

Article

Enzyme-Assisted Fermentation Using Bromelain and Laccase Enhances Phenolic Profile, Antioxidant Capacity and Bioactive Compounds of CCN-51 Cocoa Beans

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Featured Application

The enzyme-assisted fermentation strategy proposed in this study can be applied in cocoa post-harvest processing to enhance the functional quality and antioxidant properties of cocoa beans. The optimized bromelain–laccase treatment can be implemented to increase antioxidant capacity and bioactive compound content, supporting the production of high-quality cocoa and functional chocolate products for the food industry.

Abstract

Cocoa fermentation is a key post-harvest process that determines the chemical composition and functional quality of cocoa beans. This study evaluated the effect of enzyme-assisted fermentation, using bromelain and laccase, on the phenolic compounds, methylxanthines and antioxidant capacity of CCN-51 cocoa beans from northern Peru. Fresh cocoa beans were fermented in wooden boxes under ambient conditions with different enzymatic treatments based on a factorial design. Samples were collected at 0, 2, 4 and 6 days of fermentation to determine total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (DPPH, ABTS and FRAP), and the concentrations of theobromine, caffeine, catechin and epicatechin by UHPLC-MS. Significant changes in phenolic composition and antioxidant activity were observed during fermentation ($p < 0.05$), with higher values in enzyme-treated samples, particularly at day 4. Principal component analysis indicated that phenolic compounds and antioxidant activity were the main variables responsible for sample differentiation. Response surface methodology showed that bromelain had the strongest influence on most responses. Optimization using a desirability function predicted an optimal enzymatic condition of 52.19 g of bromelain and 18 g of laccase per 5 kg of cocoa beans to maximize bioactive compounds. These findings highlight that enzyme-assisted fermentation is a promising strategy to enhance cocoa functional quality.

Keywords: cocoa fermentation; enzyme-assisted processing; bromelain; laccase; phenolic compounds; response surface methodology



Academic Editor: Danila Cianciosi

Received: 20 March 2026

Revised: 13 April 2026

Accepted: 16 April 2026

Published: 17 April 2026

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1. Introduction

Cocoa (*Theobroma cacao* L.) is one of the most important agricultural commodities worldwide due to its economic relevance and its role as a source of bioactive compounds associated with human health benefits [1]. Cocoa beans are particularly rich in phenolic compounds, flavonoids and methylxanthines, which contribute to antioxidant activity and to the sensory and functional properties of cocoa-derived products [2]. These compounds have been widely studied for their potential in the development of functional foods and nutraceutical formulations [3].

Fermentation is a critical post-harvest stage in cocoa processing, as it determines the formation of flavor precursors and significantly influences the chemical composition and functional quality of cocoa beans [4]. During fermentation, complex biochemical transformations occur, including protein degradation, diffusion and oxidation of polyphenols, and structural changes in the cotyledon [5,6]. These reactions directly affect antioxidant capacity and the concentration of bioactive compounds such as catechins, epicatechins and methylxanthines [7]. Therefore, optimizing the fermentation process is essential to improve both technological and functional attributes of cocoa beans [8].

Recent studies have shown that controlled fermentation strategies, including the use of selected microbial cultures, can significantly improve the physicochemical and aromatic properties of cocoa beans and enhance fermentation efficiency [9,10]. Among these strategies, the application of exogenous enzymes has emerged as a promising technological approach to modulate biochemical reactions during fermentation [11,12]. Bromelain, a proteolytic enzyme, can promote protein hydrolysis and facilitate the release of bound phenolic compounds, while laccase, an oxidative enzyme, is capable of catalyzing the oxidation and transformation of phenolic substrates [13,14]. The combined use of these enzymes may influence the stability and availability of antioxidant compounds and modify the overall bioactive profile of cocoa beans.

Although exogenous enzymes are high-molecular-weight biomolecules and are not expected to deeply penetrate intact cocoa cotyledon tissues, they may still influence fermentation through surface and microenvironmental effects [15,16]. During cocoa fermentation, beans remain surrounded by mucilage and undergo structural and biochemical changes that can facilitate mass transfer and substrate exposure [17]. Under these conditions, bromelain may promote proteolysis at the mucilage–seed interface and contribute to the release of phenolic compounds associated with proteins or cell wall components [18], whereas laccase may modify the oxidative environment and the transformation of exposed phenolic substrates [19]. Therefore, the proposed strategy is not based on direct intracellular enzyme action but on the modulation of extracellular biochemical events that may ultimately affect the composition and functional quality of fermented cocoa beans.

Despite the growing interest in enzyme-assisted food processing, limited information is available regarding the combined effect of bromelain and laccase on cocoa fermentation and their influence on phenolic composition, methylxanthines and antioxidant activity [20,21]. In addition, studies integrating multivariate statistical analysis and response surface methodology to optimize enzymatic conditions during cocoa fermentation remain scarce.

Therefore, the aim of this study was to evaluate whether the exogenous application of bromelain and laccase during cocoa fermentation could modulate the evolution of the phenolic compounds, methylxanthines and antioxidant capacity of CCN-51 cocoa beans from northern Peru. Multivariate analysis and response surface methodology were applied to identify optimal enzymatic conditions and to understand the relationships between enzymatic treatments and bioactive compound evolution during fermentation. Although this study was conducted using Peruvian cocoa beans, the technological approach proposed

here may be applied to other cocoa-producing regions seeking to enhance the functional quality and value-added potential of cocoa through controlled enzymatic fermentation.

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents were of LC-MS grade: hexane (Merck, Darmstadt, Germany), methanol (J.T. Baker, Phillipsburg, NJ, USA), acetonitrile (J.T. Baker, Shanghai, China), and formic acid (98–100%, Merck, Darmstadt, Germany). Deionized water was obtained with a Milli-Q water purification system (Millipore Synergy® UV, Molsheim, Francia). Standard compounds such as (+)-catechin, (–)-epicatechin, caffeine and theobromine were purchased from Sigma-Aldrich (St. Louis, MO, USA), as were other reagents such as gallic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and the Folin–Ciocalteu reagent.

2.2. Pod Sampling and Bean Fermentation

Fresh cocoa pods (*Theobroma cacao* L., clone CCN-51) were harvested in the Potrerillo locality, Las Lomas district, Piura, Peru (UTM WGS84, Zone 17S: 532,847 m E; 9,483,621 m N; 240 m a.s.l.). Pods were selected based on the absence of physical damage or pest infestation and on uniform red-orange to intense red coloration, which is indicative of full maturity for the CCN-51 clone [22]. After manual opening of the pods and removal of the beans, fresh cocoa beans with mucilage were obtained for fermentation experiments.

Fermentation was conducted in wooden boxes made of *Guazuma crinita* (bolaina blanca) with dimensions of 20 × 25 × 20 cm, a wood thickness of 2.5 cm, and a capacity of 5 kg per unit. Seven fermentation boxes were used, each containing 5 kg of fresh cocoa beans. Six treatments involved the addition of bromelain (Creative Enzymes, New York, NY, USA; specific activity: 1200 GDU/g, purity ≥ 85% by SDS-PAGE, CAS No. 9001-00-7) and laccase (Creative Enzymes, New York, NY, USA; specific activity: ≥0.5 U/mg, purity ≥ 90%, CAS No. 80498-15-3) at different concentrations according to the experimental design, while one box without enzyme addition served as the control treatment (Table 1). The enzymes were first dissolved in 100 mL of freshly collected and filtered cocoa mucilage to ensure homogeneous dispersion, and the resulting solution was applied by controlled dripping over the cocoa mass. Manual mixing (20 rotations) was performed to promote uniform distribution of the enzymes within the fermenting beans. Fermentation was carried out under ambient conditions, and temperature was monitored every 12 h using a digital probe thermometer (Model TP101, stainless steel probe, measurement range –50 to 300 °C, accuracy ±1 °C, Shenzhen, China) throughout the process. From the second day on-ward, the cocoa mass was manually mixed approximately every 20 h to ensure uniform fermentation. Throughout the fermentation period, internal cocoa mass temperature ranged from 26 °C to 48 °C across all boxes: the initial anaerobic phase (days 0–2) registered temperatures between 26 °C and 33 °C, consistent with yeast-driven fermentation activity, while the aerobic acetic phase (days 2–6) reached peak temperatures of 44–48 °C as a result of exothermic oxidation by acetic acid bacteria. Ambient relative humidity at the fermentation site ranged from 72% to 88% (measured with a digital thermo-hygrometer, ±3% RH accuracy). The wooden boxes used were left open at the top throughout the process, allowing passive air exchange without forced aeration. Cocoa beans were evaluated for each treatment on fermentation days 0, 2, 4, and 6.

