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Metabolomic insights into flavour precursor dynamics during fermentation of cacao beans cultivated in diverse climatic production zones in Colombia

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ABSTRACT

The market for flavour superior quality cacao provides significant economic and non-economic benefits to farmers. Flavor precursor metabolites, formed during various post-harvest stages, are crucial for developing superior sensory attributes. However, identifying these metabolites and understanding how climate variations and post-harvest practices influence them remains a challenge. This study investigates how the fermentation methodology applied and climate conditions in different zones of the cacao beans producing region of Arauca – Colombia, influence the metabolomic profile of cacao beans and their flavour precursor metabolites.

Untargeted metabolomic analysis was performed by UHPLC-ESI-Orbitrap-MS on cacao beans fermented for 0, 24, 48, 72, 96, and 120 h from 9 production zones. The PLS-DA model highlighted that the metabolomics fingerprint changes through fermentation time. Among the most discriminant metabolites, 18 oligopeptides, sucrose, glucose, fructose, flavanols, and acids were tentatively identified. The chemometric analysis showed that fermentation time has a significant impact on the metabolomic profile of cacao beans, while agroclimatic conditions had a minor influence.

Metabolomic analyses defined 96 h as the optimal fermentation time to maximize the amount of aroma precursors. Metabolomic analyses identified 96 h as the optimal fermentation time to maximize the amount of aroma precursors across all 9 cacao production zones evaluated. This study underscores the central role of fermentation in shaping flavor precursors, and contributes to the development of new approaches for cacao processing based on the tracking of biochemical and functional compounds (quality biomarkers).

1. Introduction

Cacao is the main ingredient of chocolate, which is one of the most esteemed and globally products consumed in developed markets and is increasingly penetrating new ones, mainly in Asia and Latin America (Guzmán Penella et al., 2023). Superior quality flavor cacao can be distinguished by its special aromas, complementary sensory attributes as fresh fruits, browned fruits, floral, vegetal, woody, nutty, spice, and caramel notes as well as rich and balanced chocolate bases (Cacao of Excellence 2023). The quality of cacao is influenced by different factors, such as the climate conditions of production and genetic diversity (Kongor et al., 2016; López-Hernández et al., 2022), as well as post-harvest and processing practices (Herrera-Rocha et al., 2024), with fermentation being one of the most crucial stages (Caligiani et al., 2016; Escobar et al., 2021; Hinneh et al., 2019; Santander et al., 2021; Santander Muñoz et al., 2020). During cacao bean fermentation, bacteria and yeasts present in the cacao environment initiate a series of biochemical reactions that transform the main components of cacao seeds, primarily proteins, and carbohydrates, into simpler compounds such as peptides, amino acids, fructose, glucose sucrose, which are

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known as flavor precursor metabolites. These metabolites react during roasting to generate flavour that gives rise to the formation of some sensory attributes in chocolate (Salger et al., 2019; Santander et al., 2021). The variation in the presence and concentration of these metabolites during fermentation has allowed the understanding of biochemical processes (Xiao et al., 2012) related to the development of chocolate flavour (Balcázar-Zumaeta et al., 2023). In this sense, depending on the type of compounds generated, chocolate production can be directed in different ways to enhance specific quality-related sensory attributes (Herrera-Rocha et al., 2023; Hinneh et al., 2018; Kumari et al., 2018; Marseglia et al., 2014).

In recent years, different studies have been focused on the importance of tracking the flavour precursors formed during fermentation, due to the interest in variation dynamics observed in them, for example, some metabolites increase, such as reducing sugar and peptides (Becerra et al., 2022; D'Souza et al., 2018), while others decrease, such flavanols (Febrianto & Zhu, 2020), and depending on how this dynamic goes, the potential impact on the flavour characteristics can vary (Hinneh et al., 2018; Kongor et al., 2016; Scalone et al., 2019; Yu et al., 2018).

After carbohydrates, proteins are the second most abundant macronutrients in cacao beans, with albumins and vicilin-like globulins being the most prevalent (Caligiani et al., 2016). During fermentation, these proteins are degraded by enzymatic hydrolysis to their constituent components, amino acids, and oligopeptides, facilitated by specific enzymes such as cysteine endo-protease, leucine-p-nitroanilide cleaving seryl exopeptidase, aspartic endoprotease, and carboxypeptidase (Biehl & Ziegleder, 2003). These amino acids and oligopeptides subsequently act as flavour precursors. The Maillard reactions are crucial for flavour formation in cacao, starting with amino acids, short peptides, or amine-containing molecules reacting with aldehydes from reducing sugars. This reaction forms initial "Amadori" compounds, which then undergo further transformations, leading to diverse flavour compounds. Proteogenic molecules, especially peptides, play a particularly significant role in this process, as oligopeptides are more reactive than individual amino acids (D'Souza et al., 2018). On the other hand, methylxanthines and flavonols (caffeine, theobromine, catechin and epicatechin) contribute to the bitterness and astringent in cacao beans. During fermentation, the concentrations of these bioactive compounds should be reduced to a level that improves the palatability of the resulting chocolate (Cortez et al., 2023).

All these chemical compounds can be associated with desirable or undesirable characteristics in cocoa. Desirable flavors are pleasant and balanced characteristics that contribute to a high-quality sensory profile (Afoakwa et al., 2008; Rodriguez-Campos et al., 2011). These include sweet and fruity notes, floral and citrus aromas, caramel and nutty flavors, and balanced bitterness. Such properties are highly sought after in premium chocolates, particularly in fine or flavor-grade cocoa (Herrera-Rocha et al., 2021; Rodriguez-Campos et al., 2011, 2012). Undesirable flavors, on the other hand, are negative characteristics that can affect product acceptance. These include excessive acidity, overpowering bitterness or astringency, musty or earthy flavors, unpleasant fermented aromas, and sometimes herbaceous or vegetal notes (Rodriguez-Campos et al., 2011). These desirable or undesirable attributes are linked to specific chemical compounds. Desirable compounds include esters, aldehydes, alcohols, lactones, and pyrazines, which contribute to pleasant flavors. Conversely, undesirable compounds, when present in high concentrations, include acetic acid, lactic acid, and polyphenols (such as theobromine, catechin, and epicatechin), among others (Afoakwa, 2014; Afoakwa et al., 2009).

Several studies have indicated that genetic diversity, climate conditions, the geographical origin of cacao production zones, and post-harvest practices, especially fermentation, play a significant role in influencing cacao quality. However, the direct relationship and influence between climate conditions metabolomic profiles and the consequent dynamic behavior of the precursor metabolites, as well as sensory profiles have not been conclusively established. While there are hypotheses regarding the biochemical changes occurring during cacao fermentation, gaps remain in understanding these dynamics at the molecular level. Therefore, identifying and characterizing the metabolomic fingerprint and chemically unknown compounds associated with the aroma and flavour development of cacao derived products is essential for quality differentiation (Fayeulle et al., 2020).

Determining the chemical composition for traceability is essential for tracking the quality of chocolate. Monitoring this data during post-harvest processes like fermentation, allows for the potential maximization of desirable compounds and minimization of undesirable ones, following process variables such as fermentation time during spontaneous fermentation. This could result in chocolate with desirable flavour and aromatic characteristics while ensuring consistency in the quality of the final product. Chemometric analysis is crucial for interpreting the complexity of the chemical composition of the cacao beans.

