isoamyl alcohol, ethanol, acetaldehyde, and ethyl acetate than beans fermented without yeast growth (Elhalis, Cox, Frank, et al., 2020). On the contrary, yeast was found to lower by up to 50% of (1) N-alkanoyl-5-hydroxytryptamides (C-5HTs), (2) cafestol, and (3) kahweol, which are associated with gastric irritation and blood cholesterol increase (Tinoco et al., 2019).

Comparing wet and dry post-harvest processes (SSF), wet fermentation of Robusta beans may be advantageous to SSF, especially in terms of food safety and timing efficiency. Certain foodborne pathogens can be inhibited from growing or reproducing in the acidic environment of wet fermentation (current study pH 3.6–4.95) (Kim, Wilkins, et al., 2018). The absence of mould in the present study reduced the possibility of mycotoxin synthesis, such as ochratoxin A, which typically exists in SSF (Schwan & Fleet, 2014). In addition, the SSF process is incredibly time-consuming, requiring up to 30 days of direct sun-drying and being highly weather and environment-dependent. Also, elevated carbon dioxide levels (350–400 vs 650–1,200 ppm), temperature increases (2–5°C) and drought stress hasten coffee bean fungi deterioration (Medina et al., 2017). Regarding water-soluble polysaccharides, wet-processed coffee beans yield more than dry-process beans (Tarzia et al., 2010).

Phytochemical Constituents of Green Robusta Coffee Beans after SWF

After SWF, green coffee Robusta beans from days 0, 3, and 5 were chosen to examine the influence of phytochemical constituents, particularly caffeine and CGA, as shown

in Figure 6 and chromatogram, as shown in Figure 7. All examined phytochemicals exhibited significant differences at each fermentation stage, with caffeine and CGA showing decremental trends, whereas caffeic acids showed incremental trends, and 3,4-dihydroxybenzoic acid showed no significant changes. The caffeine concentration decreased by around 35%, whereas CGA decreased by approximately 20% at the end of SWF. The increment in caffeic acids is partly thought to be produced by CGA catabolism during SWF. It should be noted that SWF does not necessarily cause the alteration of caffeine and CGA concentrations in green coffee beans, as Joët et al. (2010) reported. However, the results indicate that SWF has successfully decreased the quantities of caffeine and CGA in green Robusta coffee beans. Further validation of the regression and correlation between SWF, caffeine and CGA was conducted using GLM (Table 4) and Pearson statical analysis (Table 5). Based on the GLM, fermentation periods were highly associated with the variation of caffeine and CGA (adjusted $R^2 > 80\%$), and both compounds showed a very strong negative correlation with fermentation periods; the longer the Robusta coffee beans fermented, the lower the CGA and caffeine contents.

