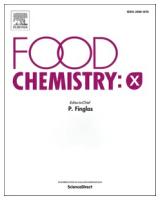
Food Chemistry: X 22 (2024) 101486

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Amino acid profile behavior during the fermentation of Criollo cocoa beans

César R. Balcázar-Zumaeta [a,b], Editha Fernández-Romero [a,c,*], Alessandra Santos Lopes [d], Nelson Rosa Ferreira [e], Gilson Celso Albuquerque Chagas-Júnior [d], Ives Yoplac [f], Heydi A. López-Trigoso [a], Mery L. Tuesta-Occ [a], Italo Maldonado-Ramirez [g], Jorge L. Maicelo-Quintana [h], Ilse S. Cayo-Colca [h], Efrain M. Castro-Alayo [a]

[a] Instituto de Investigación, Innovación y Desarrollo para el Sector Agrario y Agroindustrial (IIDAA), Facultad de Ingeniería y Ciencias Agrarias, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Chachapoyas 01001, Peru.
[b] Programa de Doctorado en Ciencias Agrarias, Escuela de Posgrado, Universidad Nacional de Piura, Piura, Jr. Tacna 748, Piura, Peru.
[c] Programa de Maestría en Cambio Climático, Agricultura y Desarrollo Rural Sostenible-MACCARD, Escuela de Posgrado, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Calle Higos Urco 342-350-356, Chachapoyas, Amazonas, Peru
[d] Graduate Program in Food Science and Technology (PPGCTA), Institute of Technology (ITEC), Federal University of Pará (UFPA), Belém, Pará, Brazil.
[e] Laboratory of Biotechnological Processes (LABIOTEC), Graduate Program in Food Science and Technology (PPGCTA), Institute of Technology (ITEC), Federal University of Pará (UFPA), Belém, 66075-110, Brazil.
[f] Laboratorio de Nutrición Animal y Bromatología de Alimentos, Facultad de Ingeniería Zootecnista, Agronegocios y Biotecnología, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Chachapoyas 01001, Peru
[g] Facultad de Ingeniería Mecánica y de Sistemas, Facultad de Ingeniería y Ciencias Agrarias, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Chachapoyas 01001, Peru
[h] Facultad de Ingeniería Zootecnista, Agronegocios y Biotecnología, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Chachapoyas 01001, Peru

ARTICLE INFO

Keywords:
Aromatic precursors
Essential
Hydrolysis
Multivariate analysis
Starter culture

ABSTRACT

The study investigated the behavior of seventeen amino acids during spontaneous (SF) and starter culture (SC) fermentation of Criollo cocoa beans from Copallín, Guadalupe and Tolopampa, Amazonas-Peru. For this purpose, liquid chromatography (UHPLC) was used to quantify amino acids. Multivariate analysis was used to differentiate the phases of the fermentation process. The percentage of essential amino acids during SC fermentation (63.4%) was higher than SF (61.8%); it was observed that the starter culture accelerated their presence and increased their concentration during the fermentation process. The multivariate analysis identified a first stage (day 0 to day 2), characterized by a low content of amino acids that increased due to protein hydrolysis. The study showed that adding the starter culture (*Saccharomyces cerevisiae*) to the fermentation mass increased the concentration of essential amino acids (63.0%) compared to the spontaneous process (61.8%). Moreover, this addition reduced the fermentation time (3–4 days less), demonstrating that the fermentation process with a starter culture allows obtaining a better profile of amino acids precursors of flavor and aroma.