This study aims to explore the impact of fermentation processes and the climatic conditions of cacao production zones in Arauca – Colombia, on the chemical transformation of cocoa beans. Through chemometric and metabolomic analyses, the research seeks to identify key biomarkers associated with cacao quality, offering insights into how fermentation and climate variables influence the development of flavour precursors.

2. Materials and methods

2.1. Chemicals

Acetone, acetonitrile (MeCN), and formic acid (HCOOH) liquid chromatography-mass spectrometer (LC-MS) grade was purchased from Merck (Darmstadt, Germany). Analytical grade fructose, glucose, and sucrose (all contents (99 %) were purchased from Sigma-Aldrich (North Harrison, USA). Acetic acid (99.5 % ACS reagent, Sigma-Aldrich, St. Louis, Missouri, USA), lactic acid (99 %, Sigma-Aldrich), citric acid ($\geq$98 %, Sigma-Aldrich), (+)-catechin (98 %, Sigma-Aldrich), epicatechin (98 %, Sigma-Aldrich), theobromine (98 %, Sigma-Aldrich) and caffeine (98 %, Sigma-Aldrich). Standards of sugars and acids were prepared in Milli-Qwater. Catequin, epicatechin, theobromine and caffeine were prepared in a mixture of MeOH: H2O (50:50). Ionized water was produced using a Barnstead™ Smart2Pure™ (Thermo Scientific™, Waltham, MA, USA) purification system and was used to prepare the mobile phase and standard.

2.2. Fermentation of cacao seeds

Cacao sourcing was carried out following a climate-based approach, based on previous studies and classifications conducted by González-Orozco et al. (Gonzalez-Orozco et al., 2023; Llano et al., 2024). This study provided a database on the spatial distribution of cocoa farms in Arauca and agroclimatic maps identifying and locating the climatic regions for cacao production in the area.

Seven climate variables were used to perform the statistical analysis against the predefined cacao regional clusters in Arauca. These variables included wind speed, relative humidity, minimum temperature, maximum temperature, average temperature, annual precipitation, and solar radiation. Additionally, 11 soil temperature variables were analyzed at a depth of 0–5 cm. These variables included mean annual temperature, average diurnal range, isothermality, temperature seasonality, maximum temperature of the hottest month, minimum temperature of the coldest month, annual temperature range, mean temperature of the wettest month, mean temperature of the driest month mean temperature of the wettest quarterly period, and mean temperature of the coldest quarterly period.

The cacao used in this study was similarly sourced from the same 9 independent climatic zones to ensure consistency and relevance to the research objectives. A total of 180 representative farms were selected, comprising 20 farms from each climatic zone. The selection included a diverse range of cacao cultivars, both local hybrids and well-known

varieties from Arauca, such as FSA13, FEAR5, FTA2, due to their success in global competitions, and commercial clones like CCN 51, ICS95, and TSH565. This approach helps to minimize the impact of genetic and heterogeneity on the potential differences that could be observed in the results, thereby isolating the effects of climatic factors on chocolate quality.

Only healthy, fully ripe cacao pods were harvested one day before fermentation and transported to a central postharvest processing facility. The fermentation process was conducted following the methodology described by (Escobar et al., 2021; González-Orozco et al., 2023; Llano et al., 2024; Santander et al., 2021), with minor modifications. Cacao pods were carefully opened, and the seeds were manually extracted and mixed to form a homogenous initial batch. From this batch, 150 kg of seeds were divided into three separate 50 kg batches. Each batch was placed in independent compartments of a validated wooden fermenter system. The first aeration of the cacao mass was performed manually after 48 h using a wooden shovel, followed by subsequent mixing every 12 h, continuing until the fermentation reached 120 h.

Cacao Sampling for metabolite dynamics analysis during fermentation:

A 50 g sample of cacao beans was manually collected from the top, middle, and bottom of each compartment at various fermentation times (0, 24, 48, 72, 96, and 120 h). The samples were immediately stored at −18 °C to preserve their integrity. Subsequently, these samples were lyophilized using an Eyela FDU-1100 (Tokyo Rikakikai, Japan). The dried cacao beans were manually deshelled and then ground into a fine powder (< 1 mm) using a Moulinex grinder (KitchenAid, China). The resulting powder was stored at −20 °C. For defatting, the powder underwent soxhlet extraction with hexane (boiling point 40–70 °C) for 4 h. The defatted powder was then stored at −20 °C.

2.3. Untargeted metabolomic

Six replicates of cacao extracts were prepared for each fermentation time evaluated.

4 g cocoa powder were defatted using a soxhlet extractor (SOX406 Hanon Group) boiling point 40–70 °C) for 4 h. The defatted powder was then stored at −20 °C. About 0.5 g of the defatted cocoa was mixed with 2.5 mL of an acetone/water/formic acid solution (70/29.5/0.5) and stirred in an Orbital Shaker at 120 rpm for 30 min. The extraction mixture was then subjected to ultrasound for 5 min at 30 °C, followed by centrifugation at 5590 RCF for 5 min, and the supernatant was collected. This process was repeated twice more, combining the three supernatants into one vessel. The combined supernatant was concentrated using a speed Vac Savant for 30 min at room temperature, then diluted with mobile phase A in a 5 mL volumetric flask. Finally, the diluted extract was filtered through PVDF membranes with a pore size of 0.22 μm, yielding the cocoa extract for untargeted metabolomic analysis by (Mayorga-Gross et al., 2016a). As part of quality control, a pooled sample was prepared by combining 100 μL from each sample. Liquid chromatography separation was performed on a Thermo Scientific™ Ultimate 3000 HPLC system (Waltham, MA, USA), equipped with a UPLC CSH C18 column (1.7 μm particle size, 2.1 mm × 100 mm, Waters Inc.) and a VanGuard CSH C18 pre-column (1.7 μm particle size, 2.1 mm × 5 mm, Waters) maintained at 30 °C. The mobile phase consisted of H_2O/HCOOH (99/1, v/v) as solvent A and CH_3CN/HCOOH (99/1, v/v) as solvent b. The flow rate was set at 0.4 mL/min, with an injection volume of 2 μL. The elution program was as follows: 0–10 % B (0–7 min), 10–95 % B (7–22 min), 95–0 % B (22–22.1 min), followed by an isocratic hold at 0 % B (22.1–26 min)

Mass spectrometry analysis was performed using an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source operated in positive ion mode. The ionization voltage was set to 3500 V, with sheath, auxiliary, and sweep gases set to 40, 10, and 2 arbitrary units, respectively. The ion transfer tube and vaporizer temperatures were set to 280 °C and 300 °C, respectively. The mass range was set from 80 to 1200 *m/z*. The RF lens was set at 50 %, and the Automatic Gain Control (AGC) target was determined using 1 microscan. The resolution was set to 70,000. The acquisition sequence began with four injections of a solvent blank at the start of the sequence followed by four injections of the pooled sample. Subsequently, a mobile phase blank and the pooled sample were injected every five sample injections for compound normalization. Additionally, the run order of experimental samples was randomized to ensure that comparisons of metabolite concentrations in the subsequent analysis were not affected by temporal drift in instrument performance.