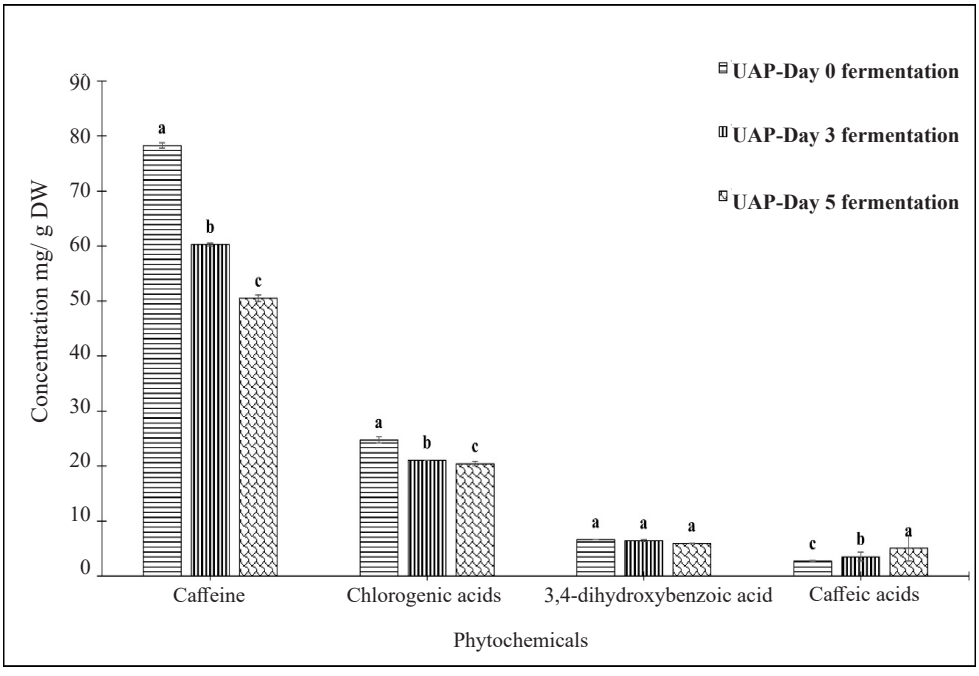

Figure 6. Comparison of phytochemical constituents of green Robusta coffee beans at certain SWF periods

Note. UAP = University Agricultural Park; [a-c] Mean values ± standard deviations without a common superscript within the group are significantly different ($p < 0.05$)