Table 1. Experimental design of cocoa bean fermentation using bromelain and laccase.

Treatment	Application of Enzymes (g/5 kg)		Bromelain	Laccase	Description
	Bromelain	Laccase			
T0	0	0	0%	0%	Enzyme-free control
T1	18	18	−1 (0.36%)	−1 (0.36%)	Low concentrations of both enzymes
T2	57	18	+1 (1.14%)	−1 (0.36%)	High bromelain, low laccase
T3	18	57	−1 (0.36%)	+1 (1.14%)	Low bromelain, high laccase
T4	57	57	+1 (1.14%)	+1 (1.14%)	High concentrations of both enzymes
T5	38	38	0 (0.75%)	0 (0.75%)	Central point
T6	38	38	0 (0.75%)	0 (0.75%)	Central point

2.3. Preparation of the Fermentation Sample

The cocoa beans were freeze-dried (0.021 mbar, −55 °C, 72 h) and ground into a fine powder. The dried and ground sample was weighed (500 mg), degreased by adding 10 mL of hexane, vortexed (2 min), and centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was discarded and the procedure was repeated three times. The defatted residue was air-dried at room temperature for 1 h to allow complete hexane evaporation before extraction. The fat-reduced cocoa powder was then extracted at room temperature with 10 mL of methanol/water (1:1, *v/v*) for 30 min in an ultrasonic bath at room temperature with a frequency of 37 kHz. After extraction, the mixture was centrifuged for 20 min at 5000 rpm at 5 °C. The supernatant was decanted and filtered through a nylon membrane (0.45 µm). Finally, the supernatant was placed in a flask and filled to obtain 10 mL of extract solution and stored in the dark at −20 °C until use in the chromatographic analysis and antioxidant evaluations. [23]. It should be noted that the extraction procedure employed primarily recovers extractable phenolic compounds, while non-extractable phenolics bound to the cocoa matrix were not evaluated in this study.

2.4. Quantification Method of Catechins and Methylxanthines

The analysis was performed on a UHPLC-PDA-MS system (LCMS-2050, Shimadzu, Kyoto, Japan). Chromatographic separations were performed using an ACQUITY UPLC[®] BEH C18 column (1.7 µm, 2.1 mm × 100 mm; Waters, Milford, MA, USA) in binary gradient mode. Mobile phase A: water acidified with 0.1% formic acid (*v/v*). Mobile phase B: acetonitrile with 0.1% formic acid. The gradient for mobile phase A was: 0–0.5 min (95–95%); 0.5–2 min (95–90%); 2–5 min (90–85%); 5–8 min (85–80%); 8–10 min (80–70%); 10–14 min (70–30%); 14–16 min (30–30%); 16–17 min (30–95%); 17–20 min (95–95%). The mobile phase flow rate was kept constant at 0.3 mL/min, and the injection volume was 2 µL. The column temperature was 30 °C, while the autosampler compartment temperature was maintained at 10 °C to preserve sample stability during analysis. Detection was performed by a photodiode array (PDA) with spectral monitoring from 190 to 600 nm, using a dual lamp system (deuterium lamp, D₂, for UV range; tungsten lamp, W, for visible range) with positive polarity, a spectral resolution of 512, a slit width of 1.2 nm, and a cell temperature of 40 °C. Mass spectrometer (MS) conditions: nebulizer gas 3.0 L/min, drying gas 5.0 L/min and heating gas 7.0 L/min, desolvation temperature 350 °C, desolvation line 150 °C, interface voltage 2.0 kV, approximate vacuum pressure (PG) of 2.6×10^2 Pa. SIM mode (Selected Ion Monitoring), in positive and negative ionization.

The quantification of catechins and methylxanthines was carried out by comparing the UHPLC-MS peak areas with those of commercially available reference standards (Table 2, Figure 1). Calibration solutions were prepared by diluting stock standard solutions in volumetric flasks, subsequently filled with methanol. For the caffeine, (+)-catechin, and (−)-epicatechin stock solutions, 10 mg of each was weighed and diluted to 5 mL with

methanol, reaching a concentration of 2 mg/mL. For theobromine, 12.5 mg was weighed and diluted to 25 mL with methanol/water (1:1) to reach a concentration of 0.5 mg/mL. From these stock solutions, successive dilutions were made in a methanol mixture: theobromine (35–130 µg/mL), caffeine (10–100 µg/mL), (+)-catechin (10–110 µg/mL), and (–)-epicatechin (20–120 µg/mL). Calibration curves ($y = ax + b$) were obtained by plotting concentration against the corresponding peak area, and correlation coefficients (R^2) were determined to evaluate the linearity of the method. Linearity was assessed in triplicate ($n = 3$). The limits of detection (LOD) and quantification (LOQ) were statistically determined from the parameters obtained for linearity. These determinations were performed considering the standard deviation of the response (S) and the slope of the calibration curve (m), applying Equations (1) and (2):

$$\text{LOD} = 3.3 * s \quad (1)$$

$$\text{LOQ} = 10 * s \quad (2)$$

where the following definition is used:

s = the intercept of the linear correlation between the standard deviations of phenolic standards and their concentrations [24].

Table 2. Retention times, MS data, and validation parameters for flavan-3-ols and methylxanthines.

Standards	RT (min)	[M + H] ⁺ or [M – H] [–] (<i>m/z</i>)	Limit of Detection (LOD µg/g)	Limit of Quantification (LOQ µg/g)	Linearity R ²
Theobromine	4.02	181.1	7.5	21.3	0.9978
Caffeine	6.24	195.1	1.3	5.0	0.9998
(+)-Catechin	5.05	289.1	7.5	23.8	0.9945
(–)-Epicatechin	6.72	289.1	1.3	2.5	0.9998

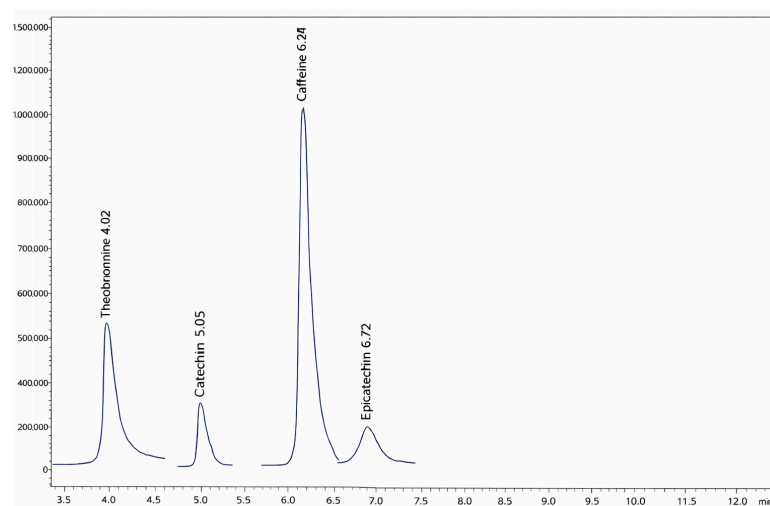


Figure 1. Chromatogram of standard mixtures of flavan-3-ols and methylxanthines analyzed by UHPLC-MS.

2.5. Antioxidant Activity

2.5.1. DPPH Scavenging Activity

The method developed by Sharma & Bhat [25] was used, with slight modifications. A volume of 100 µL of extract solution was mixed with 900 µL of methanol and 1 mL

of DPPH• radical (200 µM) dissolved in 80% methanol, the mixture was homogenized and left to stand in the dark for 30 min at 25 °C, then the absorbance was measured at 517 nm in a microplate reader (INNO-S LTEK Co., Ltd., Seongnam, Republic of Korea). The concentration of DPPH• in the reaction medium is calculated from a calibration curve obtained by linear regression. The control consisted of 1 mL of methanol and 1 mL of DPPH• solution (200 µM). The results are expressed in µmol of Trolox equivalents/g of dried cocoa sample. If a rapid discoloration of the DPPH• was observed, a new dilution of the stock solution of the extract was prepared and a new reading was carried out with this diluted solution. The reference synthetic antioxidant Trolox, at a concentration of 5–30 µM in 80% methanol solution, is tested under the same conditions.