2.4. Identification of discriminating metabolites

To identify discriminant metabolites, exact mass and fragmentation patterns were analyzed at different collision energies (10, 20, 30, and 40 eV). These patterns were matched against mass spectra and/or the exact mass of ions (with a maximum tolerance of 10 ppm) in several databases, including METLIN (metlin.scripps.edu), HMDB (https://www.hmdb.ca), Protein Prospector (prospector.ucsf.edu/prospector/mshome.htm), ReSpect for Phytochemicals (spectra.psc.riken.jp), and CFM-ID (cfmid.wishartlab.com). For peptide identification, Peaks Studio (trial version, bioinfor.com/peaks-studio) was utilized. The amino acid sequences of peptides were compared with those in the UniProt database (Switzerland) for the identification of Theobroma cacao vicilin-like globulin and albumin.

2.5. Targeted metabolomic analysis

2.5.1. Quantitation of flavan-3-ols and methylxanthines

Flavan-3-ols and methylxanthines were extracted following the protocol described by Becerra et al. (2023), with slight modifications. Briefly, 0.5 g of the sample was subjected to ultrasound-assisted extraction using 3 mL of acetone:water: formic acid (70:29.5:0.5) for 5 min in an ultrasound bath. The extract was then shaken for 5 min and centrifuged at 6000 rpm for 10 min. This extraction procedure was repeated twice more. The combined supernatants were evaporated to dryness, and the residue was redissolved in 3 % acetonitrile to a final volume of 5 mL and filtered using a 0.22 μm PVDF membrane. The resulting extracts were diluted, as necessary, to ensure their concentrations fell within the range of the calibration curve.

Liquid chromatography (LC) assays were performed using a Waters Acquity UPLC system with a Photodiode Array (PDA) detector (Waters Corporation, Milford, MA, USA). An Acquity HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm particle size) with a guard-column from Waters (Waltham, MA, USA) was used for chromatographic separation. The mobile phases were: A: 0.1 % formic acid in water; B: 0.1 % formic acid in acetonitrile. The flow rate was maintained at 0.4 mL min^{-1}, with a total run time of 19 min. The elution was conducted using a gradient as follows: 5–10 % B (0–6 min), 10–25 % B (6–10 min), 25–80 % B (10–10.1 min), 80–5 % B (10.1–11 min), isocratic 5 % B (11–12.5 min). The oven temperature was set at 40 °C, and the injection volume was 10 μL. The PDA detector was set at 280 nm for quantitative analysis, with a data acquisition rate of 20 points/sec.

Quantification was performed using external calibration. Calibration curves were constructed using six points at different concentrations (ranging from 0.1 to 100 mg/mL), with each standard solution injected three times. Samples were analyzed in triplicate, and the mean and standard deviation were reported.

2.5.2. Quantitation of organic acids and reducing sugars

Organic acids and reducing sugars were extracted following the protocol described by Ho et al. (2014), with slight modifications. Ground cacao seed samples (1.0 g) were mixed with 5 mL of MilliQ water, shaken for 5 min, and then subjected to ultrasound-assisted extraction for another 5 min. The mixture was then centrifuged at 6000 rpm for 10 min. This extraction procedure was repeated two more

times, and the combined supernatants were filtered through a 0.22 μm PVDF membrane.

For the quantification of acids, liquid chromatography (LC) assays were performed using a Waters Acquity UPLC system with a refractive index detector (RID). An Aminex HPX-87H column (Biorad, Hercules, CA) was used for chromatographic separation. The mobile phase consisted of a 0.1 mM sulfuric acid solution, with a flow rate of 0.5 mL/min in isocratic mode. The total analysis time was 20 min. The oven temperature was maintained at 45 °C, and the injection volume was 10 μL. Quantification was achieved using external calibration, with the acid calibration curve ranging from 750 mg/kg to 75000 mg/kg.

For the quantification of sugars, the same LC system, column, and chromatographic conditions were used. The sugars calibration curve covered a range from 450 mg/kg to 15000 mg/kg. Quantification was similarly conducted using external calibration standards.

2.6. Data analysis

Multivariate analyses were performed using SIMCA 13.0.2.0 software (Umetrics, Umeå, Sweden). A supervised Partial Least Squares Discriminant Analysis (PLS-DA) was applied to fermentation time groups (0, 24, 48, 72, 96, and 120 h) to identify discriminant metabolite markers. The discriminant power of each metabolite was evaluated using the Variable Importance in Projection (VIP) score, with the top 25 metabolites having VIP scores greater than 1.0 considered relevant.

The significance of the PLS-DA model was assessed using a cross-validated analysis of variance (CV-ANOVA) p-value, and the model's quality was evaluated based on the parameters R^2X, R^2Y, and Q^2X. A permutation test (n = 100) was conducted to assess data overfitting. Each variable was assigned a VIP index to indicate its discriminative power between the different groups in the PLS-DA model. Overall, the metabolites selected for further study had VIP values greater than 1 and ANOVA p-values less than 0.05.

3. Results

3.1. Untargeted metabolomic profiling of cacao beans: Insights into fermentation-driven metabolomes change

Untargeted metabolomics, coupled with multivariate statistical analysis, was employed to evaluate the impact of fermentation time on the metabolite profiles generated during the transformation of seeds into cacao beans, sourced from nine distinct climatic production zones, in order to assess how environmental factors influence the development of flavor precursor. Approximately 2,500 ions were detected in a full scan range of 70 to 1,200 Da in positive mode after processing the raw data files.

The PLS-DA model allowed discrimination of metabolites between 6 fermentation times in all the samples collected from the 9 climate cacao production zones assessed, as shown in Fig. 1. The model was statistically significant, all validity tests were deemed statistically acceptable $R^2Y = 0.717$, $Q^2Y = 0.52$. The analysis of variance of the cross-validation predictive residual (CV-ANOVA) yielded a low p-value (<0.05), and for the permutation test (n = 100) indicated a low probability of model overfitting, as shown in Fig. S1.

The results revealed a clear evolution in the chemical composition of cacao beans during the fermentation process, with significant differences in metabolomics profiles at different fermentation times, including data from the 9 production zones. In the initial phase, the metabolome, represented by PLS-DA latent variables, moves according to the vertical axis and later along the horizontal axis. All cocoa samples from 0 to 48 h of fermentation, corresponding to exothermic alcoholic fermentation, and samples from 72 to 120 h of fermentation, corresponding to post-mortem reactions, were grouped separately, showing significant differences in metabolomic composition. The 9 cocoa production zones studied presented the same metabolomic profile

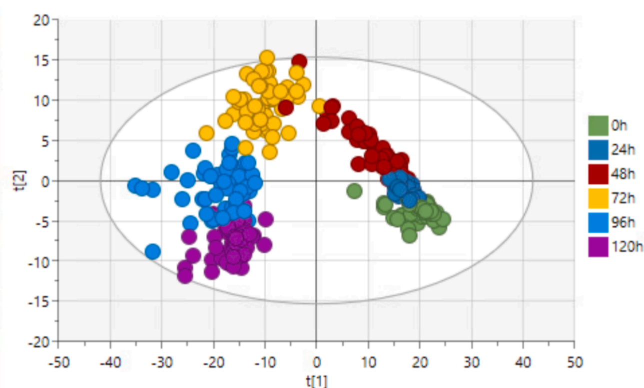

Fig. 1. Partial least square discriminant analysis (PLS-DA) score of the data set 0 h, 24 h, 48 h, 72 h, 96 h and 120 h. Permutation test for the sample dataset [n = 100; R^2 = (0.0, 0.822), Q^2= (0.0, −0.206)].

throughout the fermentation process, indicating that climatic variations did not influence the dynamic changes in the chemical composition of cocoa. Therefore, we chose to represent all the data in a single graph (Fig. 1).