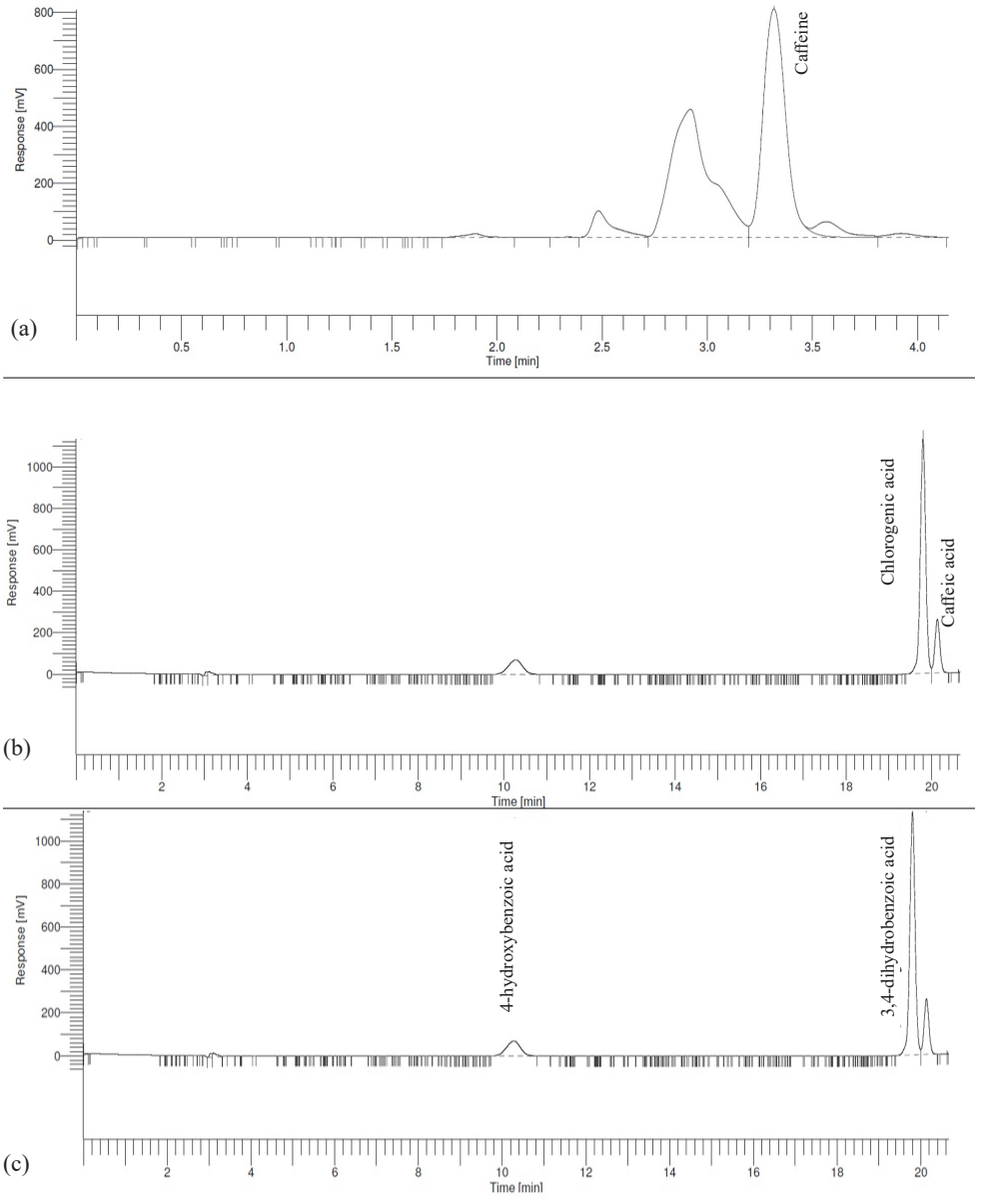

Figure 7. High-performance liquid chromatography chromatogram for fermented green Robusta coffee beans after 5 days of spontaneous wet fermentation. (a) caffeine, (b) chlorogenic acid and caffeic acid, and (c) 4-hydroxybenzoic acid and 3,4-dihydrobenzoic acid

It is deduced that *Pseudomonas* sp., *Lactobacillus* sp., and *Bacillus* sp. were present in the SWF, given that these bacteria were capable of degrading caffeine and CGA in green coffee beans and were prevalent in the soil of Peninsular and East Malaysia (Gokulakrishnan et al., 2005; Mun & Ling, 2022; Purwoko et al., 2022; Tripathi et al.,

2012; Vega et al., 2021). It also implies that comparable SWF results may be observed in other locations in Malaysia.

Without alternative nitrogen sources, such as urea and ammonium, caffeine will serve as the sole nitrogen supply for microbial survival (Hakil et al., 1999). Fungi (e.g., *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp.) can degrade caffeine into theophylline (1,3-dimethylxanthine), while bacteria (e.g., *Pseudomonas* sp. and *Serratia* sp.) can degrade caffeine into theobromine (3,7-dimethlxanthine). These molecules were then metabolised to generate xanthine, which was then transformed into carbon dioxide and ammonia by purine catabolism (Gummadi et al., 2012; Gokulakrishnan et al., 2005). While degradation of CGA by *Lactobacillus* sp. results in the synthesis of hydroxycinnamic acids such as ferulic acid, quinic acid, and caffeic acid, additional decarboxylation and reduction could lead to the production of 4-vinylcathecol and dihydrocaffiec acid (Filannino et al., 2015; Rogozinska et al., 2021).

TPC and Antioxidant Properties of Green Robusta Coffee Beans after SWF

The result of TPC across 5-day fermentation periods is presented in Table 6. The TPC of non-fermented green Robusta coffee beans (day 0) was significantly lower compared with all fermented green coffee beans. Likewise, the antioxidant properties of unfermented green Robusta coffee beans (day 0) are significantly lower than those of fermented green Robusta coffee beans. The GLM in Table 4 confirmed that the independent variable of fermentation periods can be used to explain the variation among the dependent variables of TPC and antioxidant properties. During the SWF periods, microbial metabolism resulted in the degradation of organic compounds and cell wall structure, such as (1) cellulose, (2) hemicellulose, and (3) lignin, by microorganisms, thereby increasing the solubility of the interior cell components of coffee beans, which could contribute to an increase in TPC (Kurniawati et al., 2016). The evidence of softer fermented Robusta coffee beans as a result of cell wall degradation will be addressed in the subsequent section on coffee beans' hardness. Also, microbial metabolism can alter the profile of phenolic compounds, for example, by converting them to aglycone flavonoid form, which contributes to an increase in antioxidant activity, and also by metabolising CGA to hydroxycinnamic acids, which have been observed to be substantially connected with antioxidant power (Huynh et al., 2014; Kurniawati et al., 2016; Mullen et al., 2013). The Pearson correlations in Table 5 reveal a high positive correlation between TPC and the antioxidant properties of fermented green Robusta coffee beans.