2.5.2. ABTS Radical Scavenging Capacity

The procedure described by Re et al. [26] was followed. A 0.01 M PBS buffer (pH 7.4) was prepared by dissolving 4.0 g of NaCl, 0.1 g of KCl, 0.72 g of Na₂HPO₄·7H₂O, and 0.12 g of K₂HPO₄ in 400 mL of ultrapure water. The pH was adjusted to 7.4, and the volume was then brought up to 500 mL with ultrapure water. In parallel, the ABTS stock solution (7 mM) was prepared with potassium persulfate (2.45 mM) in 10 mL of ultrapure water and allowed to stand for 16 h at 25 °C in the dark. The final solution was then prepared as ABTS (2 mL) + buffer (0.01 M PBS, 200 mL). Next, 20 µL of the extract solution was mixed with 1980 µL of ABTS solution (7 mM) + buffer (PBS 0.01 M), homogenized, and allowed to react in the dark for 7 min. Then, the absorbance was measured at 734 nm using a microplate reader (INNO-S LTEK Co., Ltd.). The results are expressed in µmol Trolox equivalents/g of dried cocoa sample. If a rapid discoloration of the ABTS•+ was observed, a new dilution of the stock solution of the extract was prepared and a new reading was carried out with this diluted solution. The calibration curve for Trolox Equivalent Antioxidant Capacity (TEAC) was constructed using different concentrations of Trolox (4–14 µM) in PBS buffer solution under the same conditions.

2.5.3. Ferric-Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method of Young et al. [27], with slight modifications. The FRAP reagent was prepared by adding 300 mM sodium acetate buffer (pH 3.6, 50 mL) to a solution of 10 mM TPTZ in 40 mM hydrochloric acid (5 mL) and 20 mM iron (III) chloride (5 mL). Twenty (20) µL of the extract solution were reacted with 150 µL of the FRAP reagent for 30 min under dark conditions. Readings of the colored product were then taken at 593 nm by microplate reader (INNO-S LTEK Co., Ltd.). If the absorbance reading exceeded 1.5 AU (outside the linear range of the detector at 593 nm), a new dilution of the extract solution was prepared and a new reading was made with this diluted solution, applying the corresponding dilution factor to the final calculation. The standard curve was linear between 5 and 30 µM Trolox. The results are expressed in µmol Trolox equivalents/g of dried cocoa sample.

2.6. Determination of Total Phenolic and Flavonoid Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu reagent, following the method described by Salih et al. [28], with slight modifications. A 50 µL volume of the extract solution was mixed with 50 µL of the Folin–Ciocalteu reagent and 1.5 mL of distilled water; the mixture was stirred and allowed to stand for 3 min. It was then neutralized with 50 µL of sodium carbonate solution (20%). The reaction mixture was incubated at 40 °C for 20 min. The absorbance of the resulting blue color was measured at 765 nm using a microplate reader (INNO-S LTEK Co., Ltd.). A calibration curve with gallic acid as standard was produced (200, 400, 600, 800 and 1000 µg/mL) ($y = 0.002x - 0.0784$;

$R^2 = 0.9987$). The TPC is expressed as milligram equivalent of gallic acid per one hundred grams (mg GAE/100 g) of dried cocoa sample.

Total flavonoid content (TFC) was determined according to the method described by Čavar Zeljković et al. [29]. In brief, 1 mL of sample solution was mixed with the same volume of 2% AlCl_3 in methanol. Absorbances were read at 415 nm after 10 min incubation at room temperature. The total flavonoid content was expressed as milligram equivalents of quercetin per one hundred grams (mg QE/100 g) of dried cocoa sample.

2.7. Statistical Analysis

All tests were performed in triplicate, and the results are presented as means $\pm$ standard deviation (SD). The statistical significance of differences between groups was evaluated by one-way ANOVA using SigmaPlot 14.0 software. Differences between means were assessed using Tukey's post hoc test, and significance was determined at a 5% level.

For the correlation analysis among variables, data distribution was first assessed using the Shapiro–Wilk test, applying $p < 0.05$ as the threshold for non-normality. Since the variables did not meet the assumption of normal distribution, Spearman's rank correlation coefficient was used. This non-parametric approach allows for the assessment of monotonic relationships without requiring normality, providing a robust estimation of the strength and direction of associations between variables. All analyses were performed using SigmaPlot 14.0 software (Systat Software Inc., San Jose, CA, USA).

For the response surface methodology, a full 2^2 factorial design with two center points was used to evaluate the main and interaction effects. Each factor was studied at two coded levels (-1 and $+1$), while the center points (0.0) were included in duplicate to estimate the pure error and assess the potential presence of curvature on the response surface. To determine the optimal experimental conditions, a numerical optimization analysis based on the desirability function was performed. Each response variable was transformed into an individual desirability function (d_i), with values ranging from 0 (completely undesirable response) to 1 (completely desirable response). Statistical analysis was performed using Design-Expert software version 25.0.7 (Stat-Ease Inc., Minneapolis, MN, USA).

In order to explore the multivariate structure of the data and analyze the relationships between the evaluated variables, Principal Component Analysis (PCA) was performed using OriginPro 2026 SR1 software (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Effect of Enzymatic Treatments on Total Phenolic and Flavonoid Contents

Phenolic compounds in cocoa beans are commonly classified into extractable and non-extractable fractions based on their solubility and association with the food matrix. Extractable phenolic compounds (EPP) are readily solubilized using aqueous–organic solvents, whereas non-extractable phenolic compounds (NEPPs) remain bound to cell wall components such as polysaccharides and proteins and require hydrolysis for their release [30]. Therefore, the phenolic content measured in this study corresponds primarily to the extractable fraction.

The evolution of total phenolic content (TPC) and total flavonoid content (TFC) during cocoa bean fermentation was significantly influenced by both fermentation time and enzymatic treatments ($p < 0.05$) (Table 3, Figure 2). At day 0, although fermentation had not yet progressed, some variability among treatments was observed. This can be attributed to the natural heterogeneity of fresh cocoa beans, including differences in mucilage content, degree of ripeness and initial biochemical composition. In addition, the application of enzyme solutions dissolved in mucilage prior to the first sampling may have contributed to slight differences in extractability of phenolic compounds and antioxidant measurements

at the initial stage. Therefore, these variations do not reflect fermentation effects but rather inherent biological variability and initial sample handling conditions [16,31].