1. Introduction

Cocoa (*Theobroma cacao* L.) is the primary raw material for chocolate production, so its aromatic characteristic is a crucial point for the development of its flavor (Hinneh et al., 2018); for this, the bean goes through different processing stages that origins the aromatic profile to be developed from precursors (volatile and non-volatile) (Afoakwa, Paterson, Fowler, & Ryan, 2008; Balcázar-Zumaeta, Castro-Alayo, Cayo-Colca, Idrogo-Vásquez, & Muñoz-Astecker, 2023; Deus, Bispo, Franca, & Gloria, 2021). Sari et al. (2023) indicate that the genotype of cocoa beans is a factor that permits differentiating the profile and concentration of amino acids, highlighting the Criollo variety for having a significant concentration of these compounds compared to other varieties (Castro-Alayo, Idrogo-Vásquez, Siche, & Cardenas-Toro, 2019; Perez,

* Corresponding author at: Instituto de Investigación, Innovación y Desarrollo para el Sector Agrario y Agroindustrial (IIDAA), Facultad de Ingeniería y Ciencias Agrarias, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas, Calle Higos Urco 342-350-356, Chachapoyas 01001, Peru.
E-mail addresses: cesar.balcazar@untrm.edu.pe (C.R. Balcázar-Zumaeta), editha.fero@gmail.com (E. Fernández-Romero), alessalopes@ufpa.br (A.S. Lopes), nelson.ufpa@gmail.com (N.R. Ferreira), chagasjunior.gca@gmail.com (G.C.A. Chagas-Júnior), ives.yoplac@untrm.edu.pe (I. Yoplac), 7439899620@untrm.edu.pe (H.A. López-Trigoso), 7703865720@untrm.edu.pe (M.L. Tuesta-Occ), Italo.Maldonado@untrm.edu.pe (I. Maldonado-Ramirez), jmaicelo@untrm.edu.pe (J.L. Maicelo-Quintana), icayo.fizab@untrm.edu.pe (I.S. Cayo-Colca), efrain.castro@untrm.edu.pe (E.M. Castro-Alayo).

https://doi.org/10.1016/j.fochx.2024.101486
Received 4 March 2024; Received in revised form 13 May 2024; Accepted 15 May 2024
Available online 18 May 2024

Lopez-Yerena, & Vallverdú-Queralt, 2022; Sari et al., 2023). Peru is one of the main areas where there are large cocoa crops with particular relevance for its economy (Cádiz-Gurrea et al., 2020), especially in the Amazonas region (Peru). One of the most important crops is Criollo cocoa, which has the denomination of origin "Cacao Amazonas Peru" and is produced in the provinces of Utcubamba and Bagua (Balcázar-Zumaeta et al., 2023).

The fermentation is a stage responsible for producing aromatic precursor compounds that regulate the astringency and flavor of the fermented cocoa pulp-bean mass (Velásquez-Reyes et al., 2023). During fermentation (takes 4–7 days), the production of ethanol, acetic acid, and heat causes the death of the bean embryos, which sets the biochemical stage for a plethora of flavor- and color-forming reactions between peptides, amino acids, carbohydrates, and other compounds (Brunetto et al., 2020a; Castro-Alayo et al., 2019; Perez et al., 2022); in particularity, the amino acids standing out for impact in the volatile flavor formation (Gutiérrez-Ríos et al., 2022; Marseglia et al., 2014). These reactions are responsible for the formation of different bioactive compounds such as reducing sugars, amino acids, peptides, acids, flavan-3-ols, and anthocyanins, among others. These bioactive compounds give chocolate its particular aroma and flavor (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Chang et al., 2024) thanks to the action of microorganisms and chemical reactions that occur inside the bean (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Escobar et al., 2021; Quelal, Hurtado, Benavides, Alanes, & Alanes, 2023). The amino acid, which results from the microbial actions during the vicilin class globulin (VCG) reaction (Castro-Alayo et al., 2019; Fang et al., 2020; Gutiérrez-Ríos et al., 2022), enters the cocoa bean by molecular diffusion and confers the final characteristics of the chocolate.

The fermentation is responsible for forming the free amino acid profile due to the protein cleavage in the cocoa bean (autolysis) (Brunetto et al., 2020b; Marseglia et al., 2014). The cocoa quality and chocolate perception in flavor and aroma depend on the amino acid profile (Dala-Paula, Deus, Tavano, & Gloria, 2021; Deus, Bispo, Franca, & Gloria, 2020). Moreover, a well-fermented cocoa bean presents a higher content of amino acids (Tchouatcheu, Noah, Lieberei, & Niemenak, 2019); in addition, they are responsible for different physiological functions for humans (Salaria, Boatwright, Thavarajah, Kumar, & Thavarajah, 2022), such as protein synthesis, repair tissues and nutrient absorption, among others (Dala-Paula et al., 2021; Lopez & Mohiuddin, 2022). However, the concentration of amino acids varies by cacao origin, environmental conditions, and agricultural practices, among others (de Araujo et al., 2021; Sari et al., 2023). Also, a standardized fermentation with a starter culture is a process that can regulate these variations (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023).