The PLS-DA model highlighted the metabolites contributing most to the observed variations, offering valuable insights into the biochemical changes occurring during the cacao fermentation process.

Table 1 shows the most discriminating metabolites found by PLS-DA across all fermentation time. Among these, 18 peptides and several other metabolites, including 4-butyrolactone, succinic anhydride, 2-hydroxyphenethylamine, aspartic acid, and eleostearic acid, were tentatively identified. Some metabolites remained unidentified. The peptides were the most significant group of discriminant metabolites, consistent with previous findings (Caligiani et al., 2016; Santander et al., 2021).

In our study, some dipeptides (VW, WF and WL), tripeptides (LAL, VLY, LSW, DVF, MSF, FLF), tetrapeptides (LAIN, TDPQ) and peptides with more than 6 units (GINDYR, VPSKLVDN, APLSPGDVF, DEEGNFKIL, IFVPHYNSKATF, DNEWAWM and DNEWAWMFK) were tentatively identified.

These peptides, with a molecular weight between 303 and 1424 Da, are crucial precursors for the development of cacao aroma (Caligiani et al., 2016). Peptides are likely to enhance some flavours during roasting (Ogasawara et al., 2006). Some of the discriminant oligopeptides found in this study have also been reported as relevant in previous works, including DEEGNFKIL, APLSPGDVF, DVF according to (Caligiani et al., 2016). The peptides LSF, DVF, WL, WF, VW were previously reported by (D'Souza et al., 2018) and WF, WL e IFVPHYN according to (Mayorga-Gross et al., 2016b; Santander et al., 2021).

In this study, we found that, after the fermentation, the number of differentiating peptides derived from vicilin was more abundant than the peptides derived from albumin (highlighted in red), at a ratio 2:1. These results are consistent with those previously reported by (Scollo et al., 2020), who showed that, compared to albumin, vicilin is more extensively degraded during fermentation, resulting in a greater abundance of its peptides. Additionally, it is noteworthy that the amino acid region of the vicilin peptides identified in this study ranged from residues 178 and 465, which is included in the 131—566 region reported by (Kratzer et al., 2009), as the origin of peptides that contribute to cacao aroma precursors. Therefore, this study allows us to reduce the range of interest to the region 131–566.

Cacao aroma is derived from amino acid precursors such as methionine (M), leucine (L), valine (V), isoleucine (I), phenylalanine (F), and alanine (A) at the N-terminus. These amino acids are highly reactive with reducing sugars during the initial stages of the Maillard reaction, serving as precursors to key aromatic volatile compounds that produce the characteristic aroma of cacao (Frauendorfer & Schieberle, 2006; Granvogl et al., 2006; Ullrich et al., 2022). In our study, six out of the

Table 1
Key discriminant metabolites as potential biomarkers of cacao quality during fermentation.

Assigned identity	Observed *m/z*	Adduct	Z	Proposed Formula	*m/z* (ppm)	RT	VIP	Ontology	Amino acid región
Aspartic acid	134.0449	$[M + H]^+$	1	$C_4H_7NO_4$	0.9	0.70	2.49		
Unknown 1	137.1074	$[M + H]^+$	1	$C_8H_{12}N_2$	0.6	7.90	2.11		
Unknown 2	137.1074					11.65	2.25		
Unknown 3	367.1684					11.45	2.18		
Unknown 4	349.2119					12.4	2.17		
GINDYR	383.1963	$[M + 2H]^+$	2	$C_{31}H_{48}N_{10}O_{11}$	0.0	7.44	2.04	Vicilin	178–183
DEEGNFKIL	532.7665	$[M + 2H]^+$	2	$C_{47}H_{73}N_{11}O_{17}$	0.2	12.14	2.02	Vicilin	158–166
Unknown 5	335.1965					10.83	2.01		
Unknown 6	160.0969	$[M + H]^+$	1	$C_7H_{13}NO_3$	0.6	0.78	1.99		
Unknown 7	383.1962					12.91	1.94		
LAL	316.2230	$[M + H]^+$	1	$C_{15}H_{29}N_3O_4$	0.3	10.90	1.93	Vicilin Albumin	258–260 195–197
Unknown 8	174.1125	$[M + H]^+$	1	$C_8H_{15}NO_3$	0.0	0.62	1.87		
WL	318.1811	$[M + H]^+$	1	$C_{17}H_{23}N_3O_3$	0.3	10.87	1.82	Peroxidase	14–15
Unknown 9	623.7758					12.75	1.81		
Unknown 10	128.1071	$[M + H]^+$	1	$C_7H_{13}NO$	0.8	11.23	1.80		
N-methylphenylalanine	180.1019	$[M + H]^+$	1	$C_{10}H_{13}NO_2$	0.0	0.83	1.78		
Succinic anhydride	101.0237	$[M + H]^+$	1	$C_4H_4O_3$	1.7	9.19	1.76		
Unknown 11	266.1232					0.62	1.75		
WF	352.1654	$[M + H]^+$	1	$C_{20}H_{21}N_3O_3$	0.6	11.82	1.72	Albumin	153–154
DNEWAWMFK	613.7680	$[M + 2H]^+$	2	$C_{58}H_{75}N_{13}O_{15}S$	1.0	13.37	1.72	Albumin	199–207
TDPQ	460.2024	$[M + H]^+$	1	$C_{18}H_{29}N_5O_9$	3.0	0.78	1.71		
MSF	384.1585	$[M + H]^+$	1	$C_{17}H_{25}N_3O_5S$	0.8	10.83	1.68	Vicilin	342–344
Unknown 12	129.0548	$[M + H]^+$	1	$C_6H_8O_3$	−2.8	9.18	1.66		
VW	304.1654	$[M + H]^+$	1	$C_{16}H_{21}N_3O_3$	0.7	8.90	1.64	Albumin	121–122
LSW	405.2130	$[M + H]^+$	1	$C_{20}H_{28}N_4O_5$	0.5	10.80	1.61	Peroxidase	719–721
Unknown 13	146.0574	$[M + H]^+$	1	$C_6H_9O_4$	−3.4	0.71	1.59		
Unknown 14	349.2120					11.91	1.58		
Unknown 15	261.6597					7.94	1.57		
IFVPHYNSKATF	475.2505	$[M + 3H]^+$	3	$C_{69}H_{98}N_{16}O_{17}$	0.0	11.35	1.57	Vicilin	394–405
LAIN	430.2659	$[M + H]^+$	1	$C_{19}H_{35}N_5O_6$	0.2	10.93	1.57	Vicilin	348–350
FLF	426.2383	$[M + H]^+$	1	$C_{24}H_{31}N_3O_4$	0.9	13.02	1.54	Vicilin	53–55
Unknown 16	232.1179					11.79	1.54		
VPSKLVDN	436.2477	$[M + 2H]^+$	2	$C_{38}H_{66}N_{10}O_{13}$	0.3	8.14	1.51	Vicilin	421–428
APLSPGDVF	451.7345	$[M + 2H]^+$	2	$C_{42}H_{63}N_9O_{13}$	0.0	12.63	1.49	Vicilin	457–465
2-Hydroxyphenethylamine	120.0809	$[M + H\text{-}H_2O]^+$	1	$C_8H_{11}NO$	3.3	9.14	1.49		
VLY	394.2335	$[M + H]^+$	1	$C_{20}H_{31}N_3O_5$	1.8	11.08	1.48	Peroxidase	171–173
Linolenic acid	279.2318	$[M + H]^+$	1	$C_{18}H_{30}O_2$	0.2	20.45	1.47		
4-Butyrolactone	87.0445	$[M + H]^+$	1	$C_4H_6O_2$	1.2	0.60	1.46		
Unknown 17	290.1835					8.69	1.44		
Unknown 18	103.0632	$[M + H]^+$	1	$C_4H_8NO_2$	4.1	0.6	1.44		
Unknown 19	452.2358					12.63	1.43		
IFK	204.1358	$[M + 2H]^+$	2	$C_{21}H_{34}N_4O_4$	−2.4	9.14	1.38		
DVF	380.1814	$[M + H]^+$	1	$C_{18}H_{25}N_3O_6$	0.5	11.18	1.38	Vicilin	463–465
DNEWAWM	476.1869	$[M + 2H]^+$	2	$C_{43}H_{54}N_{10}O_{13}S$	0.0	13.74	1.37	Albumin	199–205