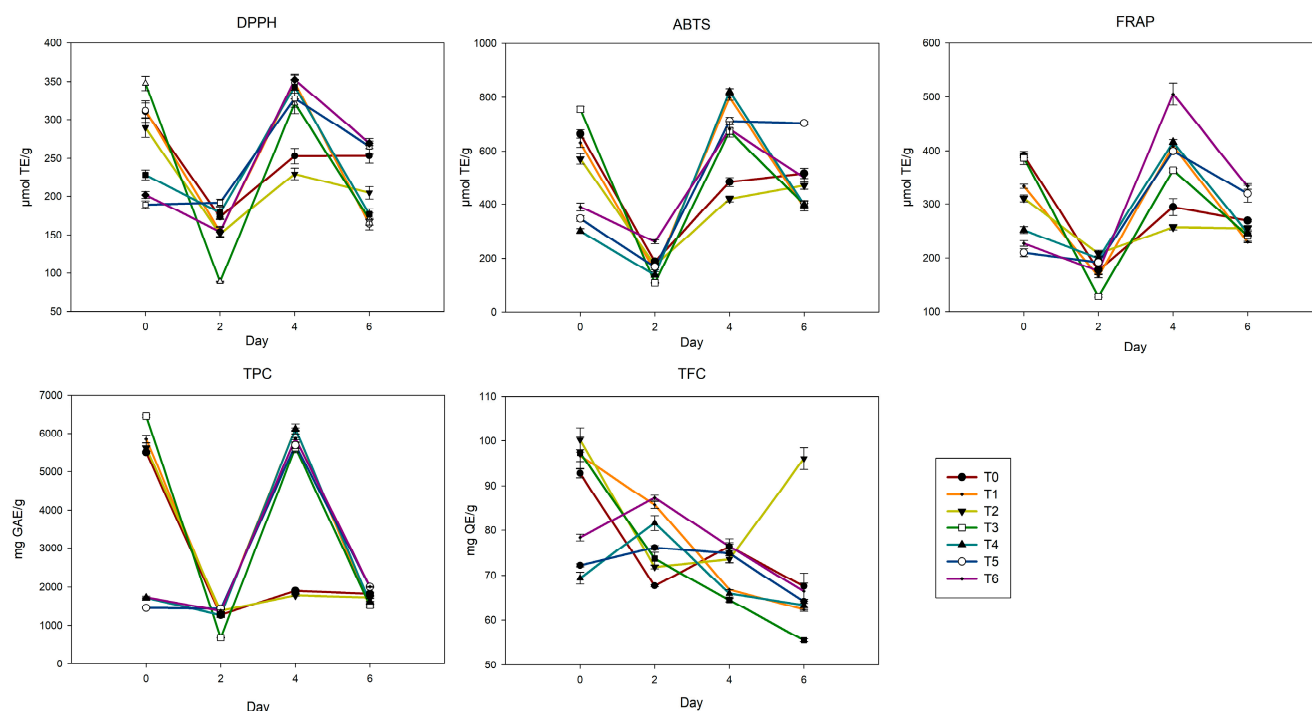


Figure 2. Temporal changes in antioxidant activity (DPPH, ABTS and FRAP) and phenolic and flavonoid contents during enzyme-assisted fermentation of CCN-51 cocoa beans under different enzymatic treatments.

Table 3. Antioxidant activity and phenolic and flavonoid contents of cocoa beans during enzyme-assisted fermentation.

Day	Treatment	TPC (mg GAE/100 g)	TFC (mg QE/100 g)	DPPH ($\mu\text{mol TE/g}$)	ABTS ($\mu\text{mol TE/g}$)	FRAP ($\mu\text{mol TE/g}$)
0	T0	5501.33 $\pm$ 23.09 ^e	92.70 $\pm$ 0.97 ^b	310.43 $\pm$ 14.08 ^{de}	662.69 $\pm$ 15.15 ^{de}	390.61 $\pm$ 2.76 ^c
	T1	5848.00 $\pm$ 105.83 ^c	96.64 $\pm$ 1.32 ^{ab}	311.99 $\pm$ 9.62 ^{de}	630.59 $\pm$ 17.01 ^e	333.27 $\pm$ 3.22 ^e
	T2	5614.66 $\pm$ 133.16 ^{de}	100.19 $\pm$ 2.74 ^a	289.35 $\pm$ 12.18 ^{ef}	570.66 $\pm$ 19.51 ^f	310.40 $\pm$ 5.26 ^{ef}
	T3	6454.66 $\pm$ 30.55 ^a	97.27 $\pm$ 3.50 ^a	347.51 $\pm$ 9.24 ^{ab}	754.65 $\pm$ 10.13 ^b	386.54 $\pm$ 10.06 ^{cd}
	T4	1713.73 $\pm$ 16.74 ^{ij}	69.34 $\pm$ 1.31 ^{hij}	228.15 $\pm$ 6.42 ^{ij}	301.50 $\pm$ 8.83 ^k	252.01 $\pm$ 5.16 ^{gi}
	T5	1445.40 $\pm$ 14.00 ^{klm}	72.17 $\pm$ 0.33 ^{ghi}	188.84 $\pm$ 4.90 ^{kl}	347.06 $\pm$ 10.10 ^j	210.03 $\pm$ 5.99 ^{kl}
	T6	1729.40 $\pm$ 29.46 ^{hij}	78.35 $\pm$ 0.77 ^{ef}	205.61 $\pm$ 7.67 ^{jk}	390.08 $\pm$ 15.03 ⁱ	227.33 $\pm$ 4.27 ^{jk}
2	T0	1266.06 $\pm$ 18.17 ⁿ	67.64 $\pm$ 0.22 ^{ijk}	173.06 $\pm$ 3.24 ^{lm}	188.13 $\pm$ 6.61 ^l	177.21 $\pm$ 1.77 ^{mno}
	T1	1289.40 $\pm$ 25.35 ^{mn}	85.74 $\pm$ 0.8 ^{cd}	153.99 $\pm$ 5.92 ^m	152.85 $\pm$ 3.01 ^{lm}	165.47 $\pm$ 2.30 ^o
	T2	1370.73 $\pm$ 20.81 ^{lmn}	71.78 $\pm$ 0.38 ^{ghi}	151.34 $\pm$ 4.17 ^m	174.75 $\pm$ 2.97 ^{lm}	208.45 $\pm$ 4.89 ^{kl}
	T3	681.06 $\pm$ 7.63 ^o	73.63 $\pm$ 1.40 ^{gh}	88.62 $\pm$ 0.68 ^u	107.83 $\pm$ 1.39 ⁿ	127.73 $\pm$ 1.88 ^p
	T4	1256.73 $\pm$ 26.72 ⁿ	81.61 $\pm$ 1.69 ^{de}	178.37 $\pm$ 7.42 ^{klm}	136.42 $\pm$ 3.45 ^{mn}	199.33 $\pm$ 3.77 ^{lm}
	T5	1428.4 $\pm$ 12.76 ^{lmn}	76.06 $\pm$ 0.46 ^{fg}	191.56 $\pm$ 4.35 ^{kl}	167.96 $\pm$ 4.70 ^{lm}	191.75 $\pm$ 2.90 ^{ln}
	T6	1367.4 $\pm$ 30.34 ^{lmn}	87.35 $\pm$ 0.55 ^c	156.03 $\pm$ 6.15 ^m	262.50 $\pm$ 7.75 ^k	175.31 $\pm$ 4.42 ^{no}
4	T0	1896.73 $\pm$ 27.06 ^{gh}	76.30 $\pm$ 0.88 ^{fg}	252.89 $\pm$ 10.12 ^{gi}	483.90 $\pm$ 17.14 ^{gh}	294.3 $\pm$ 12.81 ^f
	T1	6121.33 $\pm$ 133.16 ^b	66.81 $\pm$ 0.00 ^{jkl}	348.54 $\pm$ 9.69 ^a	798.36 $\pm$ 10.01 ^a	408.82 $\pm$ 2.61 ^{bc}
	T2	1779.40 $\pm$ 21.99 ^{hi}	73.48 $\pm$ 0.72 ^{gh}	229.40 $\pm$ 7.58 ^{hij}	419.95 $\pm$ 10.69 ⁱ	257.28 $\pm$ 4.05 ^{gh}
	T3	5621.33 $\pm$ 130.12 ^{de}	64.43 $\pm$ 0.36 ^{kl}	321.42 $\pm$ 13.52 ^{bcd}	673.67 $\pm$ 9.47 ^{cd}	363.37 $\pm$ 2.65 ^d
	T4	6114.66 $\pm$ 30.55 ^b	65.94 $\pm$ 0.81 ^{jkl}	343.27 $\pm$ 16.25 ^{ac}	821.41 $\pm$ 10.95 ^a	415.89 $\pm$ 3.78 ^b
	T5	5688.00 $\pm$ 87.17 ^{cd}	74.79 $\pm$ 2.09 ^{fg}	327.63 $\pm$ 11.46 ^{ad}	710.34 $\pm$ 13.94 ^c	399.19 $\pm$ 3.13 ^{bc}
	T6	5861.33 $\pm$ 30.55 ^c	76.35 $\pm$ 1.72 ^{fg}	354.23 $\pm$ 3.31 ^a	680.26 $\pm$ 28.61 ^{cd}	505.28 $\pm$ 16.82 ^a

Table 3. Cont.