Even though GLM and Pearson correlations supported the effects of fermentation durations on TPC and antioxidant properties, the connection was only somewhat moderately positive. Perhaps the TPC and antioxidant properties trends were associated more with microbial loads than fermentation durations, given that

Table 6
TPC and antioxidant properties of non-fermented and fermented green Robusta coffee beans

Fermentation day	Extract yield (%)	Total phenolic content (mg GAE/100 g DW)	IC_{50} (µg/ml)	TEAC (mmol TE/ 100 g DW)	Reducing power (mmol TE/ 100 g DW)
0	15.00 ± 0.00	202.70 ± 20.61 [a]	217.50 ± 3.54 [a]	14.81 ± 0.14 [a]	13.87 ± 0.60
1	19.01 ± 1.40	303.52 ± 18.76 [b]	175.00 ± 1.41 [ab]	21.60 ± 0.09 [b]	21.60 ± 1.25
2	14.28 ± 2.51	297. 53 ± 18.74 [b]	112.50 ± 37.48 [b]	21.64 ± 0.08 [b]	21.65 ± 1.05
3	18.03 ± 2.79	299.94 ± 29.23 [b]	136.00 ± 22.63 [ab]	20.99 ± 0.26 [b]	20.64 ± 0.86
4	16.27 ± 0.56	279.35 ± 43.41 [b]	145.50 ± 14.85 [ab]	21.01 ± 0.34 [b]	22.10 ± 0.99
5	14.56 ± 1.91	293.93 ± 14.47 [b]	152.50 ± 19.09 [ab]	21.07 ± 1.14 [b]	22.24 ± 0.54

Table 7
Pearson correlations of microbial loads and total phenolic content (TPC), antioxidant properties, caffeine, and chlorogenic acid (CGA) of fermented green Robusta coffee beans

Variables	LAB	Total plate count	Yeast	TPC	Reducing power
Microbiological properties					
Total plate count	0.950				
Yeast	0.877	0.914			
TPC and antioxidant properties					
TPC	0.774	0.697	0.857		
Reducing power	0.942	0.898	0.840	0.716	
TEAC	0.900	0.914	0.901	0.786	0.924
Phytochemical constituents					
Caffeine	-0.920	-0.879	-0.895		
CGA	-0.948	-0.894	-0.951		

Note. All correlations were significant ($p < 0.05$); LAB = Lactic acid bacteria; TEAC = Trolox equivalent antioxidant capacity

the microbial loads in Figure 5 exhibited an identical trend pattern. This notion was further corroborated by Table 7 Pearson correlations, which revealed a stronger positive correlation between microbial loads and TPC and antioxidant capabilities. It suggests that in order to boost TPC and antioxidant levels even further, the microbial load should be increased, which may be accomplished using a stirred-tank bioreactor that can regulate and optimise fermentation conditions such as (1) selection and combination of inoculated strains (2) appropriate culture supplementation, (3) temperature (e.g., 30°C), (4) aeration (e.g., 1 L/min), and (5) agitation (200 rpm) (de Carvalho Neto et al., 2018; de Jesus Cassimiro et al., 2022; Haile & Kang, 2019; L. W. Lee et al., 2017; Martinez et al., 2019; Wang et al., 2020). This observation differed slightly with caffeine and CGA, as both phytochemicals strongly correlated with fermentation periods and microbial loads (Tables 5 and 7).