The effect of the fermentation process on amino acids results in the development of key volatile fractions (mainly alcohols and esters) (Utrilla-Vázquez, Rodríguez-Campos, Avendaño-Arazate, Gschaedler, & Lugo-Cervantes, 2020), which results in the variation of aroma precursors (Rawel, Huschek, Sagu, & Homann, 2019). Therefore, determining the amino acid profile can help us to understand the behavior of these compounds during fermentation (Brunetto et al., 2020a) since fermentation is currently a non-standardized (spontaneous) process that occasions differences in cocoa bean quality (Balcázar-Zumaeta, Castro-Alayo, et al., 2023). Given this, the use of inoculums during fermentation allows the synthesis of flavor compounds and derivatives (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023); inoculums reported were yeasts (*Kluyveromyces marxianus, Pichia kluyveri*, and *Saccharomyces cerevisiae*), acetic acid bacteria (*Acetobacter pasteurianus, A. aceti,* and *A. tropicalis*), and lactic acid bacteria (*Lactobacillus fermentum* and *L. plantarum*) (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023; Ooi, Ting, & Siow, 2020). In addition, the use of starter cultures is a method that improves the chemical composition of the fermented cocoa bean (with emphasis on amino acids) (Febrianto, Wang, & Zhu, 2022), can modulate the fermentation time and influence the amino acids concentration (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Castro-Alayo et al., 2019; Korcari et al., 2023; Sande et al., 2020; Schlüter, André, Hühn, Rohn, & Chetschik, 2022).

It is possible to obtain a high concentration of amino acids and aromatic compounds during roasting, as long as the fermentation is carried out efficiently (Castro-Alayo et al., 2019); for this, considering an adequate time and the use of starter cultures (such as *Saccharomyces cerevisiae*) contribute to improving the conditions of this process. Therefore, in this study, we report the reduction in spontaneous (SF) and starter culture (*S. cerevisiae*, SC) fermentation time based on the amino acid profile behavior, as we previously reported in another study based on the antioxidant profile characterization of cocoa beans harvested in Amazonas, Peru (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023).

2. Materials and methods

2.1. Biological materials and reagents

Criollo cocoa bean varieties were collected from three localities located in Bagua province (Amazonas region, Fig. 1); Guadalupe (5°33′45.14″ S, 78°32′50.53″ W, 420 masl), Tolopampa (5°39′21. 25″ S, 78°29′40.44″ W, 514 masl), and Copallín (5°41′13.70″ S, 78°24′20.58″ W, 869 masl) (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023).

The starter culture *Saccharomyces cerevisiae* was from ATCC® 18,824™ (01066 K, Microbiologics, Saint Cloud, MN, USA). Other reagents and materials were trichloroacetic acid and 0.22 μm Millex-GP syringe filters (Merck, Darmstadt, Germany). Methanol HPLC grade and sodium phosphate - NaH_2PO_4 were purchased from JT Baker (Deventer, The Netherlands). The hydrochloric acid (HCl), petroleum ether, acetonitrile, YPD agar, peptone wáter, and phosphoric acid were from Sigma Aldrich (St. Louis, MO, USA). The glass vials (1.5 mL) were from Wheaton (Millville, NJ, USA). The solutions of 17 amino acids (AA), o-phthaldialdehyde (OPA), 9-fluorenyl methyl chloroformate (FMOC), borate buffer, filter membrane, Nylon 47 mm, pore size 0.20 μm were purchased from Agilent Technologies (Santa Clara, CA, USA).