Day	Treatment	TPC (mg GAE/100 g)	TFC (mg QE/100 g)	DPPH (μ mol TE/g)	ABTS (μ mol TE/g)	FRAP (μ mol TE/g)
6	T0	1818.40 $\pm$ 43.86 ^{hi}	67.54 $\pm$ 2.91 ^{ijk}	253.24 $\pm$ 9.53 ^{gh}	516.7 $\pm$ 19.09 ^g	269.17 $\pm$ 1.74 ^g
	T1	1472.06 $\pm$ 18.87 ^{kl}	62.29 $\pm$ 0.38 ^l	163.89 $\pm$ 7.44 ^{lm}	389.47 $\pm$ 14.67 ⁱ	229.89 $\pm$ 1.12 ^{ijk}
	T2	1720.06 $\pm$ 20.23 ^{ij}	96.01 $\pm$ 2.45 ^{ab}	204.61 $\pm$ 8.16 ^{jk}	470.73 $\pm$ 13.83 ^h	255.17 $\pm$ 4.39 ^{gh}
	T3	1515.40 $\pm$ 19.46 ^{kl}	55.52 $\pm$ 0.33 ^m	170.18 $\pm$ 7.52 ^{lm}	400.65 $\pm$ 13.21 ⁱ	241.63 $\pm$ 1.12 ^{hij}
	T4	1604.06 $\pm$ 18.87 ^{jk}	63.26 $\pm$ 0.94 ^{kl}	176.50 $\pm$ 6.80 ^{lm}	398.93 $\pm$ 12.97 ⁱ	246.74 $\pm$ 4.57 ^{gi}
	T5	1999.73 $\pm$ 15.01 ^{fg}	64.14 $\pm$ 0.44 ^{kl}	265.53 $\pm$ 10.09 ^{fg}	702.26 $\pm$ 5.98 ^{cd}	319.28 $\pm$ 12.88 ^e
	T6	2001.06 $\pm$ 14.01 ^f	66.42 $\pm$ 1.78 ^{jkl}	271.52 $\pm$ 4.03 ^{fg}	501.09 $\pm$ 13.92 ^{gh}	332.97 $\pm$ 4.27 ^e

Values with different letters in the same column are significantly different ($p < 0.05$) according to Tukey's test.

A general decrease in TPC and TFC was observed at day 2 across all treatments, which is consistent with the intense biochemical activity occurring during the early fermentation stage, including polyphenol oxidation and diffusion into the surrounding pulp. Similar trends have been reported during conventional cocoa fermentation and are associated with enzymatic browning and polymerization reactions [32].

Interestingly, a marked increase in TPC was observed at day 4 in treatments containing bromelain and laccase, particularly in treatments combining both enzymes at medium and high concentrations. The advantage of selected enzymatic treatments over the control became evident. For example, T1 and T4 showed TPC values above 6100 mg GAE/100 g, whereas the control remained below 1900 mg GAE/100 g. This increase suggests that bromelain-mediated proteolysis may facilitate the release of phenolic compounds previously bound to proteins or cell wall components [13,33], while laccase may selectively oxidize low-molecular-weight phenolics [34], leading to the formation of more stable phenolic structures detectable by the Folin–Ciocalteu assay. The contribution of flavan-3-ols to this increase in TPC is supported by the data in Table 4. At day 4, epicatechin concentrations in T1 (5.496 mg/g), T4 (5.681 mg/g) and T5 (4.436 mg/g) were 2.2–2.8-fold higher than in the control (T0, 2.489 mg/g), while catechin showed a similar pattern, reaching 0.649 and 0.647 mg/g in T1 and T4, respectively, compared to 0.287 mg/g in T0. In addition, strong positive correlations were observed between TPC and catechin ($r_s = 0.929$, $p < 0.01$) and epicatechin ($r_s = 0.952$, $p < 0.01$) (Table 5), supporting their role as key contributors to the observed TPC rebound.

However, it should be noted that the UHPLC-MS analysis in this study was targeted to flavan-3-ols and methylxanthines and does not represent a comprehensive phenolic profile. Therefore, the contribution of other phenolic classes—such as procyanidins, phenolic acids or other polymeric structures—cannot be fully assessed and should be addressed in future studies using untargeted UHPLC-MS/MS approaches.

At day 6, TPC and TFC values tended to stabilize or slightly decrease, indicating that prolonged fermentation may promote further oxidation or degradation of phenolic compounds. This aligns with the findings reported by Pallares Pallares et al. [35], who state that fermentation has the greatest impact on the variation in total polyphenol content and antioxidant capacity of the CCN-51 cocoa clone. These results demonstrate that enzyme-assisted fermentation can modulate phenolic composition, with day 4 emerging as a critical point for maximizing phenolic content.

Table 4. Flavan-3-ols and methylxanthine contents in cocoa beans during enzyme-assisted fermentation.

Day	Treatment	Theobromine (mg/g)	Caffeine (mg/g)	Catechin (mg/g)	Epicatechin (mg/g)
0	T0	8.410 ± 0.09 ijkr	1.469 ± 0.02 ^r	0.316 ± 0.01 ⁱ	2.868 ± 0.10 ⁱ
	T1	8.453 ± 0.14 ijkp	2.021 ± 0.06 jk	0.440 ± 0.01 ^f	3.970 ± 0.03 ^f
	T2	8.640 ± 0.11 hijkl	1.911 ± 0.01 ^{ln}	0.334 ± 0.00 ⁱ	3.531 ± 0.05 ^g
	T3	9.486 ± 0.13 ef	1.660 ± 0.02 q	0.588 ± 0.00 ^b	5.254 ± 0.04 ^c
	T4	8.801 ± 0.08 gi	1.794 ± 0.01 ^p	0.152 ± 0.00 ⁿ	1.930 ± 0.04 ^k
	T5	8.967 ± 0.15 gh	1.443 ± 0.01 ^r	0.041 ± 0.00 q	0.505 ± 0.02 ^{op}
	T6	9.195 ± 0.11 fg	1.792 ± 0.01 ^p	0.103 ± 0.00 ^o	1.295 ± 0.04 ⁿ
2	T0	8.799 ± 0.12 gj	2.011 ± 0.01 jkl	0.004 ± 0.00 ^s	0.103 ± 0.00 ^r
	T1	8.597 ± 0.13 hikn	1.909 ± 0.02 lo	0.043 ± 0.00 q	0.457 ± 0.02 ^{pq}
	T2	9.469 ± 0.12 ef	2.235 ± 0.01 fh	0.039 ± 0.00 q	0.372 ± 0.00 q
	T3	8.528 ± 0.06 hijko	1.887 ± 0.03 mnop	0.039 ± 0.00 qr	0.464 ± 0.01 ^{pq}
	T4	8.048 ± 0.30 pr	2.075 ± 0.06 jk	0.021 ± 0.00 rs	0.133 ± 0.01 ^r
	T5	8.738 ± 0.30 gk	2.183 ± 0.07 ghi	0.072 ± 0.00 p	0.592 ± 0.02 ^o
	T6	8.230 ± 0.24 lmnop	1.932 ± 0.06 lm	0.053 ± 0.00 q	0.531 ± 0.01 ^{op}
4	T0	9.572 ± 0.11 ef	1.903 ± 0.03 mno	0.287 ± 0.00 j	2.489 ± 0.02 j
	T1	10.304 ± 0.14 bc	2.082 ± 0.05 ik	0.649 ± 0.00 a	5.496 ± 0.04 b
	T2	9.681 ± 0.19 de	1.690 ± 0.02 q	0.167 ± 0.00 n	1.443 ± 0.00 m
	T3	10.242 ± 0.10 bc	2.656 ± 0.04 bc	0.504 ± 0.01 d	4.285 ± 0.03 e
	T4	10.338 ± 0.05 bc	2.781 ± 0.02 a	0.647 ± 0.01 a	5.681 ± 0.10 a
	T5	10.609 ± 0.14 ab	2.341 ± 0.01 de	0.503 ± 0.00 d	4.436 ± 0.03 d
	T6	10.824 ± 0.13 a	2.701 ± 0.04 ac	0.551 ± 0.00 c	4.372 ± 0.04 de
6	T0	10.438 ± 0.10 ac	2.357 ± 0.03 d	0.360 ± 0.00 h	3.101 ± 0.02 h
	T1	9.660 ± 0.20 df	2.249 ± 0.02 efg	0.229 ± 0.00 l	1.755 ± 0.01 l
	T2	10.133 ± 0.03 cd	2.724 ± 0.02 ab	0.416 ± 0.01 g	2.819 ± 0.03 i
	T3	9.476 ± 0.07 ef	2.105 ± 0.01 ij	0.202 ± 0.02 m	1.693 ± 0.02 l
	T4	8.620 ± 0.13 hijkm	2.310 ± 0.00 df	0.247 ± 0.00 k	1.718 ± 0.01 l
	T5	9.503 ± 0.19 ef	2.208 ± 0.03 gh	0.559 ± 0.01 c	4.081 ± 0.03 f
	T6	10.199 ± 0.08 bc	2.643 ± 0.03 bc	0.474 ± 0.01 e	3.527 ± 0.07 g

Values with different letters in the same column are significantly different ($p < 0.05$) according to Tukey's test.