seven tripeptides identified contained the N-terminus amino acids.

3.1.1. *Dynamics of key discriminant metabolites during cacao fermentation*

The dynamics of the metabolomic fingerprints were analyzed from day zero (fresh seeds) to day six of the fermentation of the cacao sourced from 9 different cacao production zones. Fermentation time significantly influences the variation in the metabolome of the cacao beans, which in turn impacts the chocolate volatilome and flavour profile (Llano et al., 2024). As metabolites are closely related to aroma formation, they can serve as indicators of the optimal transformation time needed to produce cacao beans of superior quality.

The behavior of discriminant metabolites throughout the fermentation process correlates with the dynamics of the physical phenomena that govern this process, primarily pH and temperature changes. The pH decreased from 6.4 to 4.5–4.2 between 0 and 72 h for most climatic production zones, with a slight increase at 120 h, as shown in Fig. S2. Simultaneously, the temperature increased from 25 °C to 45–48 °C. At 72 h, the temperature of cocoa beans in all nine agroclimatic groups reached 45–47 °C, and at 96 and 120 h it began to decrease, as depicted in Fig. S2. As pH and temperature increased, there was a corresponding rise in the intensity of most peptides, especially those with more than three amino acids. The pH and temperature conditions observed between 72 and 120 h align with the optimal values reported in the literature for the activity of endogenous cacao enzymes such as aspartic endoprotease, invertase, and carboxypeptidase (Hansen et al., 1998; Sousa et al., 2016). These conditions facilitate biochemical changes during this period, marking it as the active phase of cacao fermentation.

Fig. 2 illustrates a dynamic evolution of discriminant metabolites in two phases: an initial phase characterized by minimal biochemical changes during the first days of fermentation, followed by an active phase starting after 48 h of fermentation.

This trend was observed in all the fermentations of cacao sourced from the 9 climatic zones of cocoa production evaluated. In the first 24 h of fermentation, almost no discriminating peptides are detected.

However, after 24 h and primarily at 48 h of fermentation, peptides of various lengths became observable. This finding corroborates previous reports that protein degradation occurs only during the fermentation (D'Souza et al., 2018a; Herrera-Rocha et al., 2023; Kumari et al., 2018), which concluded that the peptide profile can represent the level of physicochemical transformation of cacao seeds.

In general, the larger discriminant peptides reached maximum intensities at 72 h of fermentation for the cacao sourced from the 9 production zones, then tended to decrease to very low concentrations by 120 h. The analysis of tripeptide dynamics revealed that some metabolites peaked at 72 h (MSF, FLF, LAL, VLY), while others peaked after 96 h (DVF, LSW), as shown in Fig. 2. The smaller peptides (composed of two

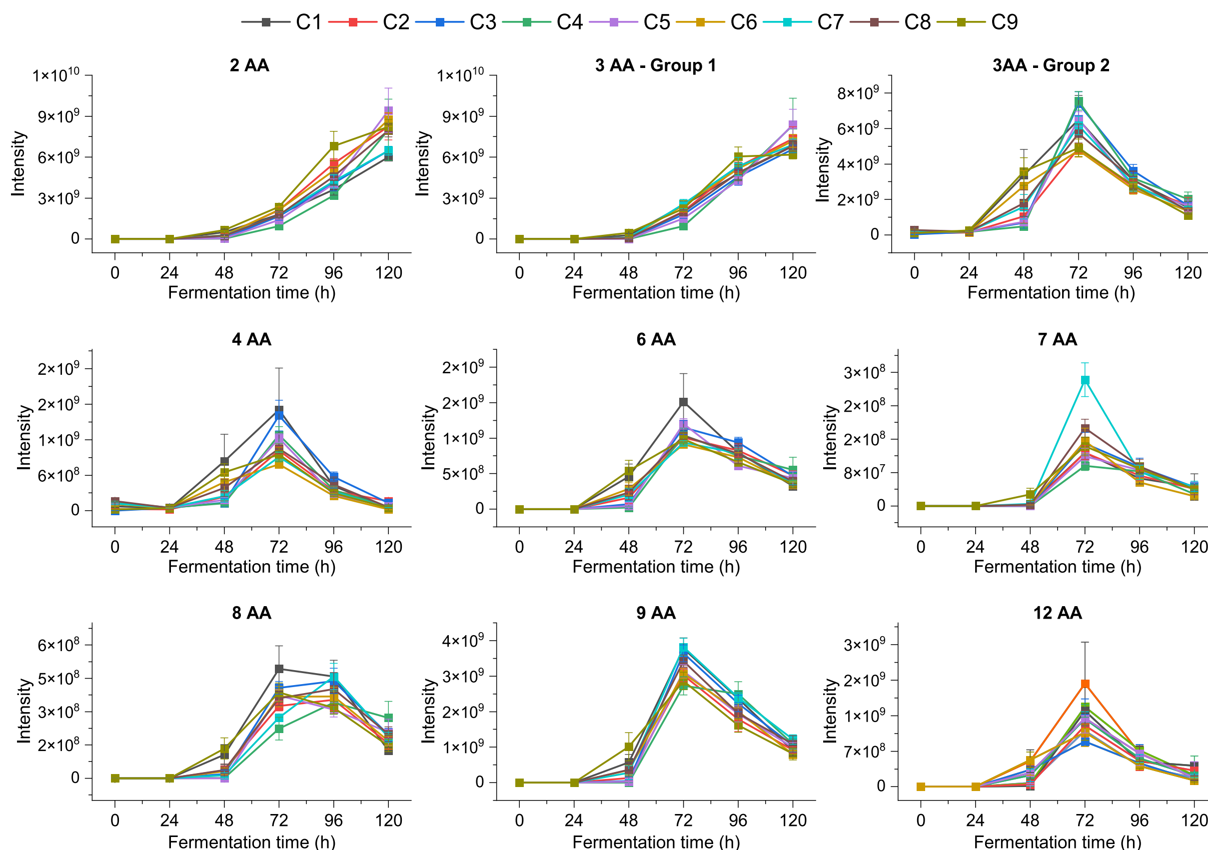

Fig. 2. Intensity dynamics of discriminant peptides (4, 6, 7, 8, 9, and 12 amino acids: respectively 4AA, 6AA, 7AA, 8AA, 9AA, and 12AA)) during cacao fermentation across the nine distinct production zones evaluated (C1 to C9).