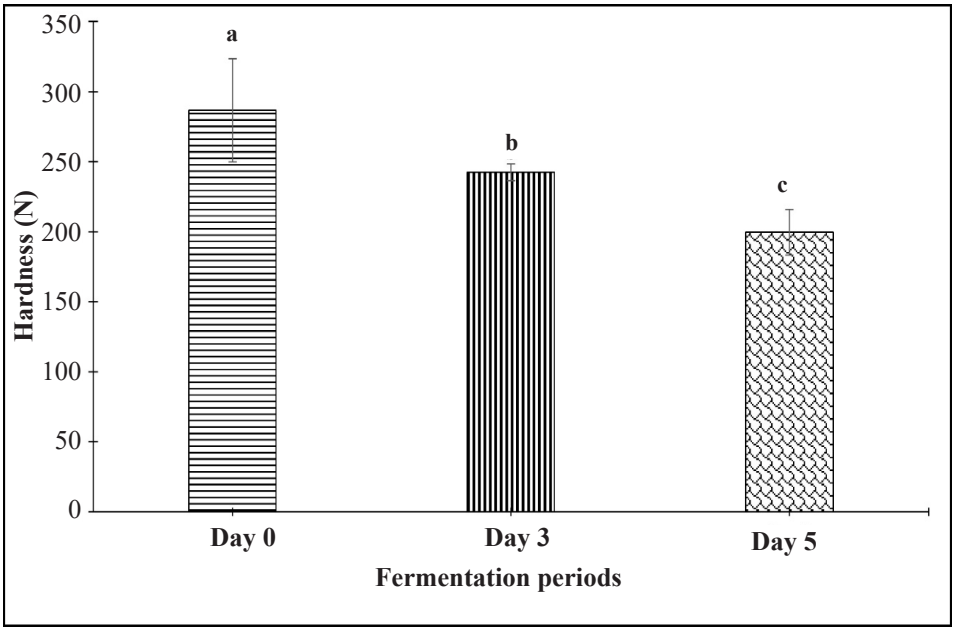

Figure 8. The hardness of fermented green Robusta coffee beans from University Agricultural Park after spontaneous wet fermentation

Note. [a-c] Mean values ± standard deviations without a common superscript are significantly different ($p < 0.05$)

The Hardness of Green Robusta Coffee Beans after SWF

The hardness of green Robusta coffee beans after SWF was compared and presented in Figure 8. Compared to non-fermented beans, fermented Robusta coffee beans were significantly softer, with the beans that underwent 5 days of SWF being the softest by 30%. As previously discussed, this could be evidence of microbial pectinase, amylase,

cellulase, and protease activities from SWF that break down cell wall components (Elhalis et al., 2021). Germination during SWF may also contribute to the softer, fermented green Robusta coffee beans. During germination, α-galactosidase combines with (1→4)-*β*-mannan (endo-*β*-mannanase) and *β*-mannosidases to breakdown galactomannans, which are the principal component of the cell wall responsible for the hardness of green Robusta coffee beans (L. W. Lee et al., 2015).

CONCLUSION

This study compared physical and chemical characteristics, including phytochemicals and antioxidations, during the preliminary screening of green Robusta coffee beans from four locations in Malaysia, i.e., the north, centre, south, and east. The coffee plantation's agroclimatic conditions exert a substantial effect. Washed green Robusta coffee beans at a higher altitude showed greater size but contained less caffeine, CGA, TPC, and antioxidant properties. In comparison, the washed green Robusta coffee beans from UAP were the smallest in size but contained the highest levels of caffeine and CGA. Such characteristics typically have disadvantages in trading, as they are judged to be of low quality and to have a poor sensory profile. Due to its high caffeine and CGA concentration, Robusta coffee from UAP was chosen for further SWF study to determine whether its caffeine and CGA content could be reduced while retaining or improving its antioxidant properties. The five-day study of SWF revealed that SWF happened satisfactorily at ambient temperature without additional specified controls like fermentation in a bioreactor. LAB and yeast dominated the SWF throughout the five-day SWF period, reaching up to 7 $\log_{10}$ CFU/g. The pH dropped as low as 3.6, providing an essential medium for mucilage breakdown. The fermented beans were softer than the unfermented beans. The current SWF has decreased the caffeine concentration by 35% and the CGA content by 20% while increasing the TPC and antioxidant qualities by approximately 50% in fermented green Robusta coffee beans. The statistical analysis additionally revealed that fermentation periods may not be as impactful as microbial loads in enhancing TPC and antioxidant properties, whilst fermentation periods and microbial loads are equally important in reducing caffeine and CGA levels.

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