Table 5. Spearman's correlation coefficients between antioxidant activity, phenolic compounds, flavan-3-ols and methylxanthines in cocoa bean.

	ABTS	FRAP	TPC	TFC	Theobromine	Caffeine	Catechin	Epicatechin
DPPH	0.907 **	0.944 **	0.952 **	0.126	0.548 **	0.205	0.864 **	0.901 **
ABTS		0.955 **	0.959 **	−0.053	0.626 **	0.306	0.951 **	0.953 **
FRAP			0.963 **	−0.025	0.619 **	0.322	0.917 **	0.938 **
TPC				0.073	0.577 **	0.224	0.929 **	0.952 **
TFC					−0.407 *	−0.424 *	−0.098	−0.049
Theobromine						0.576 **	0.655 **	0.625 **
Caffeine							0.447 *	0.382 *
Catechin								0.979 **

* $p < 0.05$; ** $p < 0.01$. Values represent Spearman's correlation coefficients (r_s).

3.2. Changes in Antioxidant Activity During Fermentation

Antioxidant activity evaluated by DPPH, ABTS and FRAP assays followed trends closely related to the evolution of phenolic content (Table 3, Figure 2). Across all treatments, antioxidant capacity decreased during the first two days of fermentation, followed by a significant increase at day 4 ($p < 0.05$), especially in enzyme-treated samples.

Treatments combining bromelain and laccase exhibited the highest antioxidant activity values at day 4, particularly in ABTS and FRAP assays. ABTS values reached 798.36–821.41 $\mu\text{mol TE/g}$ in T1 and T4, compared with 483.90 $\mu\text{mol TE/g}$ in the control.

Similar trends were observed for FRAP, supporting that the enzymatic treatment produced measurable improvements in functional parameters, although these effects were treatment- and time-dependent rather than uniformly sustained across the entire fermentation process. This behavior suggests that enzymatic treatments can promote the release or transformation of antioxidant-active compounds during fermentation [21]. The stronger response observed in FRAP may be associated with the presence of phenolic compounds capable of electron donation, while ABTS results indicate enhanced radical scavenging capacity [36].

By day 6, antioxidant activity showed variable behavior depending on the treatment, but values generally remained higher in enzymatically treated samples compared to the control. These findings confirm that enzyme-assisted fermentation can enhance the antioxidant potential of cocoa beans when fermentation time is adequately controlled [37].

3.3. Evolution of Catechins and Methylxanthines During Fermentation

The concentrations of theobromine and caffeine increased progressively during fermentation, contrary to what was reported by Cortez et al. [38] and Ruiz-Santiago et al. [39], for a fermentation process without the use of enzymes. The concentrations reached their highest levels at days 4 and 6 (Table 4, Figure 3). This discrepancy can be mechanistically explained by the specific action of bromelain on the structural organization of the cocoa cotyledon. In fresh cocoa beans, theobromine and caffeine are primarily stored in vacuolar compartments associated with protein bodies, where they form supramolecular complexes with storage proteins and condensed tannins, limiting their extractability in aqueous or hydroalcoholic systems [7,33]. Bromelain-mediated proteolysis destabilizes these protein matrices, disrupting protein–methylxanthine interactions and facilitating the release of methylxanthines into the soluble fraction [33,40]. This effect is further enhanced by the progressive loss of membrane integrity during fermentation, driven by microbial acidification and embryo death [5,6], which increases cellular permeability. In contrast, during enzyme-free fermentation, structural disruption is less pronounced, and the diffusion of methylxanthines into the surrounding pulp during the aerobic phase may partially counterbalance their release, resulting in the net decrease reported in previous studies.

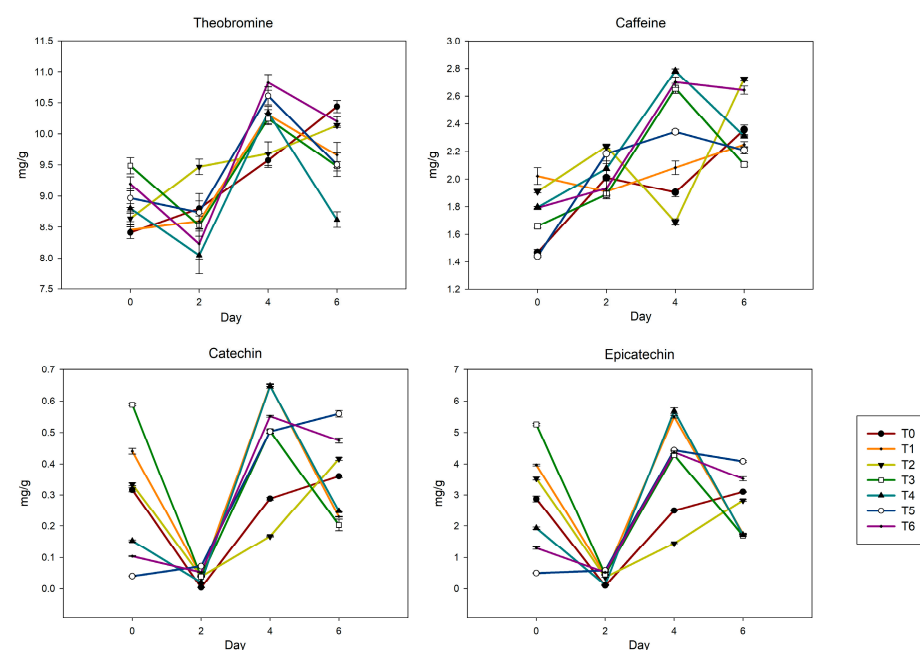


Figure 3. Temporal evolution of bioactive compounds, including methylxanthines (theobromine and caffeine) and flavan-3-ols (catechin and epicatechin), during enzyme-assisted fermentation of CCN-51 cocoa beans.

Catechin and epicatechin contents showed distinct behavior during fermentation. A pronounced decrease was observed at day 2, followed by a significant increase at day 4 in treatments containing bromelain and laccase. Epicatechin reached 5.496 and 5.681 mg/g in T1 and T4 respectively, while the control showed 2.489 mg/g, indicating that the enzymatic treatment improved the retention or recovery of this bioactive compound in intermediate stages of fermentation. This behavior contrasts with conventional cocoa fermentation, in which epicatechin is generally reduced as fermentation progresses due to oxidation and diffusion-related losses [40].

At day 6, both catechin and epicatechin levels tended to decrease, likely due to oxidative degradation and polymerization reactions. These results indicate that enzymatic treatments influence flavan-3-ol stability and availability during fermentation, with optimal levels achieved at intermediate fermentation stages.

The values reported for methylxanthines and flavan-3-ol in our study are similar to those reported by Carrillo et al. [23] for cacao beans from different cocoa-growing areas in Colombia, but the values are higher than those reported by Polanía-Hincapié et al. [41] for theobromine and epicatechin in different cocoa clones, including CCN-51.

It should be noted that the present results do not demonstrate a reduction in total fermentation time. Rather, they indicate that enzyme application modulated the temporal evolution of functional compounds, with the most favorable responses observed at intermediate stages, particularly day 4.

3.4. Correlation Analysis Between Total Phenolic Content, Antioxidant Activity, Flavan-3-Ol and Methylxanthines

Spearman's correlation analysis revealed strong positive correlations between TPC and antioxidant activity measured by DPPH, ABTS and FRAP (r_s values ranged from 0.907 to 0.963), confirming that phenolic compounds are major contributors to the antioxidant capacity of fermented cocoa beans [42,43]. Highly positive correlations were also observed between catechin, epicatechin and antioxidant assays (r_s values ranged from 0.864 to 0.953), highlighting the role of flavan-3-ol in radical scavenging and reducing power [44] (Table 5).