amino acids) exhibited increased intensities after 96 h, peaking at 120 h of fermentation, indicating their production during the final stages of fermentation. The evolution of the discriminant peptides suggests that at different fermentation times, flavour development could be influenced by a combination of large and short peptides serving as flavour precursors. Specifically, long peptides tend to decrease between 72 and 120 h, whereas short peptides tend to increase during the same period. This phenomenon is particularly significant because peptides play an important role in the Maillard reaction, thus contributing to flavour-modulating properties (Salger et al., 2019).

These findings align with the sensory analysis conducted in a complementary study (Llano et al., 2024), where flavour distinctions were observed among different fermentation times. Pronounced fruit profiles peaked at 96 h of fermentation, while nutty notes were predominant at 96 and 120 h of fermentation. Interestingly, these fermentation times coincide with the peaks in the intensity of peptides, which are crucial precursors of compounds associated with the flavour and aroma of high-quality chocolate. Consequently, the 18 discriminant peptides identified with the highest VIP scores could potentially be considered as potential biomarkers to explain the increase in sensory quality. Irrespective of the origin of the cocoa from the 9 different cacao production regions, the same trend was observed for all the discriminant peptides. Therefore, we can affirm that the fermentation process itself has a significant impact on the dynamics observed for potential peptide biomarkers, while the influence of the agroclimatic conditions across the 9 cocoa producing zones was minimal. Additionally, other metabolites were tentatively identified, as shown in Table 1. Butyrolactone, linolenic acid and aspartic acid were all tentatively identified based on their similarity to fragmentation patterns MS/MS and those reported in the literature or by in-silico prediction (CFM-ID). The corresponding MS/MS spectra are provided in the supplementary data in Fig. S3.

Butyrolactone, associated with the sweet and caramel flavour of cacao (Akoa et al., 2023; Guzmán Penella et al., 2023), emerged as a discriminating metabolite. Aspartic acid also appeared as a discriminating metabolite, exhibiting increased intensity at 120 h of fermentation across the 9 climatic production zones evaluated. This observation aligns with previous studies indicating an increase in amino acid concentration as fermentation progresses, as shown in Fig. 3. Succinic anhydride, formed through an indirect biological route involving the dehydration of succinic acid produced during fermentation, was identified but not previously reported as a differentiating metabolite in cacao fermentation processes. Butyrolactone showed variation for most production zones between 24 and 72 h of fermentation. However, no significant differences in butyrolactone content were found between early fermentation times of beans and those fermented for 120 h, as illustrated in Fig. 3. While the presence of butyrolactone in cocoa beans before roasting has been documented, its dynamics during the fermentation process have not been extensively studied (Marseglia et al., 2020).

The dynamics of linolenic acid and aspartic acid are shown in Fig. 3. Linolenic acid exhibited its highest concentrations within the first 24 h of fermentation, followed by a decline after 48 h, and stabilized at low levels through the 120-hour fermentation period. Although limited research exists on the variation of linolenic acid during fermentation, previous studies, in contrast to our findings, report no significant changes in its levels during spontaneous fermentation (Servent et al., 2018). Furthermore, comparative studies between unfermented and solid-state fermented beans have shown an increase in linolenic acid content. These discrepancies in linolenic acid behavior during fermentation suggest that its variation may be influenced by the type of fermentation (Lessa et al., 2018). Aspartic acid showed maximum intensity at 120 h of fermentation, remaining constant from the beginning of fermentation until 96 h.

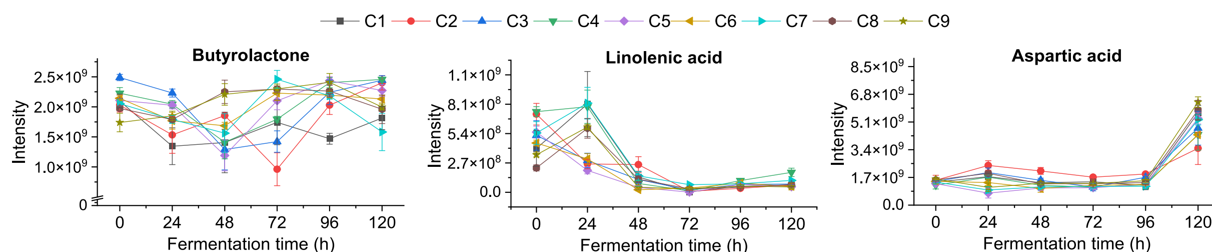

Fig. 3. Dynamics of key discriminant metabolites (butyrolactone, linolenic acid, and aspartic acid) across different fermentation times in the nine distinct cacao production zones evaluated (C1 to C9).

3.2. Targeted Metabolomics: Focused analysis of key metabolites during cacao fermentation

Acids, polyphenols, and sugars in cacao beans play an essential role in the development of the sensory profile of chocolate, influencing flavour development, and primarily affecting the complementary attributes such as fresh and browned fruits, floral, vegetal, spices, and caramel/panela, and modifying also the balance of the core attributes bitterness, acidity, astringency, sweetness, (Santander et al., 2021). The following sections present the dynamics of these compounds during fermentation.

3.2.1. Dynamics of sugars during cacao fermentation: Transformation of sucrose, glucose, and fructose

The concentration of sucrose and reducing sugars (glucose and fructose) was analyzed in relation to fermentation time for the cacao beans sourced from the 9 cacao production zones, as shown in Fig. 4. It is important to note that the concentrations reported refer specifically to the internal content within the cacao seeds. In general, raw cacao beans had higher sucrose concentration and lower concentration of reducing sugars. During fermentation, sucrose concentration exhibited a similar decreasing pattern over time across all nine fermentation of the cacao production zones evaluated, with no significant differences observed between the zones at the end of the process, as previously shown by other studies (Misnawi et al., 2002; Rottiers et al., 2019a).

This decline is attributed to the hydrolysis of sucrose into glucose and fructose, catalyzed primarily by endogenous invertase enzymes during fermentation (Becerra et al., 2022; Hashim et al., 1998; Rottiers et al., 2019a). The rate of sucrose hydrolysis was highest between 48 and 96 h of fermentation. Consequently, the content of glucose and fructose significantly increased, with glucose reaching its maximum concentration at 96 h of fermentation.