2.2. Activation of starter culture Saccharomyces cerevisiae

The activation process of *S. cerevisiae* (derived from ATCC®18824TM) was carried out according to the method described by Chagas, Ferreira, Gloria, Martins, and Lopes (2021); for this, the colonies were deposited in a sterile YPD (Yeast Extract Peptone Dextrose) agar plates, incubated at 28 °C for 72 h, and transferred to new plates with peptonized water.

After the growth of the colonies, the culture was prepared in an Erlenmeyer flask with 500 mL of peptone water; 16 plates containing the activated strains were used, adding 2 mL of peptone water to each plate (previous cleaning with a sterile swab). The content of each one was transferred to the flask with peptone water (cell growth $\approx 10^8$ cells/mL). Strain activation was performed in the Laboratory of Chemistry of the Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas (UNTRM). Finally, the media with the strains were transferred to the APROCAM cooperative to add to the cocoa fermentation processes.

2.3. Cocoa fermentation

The Criollo cocoa beans harvested from three population centers were transported and processed at the APROCAM Multiple Services Cooperative, located on Car. Bagua- Copallín Km.4 - Province of Bagua.

Two types of fermentation were used, spontaneous (SF) and fermentation with a starter culture (SC), following the procedure described by Castro-Alayo, Torrejón-Valqui, Medina-Mendoza, Cayo-Colca, and Cárdenas-Toro (2022). Both fermentation procedures were similar; for this, 40 kg of flask cocoa was covered with polyethylene bags. The turning and the box change were every 48 h; the fermentation was carried out for one week; in the case of the SC, the cocoa beans were mixed with $\approx 10^6$ cells of *S. cerevisiae* /gram of cocoa at the beginning of