These correlations support the hypothesis that enzyme-assisted fermentation enhances antioxidant activity primarily through the modulation of phenolic composition rather than changes in methylxanthine content.

3.5. Principal Component Analysis (PCA)

Principal component analysis (PCA) was performed to explore the variability in the chemical and antioxidant profiles of the cocoa samples (Figure 4). The first two principal components explained 86.8% of the total variance, with PC1 accounting for 67.7% and PC2 for 19.1%, indicating that the two-dimensional model adequately represents the relationships among the evaluated variables.

The PCA biplot revealed a clear separation of samples according to enzymatic treatment and fermentation time, suggesting that the experimental conditions significantly influenced the biochemical composition of the cocoa beans. Samples located in the upper-right quadrant of the plot were associated with higher levels of total phenolic content (TPC) and antioxidant activity (DPPH and ABTS), whereas those positioned in the lower-right quadrant showed stronger associations with methylxanthines, particularly caffeine and theobromine. In contrast, samples distributed toward the left side of the plot were characterized by comparatively lower concentrations of bioactive compounds.

The close angular proximity between the vectors of TPC, DPPH and ABTS indicates a strong positive correlation between total phenolic content and antioxidant capacity, confirming that phenolic compounds are the primary contributors to the antioxidant activity of cocoa during fermentation. Similarly, catechin and epicatechin exhibited closely

aligned vectors, reflecting their related chemical behavior as flavan-3-ols. Caffeine and theobromine also showed a strong positive association, consistent with their structural similarity and shared metabolic origin.

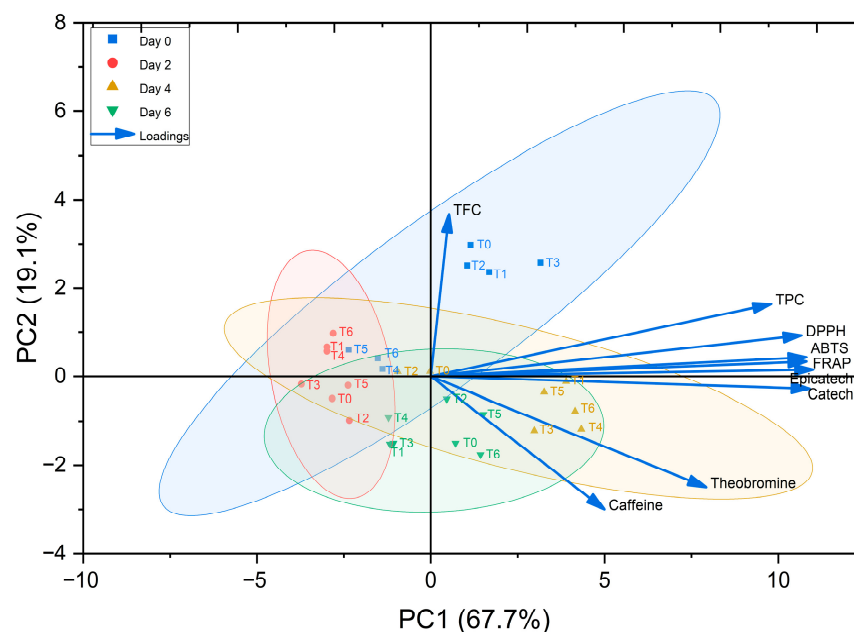


Figure 4. PCA biplot showing sample scores and variable loadings for phenolic compounds, methylxanthines and antioxidant activity during enzyme-assisted fermentation of cocoa beans.

3.6. Response Surface Methodology (RSM)

A full factorial 2^2 experimental design with two central points was applied to evaluate the combined effects of bromelain (factor A) and laccase (factor B) on antioxidant activity and bioactive compounds during cocoa fermentation. The central points were included to assess the presence of curvature in the experimental domain.

3.6.1. Effects of Bromelain and Laccase

Figure 5 presents the standardized Pareto charts showing the effects of both enzymes on antioxidant activity (DPPH, ABTS and FRAP), phenolic composition (TPC and TFC) and the concentrations of theobromine, caffeine, catechin and epicatechin. In these charts, the height of each bar represents the absolute value of the estimated effect, while the color indicates its direction: positive effects (orange) correspond to an increase in the response variable, whereas negative effects (blue) indicate a reduction. The vertical reference line corresponds to the statistical significance threshold ($\alpha = 0.05$), and bars exceeding this limit represent statistically significant effects.

The Pareto diagrams indicate that bromelain was the most influential factor for most of the evaluated responses, particularly antioxidant activity (DPPH and FRAP), total phenolic content and methylxanthine concentrations. In contrast, laccase showed a less consistent influence and was generally not statistically significant for most variables. However, the interaction between bromelain and laccase (AB) became relevant for specific responses such as total flavonoid content and epicatechin, suggesting that combined enzymatic treatments may modulate particular components of the cocoa bioactive profile.

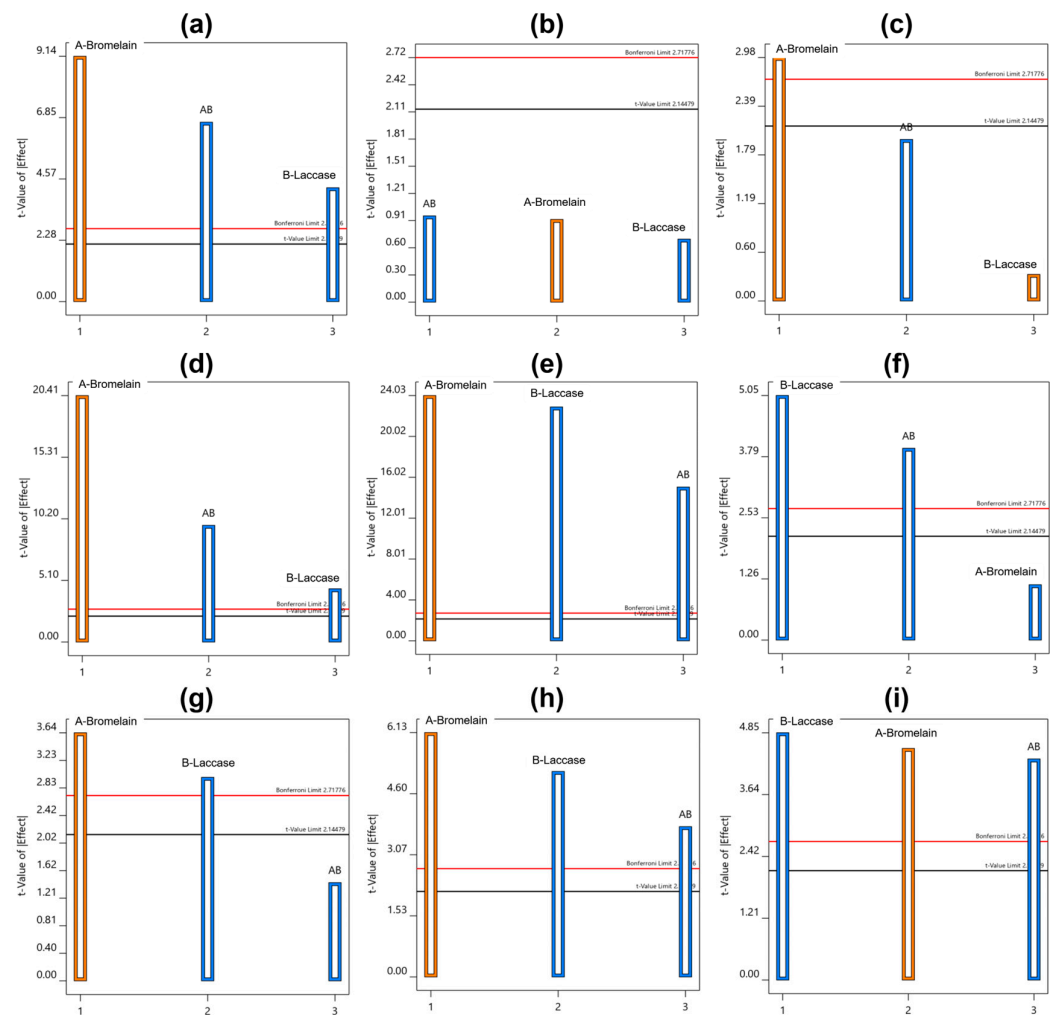


Figure 5. Standardized effects for (a) DPPH, (b) ABTS, (c) FRAP, (d) TPC, (e) TFC, (f) Theobromine, (g) Caffeine, (h) Catechin and (i) Epicatechin.