The sucrose concentration at 120 h of fermentation in the 9 production zones was < 1 mg/g, indicating complete fermentation according to (Ziegleder, 2017). At 96 h almost all the fermentations of the cacao sourced from the 9 climate production zones still presented sucrose concentration > 1 mg/g. Furthermore, the results suggest that fermentation times longer than 120 h will not favor the formation of reducing sugars following sucrose hydrolysis, since sucrose was almost entirely consumed. The sucrose content in raw beans and at the end of fermentation showed no significant differences between the climatic zones of production.

Fructose content was higher than glucose content throughout the fermentation process. This predominance of fructose over glucose has been previously observed in previous studies (Hinneh et al., 2018; Reineccius, Keeney, et al., 1972). Some findings suggest that the fructose-glucose ratio could influence the outcome of the Maillard reaction during the roasting of fermented beans (Akoa et al., 2023; Hinneh et al., 2018). The fructose:glucose ratio remained around 2 during the 48, 72, 96, and 120 h fermentations in the 9 climate production zones, showing no increase over time. This pattern aligns with previous results where fructose content exceeds glucose content (Akoa et al., 2023; Hinneh et al., 2018; Rottiers et al., 2019a). A higher concentration of fructose is important because it has been reported to be more reactive than glucose in forming volatile compounds during Maillard reactions (Matmaroh et al., 2006; Reineccius, Andersen, et al., 1972). However, there is probably a threshold beyond which a higher concentration of fructose offers no additional benefits. While this has not been reported in cocoa, it has been observed in model systems of the Maillard reaction (Matmaroh et al., 2006).

The dynamics of fructose and glucose indicate that the maximum concentrations of both sugars are generally reached after 96 h of fermentation across the nine distinct cacao production zones, highlighting a critical phase in the sugar transformation process during fermentation.

3.2.2. Dynamics of methylxanthines and flavonols during cacao fermentation

Theobromine and caffeine were the primary methylxanthines evaluated. As it is shown in Fig. 5, their concentrations remained relatively stable throughout the fermentation period ($p > 0.05$). Previous studies have indicated that theobromine and caffeine levels generally remain constant after 72 to 96 h of fermentation, with a decrease typically observed at the end of extended fermentation periods, around 240 h (Febrianto & Zhu, 2020). Since the maximum fermentation time in this study was 120 h, our findings are consistent with the stability reported at 72, 96, and 120 h. The methylxanthines content is a characteristic of unfermented cocoa beans that showed some variability between climatic zones of production, but this was considerably reduced during the process, and no significant differences were observed between the 9

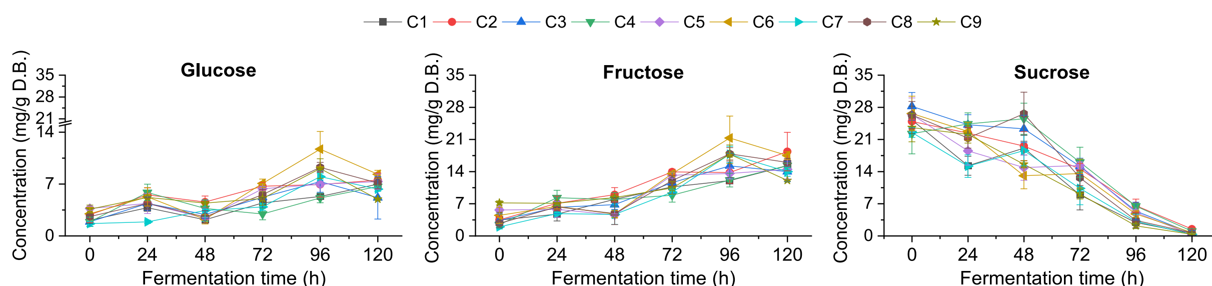

Fig. 4. Dynamics of glucose, fructose, and sucrose concentrations during cacao fermentation across the nine distinct climate zones evaluated (C1 to C9).

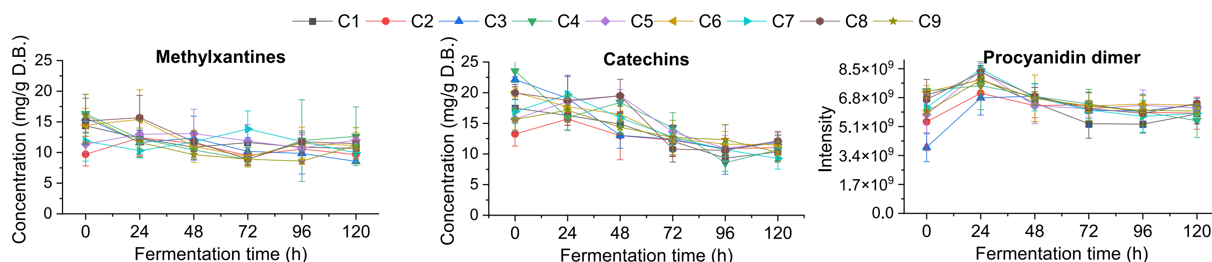

Fig. 5. Dynamics of methylxanthines, catechins, and procyanidin dimer during cacao fermentation across the nine climate zones evaluated (C1 to C9).

cacao production zones at the end of fermentation.

Catechin and epicatechin (catechins) were the main flavonols evaluated. As depicted in Fig. 5, the initial concentrations of these flavonols in unfermented beans varied among the climatic production zones, ranging from a low of 13.3 mg/g D.B. to a high of 23.5 mg/g D.B. By the end of fermentation, after 120 h, catechin concentrations had decreased to approximately 9.3 to 12.1 mg/g in the nine climatic production zones, as detailed in the Supplementary Table 1. A significant reduction in catechin content was observed throughout the fermentation period, with reductions ranging from 13 % to nearly 50 %, depending on the region. These reductions were more pronounced than those reported by Nazaruddin et al. (2006), Rodriguez-Campos et al. (2011), and Wollgast & Anklam (2000). The most notable decrease in catechin concentration occurred between 48 and 72 h, when aeration began, initiating the oxidation and subsequent condensation of catechins. After 96 h, no significant decrease in catechin content was observed in any of the nine climatic production zones studied. This pattern aligns with previous research indicating significant reductions within the first 48 h of fermentation, with no further significant changes beyond 96 h (Febrianto & Zhu, 2020). Additionally, Herrera et al. (Herrera-Rocha et al., 2021) noted that significant variations in flavonoid content occur during the first four days of fermentation, after which metabolic changes are minimal.

During cocoa fermentation, the dynamics of the procyanidin dimer showed a slight increase at 24 h, followed by stabilization in its concentration throughout the fermentation process, as depicted in Fig. 5. This behavior was consistent across the nine production zones evaluated, suggesting that, regardless of climatic variations among the different areas, the procyanidin dimer remained stable during fermentation. This is in agreement with previous studies (Balcázar-Zumaeta et al., 2023; Melo et al., 2021). However, other studies have reported a decrease in procyanidin content after 4 days of fermentation (Mayorga-Gross et al., 2016b). Although a decrease in catechin content is observed during fermentation, the expected increase in dimeric procyanidins does not occur significantly. This may be because procyanidin dimers also undergo condensation and polymerization processes, forming procyanidins of a higher degree of polymerization or even participating in oxidation reactions (Ioannone et al., 2015).