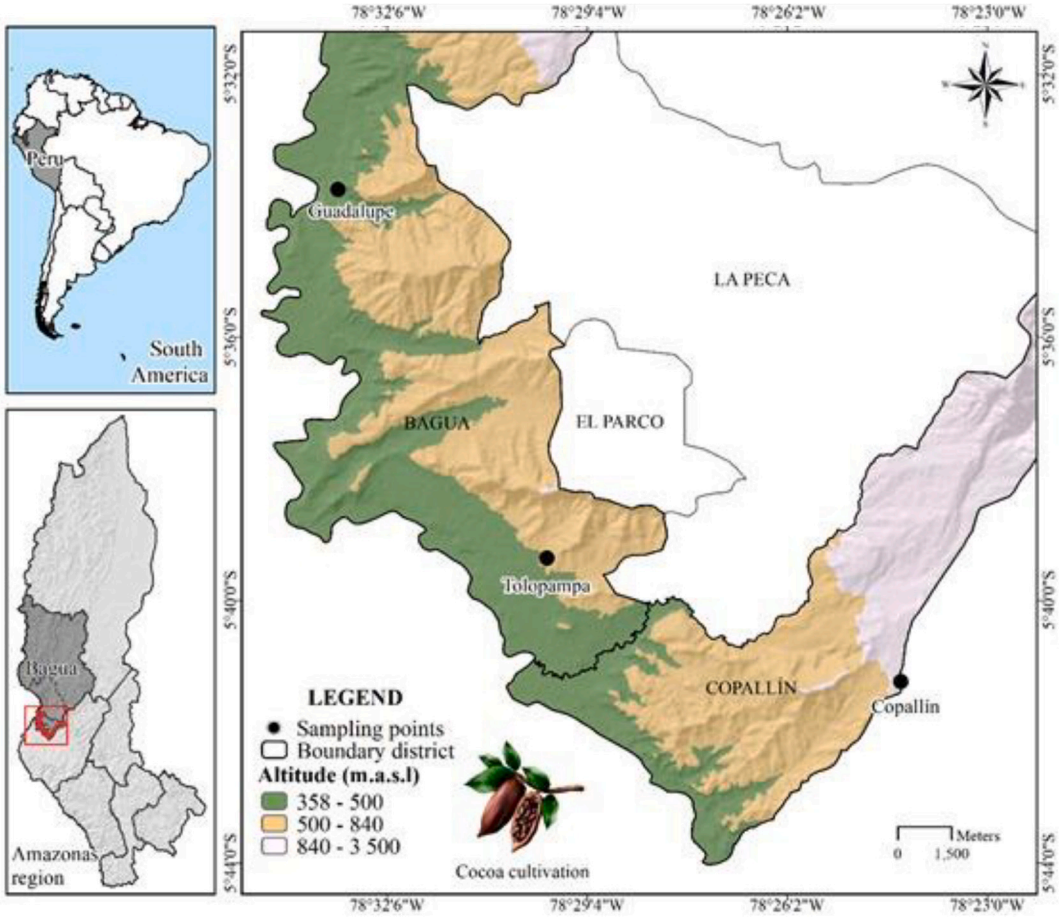


Fig. 1. Criollo cocoa bean origins (Amazonas, Perú).

the fermentation process.

The sampling procedure (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023) was as follows: 150 g of cocoa beans (separated from the pulp, under aseptic conditions) were extracted every 24 h, deposited in sterilized bags and subjected to freeze shock with liquid nitrogen from the beginning of fermentation (0 h) until the end of fermentation (168 h), to preserve the conditions of the bean at each sampling time. The samples were transported to the Food Engineering and Post-Harvest Research Laboratory of the UNTRM for conservation under deep-freezing conditions (Eppendorf, Premium 0410, Hamburg, Germany) until further analysis.

2.4. Sample treatment

The collected samples were freeze-dried as described by Balcázar-Zumaeta, Pajuelo-Muñoz, et al. (2023); the beans were placed in Falcon tubes (50 mL) at 0.008 bar and - 84 °C for 18 h in a freeze-dryer (Labconco, model 710,402,010, Kansas City, MO, USA). Subsequently, the freeze-dried cocoa beans were defatted (Hernández-Hernández, Viera-Alcaide, Morales-Sillero, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2018) with a Soxhlet extractor (Daihan Scientific, Seoul, Republic of Korea) using petroleum ether (solvent) and controlling eight siphons. Finally, the defatted samples were left at room temperature to evaporate solvent residues and stored in a dry place.

2.5. Determination of amino acids

The quantification of amino acids was determined by ultra-high performance liquid chromatography (UHPLC); for this, the analysis was performed according to the modified method of Hinneh et al. (2018). Samples of 1 g of cocoa with 15% trichloroacetic acid were centrifuged at 11800 RPM for 10 min at 4 °C; then, 1.5 µL of this solution were filtered with a 0.22 µm Nylon filters (Merck, Millex, Germany).

Separation and quantification were performed by UHPLC (Agilent Technologies, 1290 Infinity II, Waldbronn, Germany), coupled with a multisampler (G7167B), a flexible pump (G7104A), a column oven (G7116B) and diode array detector (G7117B). The separation was performed with a Zorbax Eclipse-AAA 4.6 × 150 mm, 3.5 µm column (Agilent PN 963400–902). Ortho-phthalaldehyde (OPA; Agilent PN 5061–3335) and 9-fluorenyl methyl chloroformate (FMOC; Agilent PN 5061–3337) were used for amino acid derivatization, while 0.4 mol*L-1 borate buffer (Agilent PN 5061–3339) (pH 10.2) was used as a buffer solution. The following phases were used: (A) NaH2PO4 and ultrapure water (5.5 g / 1 L, pH 7.8); (B) acetonitrile (ACN)-methanol (MeOH)-water solution (45:45:10, *v*/v/*v*/v), both solutions filtered on 0.22 Millex LCR membranes. A mix of amino acid standards was used and dissolved in 25 mL with HCl (0.1 N) for identification. The flow rate was 2 mL/min for 16 min each injection, and the column temperature was 40 °C. The quantification of amino acids was performed by comparing the sample peak areas with each standard peak using the ChemStation control software (version A.02.14 05–16, OpenLAB). The results were expressed as mg amino acid/100 g sample.