3.6.2. Optimization Criteria

The use of response surface methodology has been previously reported as an effective tool to optimize cocoa fermentation processes and improve product quality [45]. In the present study, response surface analysis and desirability function optimization were used to determine the enzymatic conditions that maximize bioactive compound content while minimizing enzyme usage. As shown in Figure 6, red points represent the experimental conditions evaluated, whereas the dashed cross indicates the optimal condition predicted by the model.

The response surface analysis revealed a significant quadratic effect of bromelain on desirability, with an optimal region at intermediate levels, whereas laccase exhibited a predominantly negative linear effect across the experimental domain. The contour and 3D surface plots indicated a moderately interactive system, where maximum desirability was achieved under conditions of high bromelain and low laccase concentrations. These findings suggest that bromelain plays a dominant role in enhancing the response, while excessive laccase levels may induce detrimental effects, likely associated with oxidative reactions.

The desirability function predicted an optimal enzymatic combination of 52.19 g of bromelain and 18 g of laccase per 5 kg of cocoa beans, maximizing the overall response across bioactive parameters. This optimum lies within the experimental domain (bromelain: 18–57 g; laccase: 18–57 g per 5 kg), supporting the validity of the interpolation. The

predicted values at this condition were: TPC = 5980 mg GAE/100 g, DPPH = 342 μ mol TE/g, ABTS = 795 μ mol TE/g, FRAP = 405 μ mol TE/g, and epicatechin = 5.38 mg/g.

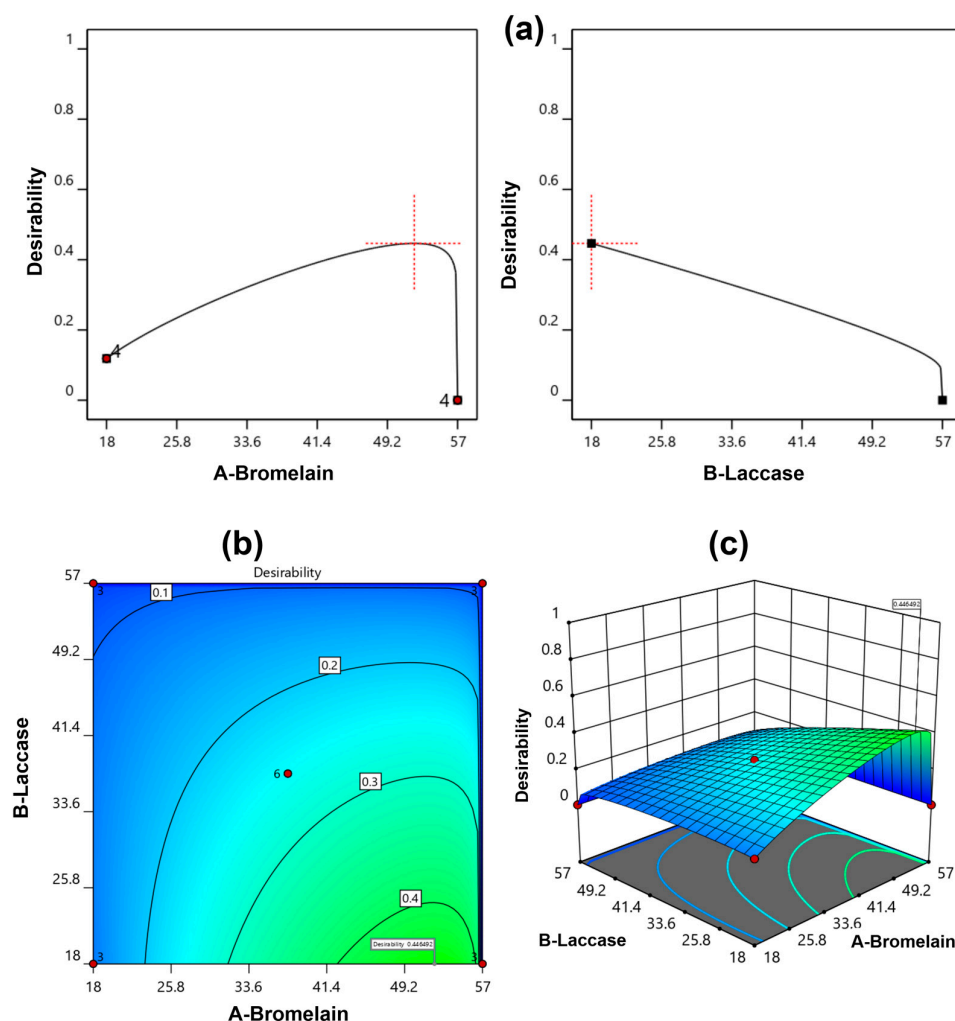


Figure 6. Response Surface for optimization based on the desirability function: (a) individual desirability functions, (b) contour surface, (c) and response surface.

Experimental validation of this predicted optimum was not performed within the scope of the present study, and this is acknowledged as a limitation. Validation of RSM-derived conditions is recommended to assess prediction accuracy and model reliability [45]. Therefore, future studies should include confirmatory fermentation trials under the predicted optimal conditions and report the deviation between predicted and experimental values.

This study demonstrates that enzyme-assisted fermentation represents a feasible technological strategy to enhance the functional quality of cocoa beans. The combined application of bromelain and laccase modulated phenolic release, antioxidant capacity and bioactive compound profiles, while response surface methodology allowed the identification of optimal enzymatic conditions. These findings provide a scientific basis for the controlled use of enzymes as an innovative approach to improve cocoa processing and value-added functional properties.

A limitation of the present study is that flavor-related attributes, such as volatile composition, sensory properties or the fermentation index, were not assessed. Therefore, although the enzymatic treatment improved several functional and bioactive parameters,

its impact on organoleptic quality should be confirmed in future studies integrating sensory and aroma analyses.

4. Conclusions

This study demonstrated that enzyme-assisted fermentation significantly influences the biochemical composition and antioxidant properties of CCN-51 cocoa beans. The application of bromelain and laccase modified the evolution of phenolic compounds, methylxanthines and antioxidant activity during fermentation, with enzyme-treated samples showing enhanced bioactive profiles, particularly at intermediate fermentation stages. Multivariate analysis confirmed that phenolic compounds were the main contributors to antioxidant capacity, highlighting their key role in the functional quality of cocoa beans. Response surface methodology revealed that bromelain was the most influential factor affecting most response variables, while laccase showed a less consistent effect but contributed to specific interactions. Optimization using the desirability function predicted an optimal enzymatic combination of 52.19 g of bromelain and 18 g of laccase per 5 kg of cocoa beans, which maximized bioactive compound levels while minimizing enzyme use. These findings indicate that controlled enzymatic treatments represent a promising technological strategy for improving cocoa fermentation and enhancing the functional value of cocoa-derived products. Although the treatment did not shorten the overall fermentation period under the tested conditions, it improved selected functional parameters at intermediate stages, supporting its potential as a complementary strategy for modulating cocoa bioactive quality. Future studies may explore the scalability of enzyme-assisted fermentation and its potential impact on industrial cocoa processing.

Author Contributions: Conceptualization, G.V.-A., H.M.-V. and W.C.; methodology, G.V.-A., W.C. and C.M.A.-E.; software, J.O. and S.F.; formal analysis, J.O., C.M.A.-E. and S.F.; writing—original draft preparation, G.V.-A. and J.O.; writing—review and editing, G.V.-A., W.C. and H.M.-V. All authors have read and agreed to the published version of the manuscript.

Funding: G.V.-A. acknowledges funding from the research circle: Evaluation of bioactive compounds and functional foods—IAP. COD. CEPLAN AOI00005300088.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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