Based on the dynamics shown in Fig. 5, the enzymatic activity that reduces catechin levels appears to be most active between 48 and 72 h of fermentation. This activity declines after 72 h, likely due to the increasing temperature and the inhibitory effect of tannins (Le Bourvellec et al., 2004). Additionally, the variability in the initial catechin content is significantly reduced by the end of the fermentation process across all climatic cacao production zones.

Higher levels of catechin, epicatechin, and caffeine contribute to the bitterness and astringency of the final product. However, the attributes of bitterness and astringency did not significantly change between 72 and 96 h, as reflected in the concentration levels in samples from the nine production zones, consistent with findings by Llano et al. (2024). It is important to note that the sensory attributes of bitterness and astringency are not solely determined by the concentrations of methylxanthines and flavonoids. The specific concentration levels at which changes in catechin, epicatechin, and caffeine become perceptible to a trained sensory panel are not well defined, particularly given their synergistic interactions with numerous other compounds in chocolate. Nevertheless, an adequate fermentation time is essential to reduce catechins and enhance aroma precursors

3.2.3. *Dynamics of acids during cacao fermentation: Impact on fermentation progress*

Acetic, lactic, and citric acids were quantified in the beans throughout the fermentation process for cacao from the 9 production zones. Lactic acid concentration across all climate production zones and fermentation times evaluated was below 1 mg/g, which corresponds to the limit of quantification of the method used. Previous studies on cacao beans at various fermentation times reported lactic acid concentrations ranging from 0.4 to 0.6 mg/g, without drastic changes over time (Ho et al., 2018). The consistently low lactic acid concentration throughout the fermentation process is favorable for chocolate quality, as lactic acid negatively impacts cacao quality. The non-volatile lactic acid content generated during fermentation persists in the chocolate after roasting, imparting an undesirable flavour (Rodriguez-Campos et al., 2011).

Citric acid did not show significant changes throughout the fermentation time in any of the 9 climate production zones evaluated, as shown in Fig. 6. However, the concentration of acetic acid changed with the fermentation time, reaching a maximum concentration between 72 and 96 h of fermentation. This pattern was consistent across all 9 climate production zones.

The observed dynamics of acetic acid show a correlation with the behavior of reducing sugars throughout fermentation. Both show a peak at the same time after 96 h of fermentation. This correlation supports previous findings that suggest the acidification of the cacao seeds during fermentation is crucial for both embryo death and the initiation of enzyme-substrate interactions producing various reducing sugars (Beckett, 2009). Therefore, it can be inferred that the concentration of acids, primarily acetic acid, influences the levels of glucose and fructose released at different fermentation times. Moreover, the increase in acetic acid concentration during fermentation, reaching its highest levels between 72 and 96 h, coincides with the decrease in pH described earlier in the fermentation process across all the 9 production zones. These high levels of acetic acid concentration correspond to the times when the concentration of most of the peptides observed also increases. Notably, after 96 h, the acetic acid concentration begins to decline, with no significant increase in reducing sugars. A fermentation time of 96 h was also previously reported as optimal by (Llano et al., 2024), resulting in a sensory profile of chocolate that achieves a quality profile dominated by complementary attributes (fruitiness and nuttiness) and a good balance between core attributes.

4. Conclusion

The results of this study clearly demonstrate that fermentation time is the primary factor influencing the chemical profile of cacao, with agroclimatic conditions showing no significant effect on the metabolomic fingerprint. Through comprehensive chemometric analysis, we

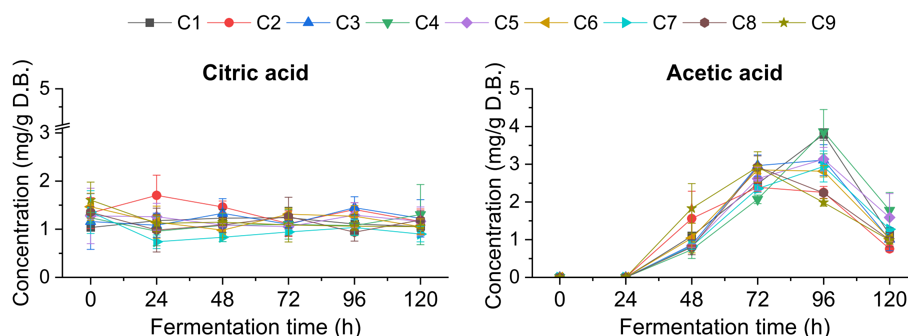

Fig. 6. Dynamics of citric and acetic acid concentrations during cacao fermentation across the nine climate zones evaluated (C1 to C9): key drivers of flavor development.

found that the biochemical changes observed in cacao are driven predominantly by the fermentation process. Identifying potential biomarkers associated with cacao quality, our findings underscore that the duration of fermentation plays a critical role in shaping the chemical composition of cacao, whereas climatic variations in the production zone had a negligible impact.

This study provides groundbreaking insights into the dynamic biochemical processes occurring during cacao fermentation, with a focus on optimizing flavor precursor formation through a detailed metabolomic approach. By combining untargeted and targeted metabolomics, coupled with multivariate statistical analysis, this research has elucidated the significant role that fermentation time plays in shaping the metabolomic fingerprint of cacao beans. Our study not only confirms the critical influence of fermentation on key flavor precursor metabolites but also highlights the limited impact of geographic and climatic differences across the production zones in the department of Arauca, Colombia, on metabolite dynamics when fermentation processes are carefully controlled.

This study advances our understanding of cacao fermentation by identifying fermentation time as the primary determinant of flavor precursor formation, with 96 h emerging as the optimal duration to maximize key aroma compounds. Through the identification of 18 discriminant metabolites as potential biomarkers of quality, the research offers new indicators for monitoring fermentation progress. In the future, these compounds could be analyzed using colorimetric tests or pH measurements, establishing correlations between these compounds and pH or color. This could lead to the development of easy-to-use field tools. Notably, the minimal influence of climatic zones on metabolite dynamics suggests that controlled fermentation processes can standardize cacao quality across diverse production regions. Key compounds such as sugars, organic acids, and methylxanthines displayed distinct dynamics during fermentation, impacting flavor development and highlighting the complex biochemical changes that occur. The practical implications for the cocoa industry are significant, as these findings enable producers to standardize fermentation techniques, achieving more homogeneous flavor profiles. Future research should focus on integrating sensory analysis to fully link metabolomic changes with the final product quality as perceived by the sensory panel. Additionally, it should aim to develop practical tools that facilitate the easy monitoring of these compounds, which serve as biomarkers of the fermentation process and act either as flavor precursors or as compounds impacting aroma, through correlations with simple measurements such as pH or color.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 4o in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

CRediT authorship contribution statement

Sandra Llano: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andrés Zorro-González:** Writing – original draft, Investigation, Formal analysis, Data curation. **Margareth Santander:** Methodology, Formal analysis, Conceptualization. **Fabrice Vaillant:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Renaud Boulanger:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Diana Marcela Ocampo Serna:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **Sebastián Escobar:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.115978.

Data availability

Data will be made available on request.

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