2.6. Data analysis

The results were obtained in triplicate and subjected to analysis of variance (ANOVA) for each type of cocoa fermentation and origin (previously verifying three ANOVA assumptions: normality, homogeneity, and independence); then, a multiple comparisons test (Tukey, 95%) was performed. For the average values, a multivariate analysis was

performed according to the quantified amino acids, for which a cluster analysis (k-means) and principal component analysis (PCA) were employed, using the complimentary software RMarkdown (RStudio, v. 2022.07.2 + 576, Boston, MA, USA).

3. Results and discussion

3.1. Amino acids quantification in Criollo cocoa beans SF and SC

The fermentation processes in Criollo cocoa under Amazonas conditions average one week (seven days). However, previous studies under similar conditions (Balcázar-Zumaeta, Castro-Alayo, et al., 2023, Balcázar-Zumaeta, Pajuelo-Muñoz, et al. (2023); Castro-Alayo et al., 2019, 2022) suggest reducing the fermentation days to optimize the presence of precursor compounds. Since amino acids are responsible for the aroma, it is necessary to study them to observe their variations during the fermentation process (Tables 1, 2 and 3). These variations have been previously reported by Calvo et al. (2021) and Sande et al., 2020) and could be used to optimize the fermentation process (Deus et al., 2021). Studies show that parts of the adhering pulp are removed during this process, which promotes microbial activity and allows the formation of amino acids (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Haruna et al., 2024). Moreover, in this study, the analysis of K-means and PCA helped better understand the fermentation time's effect on the cocoa bean's amino acid profile.

Tables 1, 2, and 3 show the concentration of 17 amino acids in the cocoa bean. It can be observed that the fermentation time affected the amino acids content ($p < 0.05$), similar to that reported by Rottiers et al. (2019), evidencing that the fermentation process degrades the proteins due to the presence of microorganisms and enzymes (Domínguez-Pérez, Beltrán-Barrientos, González-Córdova, Hernández-Mendoza, & Vallejo-Cordoba, 2020; Febrianto et al., 2022), which contribute to the formation of aromas during the cocoa bean processing (Schlüter et al., 2022; Tamimi, Hidayat, Utami, & Witasari, 2023). An atypical behavior was observed in the identification of amino acids in SF in cocoa beans (day 3, Guadalupe), which could be related to the microbial behavior (lactic acid bacteria and nitrogenous yeasts) and carbon metabolism in a spontaneous (uncontrolled) fermentation (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Deus et al., 2021) however, further studies are needed to explain this behavior.

It can be observed in the SF and SC that the main hydrophobic amino acids precursors of aroma, such as alanine (Ala), had the highest concentration of 64.23 and 30.56 mg/100 g, respectively, in cocoa from Guadalupe (Table 2), values that are within the range reported by Deus et al. (2021). On the other hand, the content of phenylalanine (Phe) was 34.60 and 34.78 mg/100 g in the SF and SC in cocoa from Copallín; correspondingly (Table 1), these two amino acids were the greatest between the third and fourth day of fermentation, similar to what was reported by Rottiers et al. (2019) who mentioned that during the first days of fermentation develops a high concentration of these amino acids due the proteolysis stimulated by lactic and acetic acid (Febrianto et al., 2022; Gutiérrez-Ríos et al., 2022). In this hydrophobic group, tyrosine (Tyr) reached concentrations of 16.59 mg/100 g (Copallín, Table 1) in the SF and 17.38 mg/100 g (Tolopampa, Table 3) in the SC from the third day of fermentation, and leucine (Leu) reached content of 37. 35 and 43.32 mg/100 g in the SF and SC, respectively (Tolopampa, Table 3).

In the case of valine (Val, hydrophobic), a concentration of 15.74 and 18.63 mg/100 g in cocoa from Tolopampa at the end of fermentation (Table 3), similar to that reported by Olugosi, Agbede, Adebayo, Onibi, and Ayeni (2019); however, its concentration increased after the second day only for SC unlike what was said by Rottiers et al. (2019) who observed same after the first day, possibly due to the genetic variation of the crop (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Servent et al., 2018). In the case of isoleucine (Iso) concentration, it was detected up to 6.90 mg/100 g (Copallín) in the SF and between 0.38 (Guadalupe) and 7.57 mg/100 g (Tolopampa) in the SC of cocoa bean (criollo). It can also be observed that from the third day of fermentation, high concentrations of this amino acid are achieved, which agrees with what was reported by Rottiers et al. (2019), where its formation is due to the interaction of Val and Leu with the acetohydroxy acid synthase that acts on pyruvate and generates acetolactate (Salaria et al., 2022).

Umami amino acids induce a palatable flavor due to aspartic and glutamic acids (Dala-Paula et al., 2021). In the SF, glutamic acid (Glu) disappeared after the third day in the bean (Tables 1, 2, and 3); this decrease is a disadvantage since it is a desirable compound of fermented cocoa (Deus et al., 2021). However, though it decreased in the SC (except for Tolopampa that disappeared after day 5, Table 3), it remained at a considerable concentration in the bean, which may be due to the metabolism of *S. cerevisiae* during fermentation (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023; Castro-Alayo et al., 2019), demonstrating that it is possible to modulate the fermentation process by inoculating with this yeast (Korcari et al., 2023; Ooi et al., 2020). In the case of aspartic acid (Asp), a significant concentration could be detected from the second day in the SF, reaching a maximum of 2.12 mg/100 g (Tolopampa, Table 3). In contrast to this, this amino acid was not present in cacao from Guadalupe (Table 2); on the other hand, the concentration of the amino acids in the SC varied among each place, highlighting that only in cacao from Guadalupe a concentration of 12.85 mg/100 g was found in the first days of fermentation, which disappeared on day 4, behavior similar to that reported by Brunetto et al. (2020b), who indicated that its degradation (or possible absence) favors the formation of hydrophobic amino acids (John et al., 2019), in addition the low concentration reported is characteristic of a well-fermented bean (de Araujo et al., 2021).

The concentration of arginine (Arg) was similar to that reported by Dala-Paula et al. (2021); in addition, it was observed that in the SF, a higher concentration of 54.20 mg/100 g was achieved (Guadalupe, Table 2), similar concentration in the same fermentation time (day 4) was reported by Deus et al. (2021), in contrast to SC, which was 34.18 mg/100 g on day 7 (Tolopampa, Table 3). Arg ensures a slightly bitter taste in cocoa (Deus et al., 2021); its content increases considerably during the fermentation of Criollo cocoa, in a higher proportion than what was reported in cocoa clones (Deus et al., 2020), which demonstrates its resistance to degradation by carboxypeptidases (Tamimi et al., 2023). Regarding glycine (Gly) content, its concentration was 5.55 mg/100 g in the SF, lower than in the SC, which reached up to 6.04 mg/100 g (Copallín, Table 1), similar to what was reported by Brunetto et al. (2020a); likewise, the presence of this amino acid is due to enzymatic hydrolysis product of the Maillard reaction (John et al., 2019; Purbaningrum, Hidayat, Witasari, & Utami, 2023). Gly recorded high concentrations from day 2 to day 5, a period that is close to that reported by Deus et al. (2021), evidencing the increase during fermentation (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Calvo et al., 2021; Fang et al., 2020), which reinforces the formation of pyrazines due to the presence of amino acids (Purbaningrum et al., 2023).

Proline (Pro) is an amino acid characteristic of Criollo cocoa (Balcázar-Zumaeta, Castro-Alayo, et al., 2023); it was observed at a concentration of 1.73 mg/100 g in the SC (Guadalupe, Table 2), slightly higher compared to SF, which was 1.59 mg/100 g (Copallín, Table 1). According to Gutiérrez-Ríos et al. (2022), this difference is due to the starter culture's metabolism, which transports a fraction of the carbon to the Krebs cycle, forming aromatic precursors associated with amino acids. Proline (Pro) gives rise to 2-acetyl-1-pyrrole (De Vuyst & Leroy, 2020), which in turn provides caramel, sweet and toasted notes (Sari et al., 2023). The concentration of Lysine (Lys) increases considerably from the second fermentation day, reaching up to 25.21 and 25.78 mg/100 g in the SF and SC, correspondingly (Copallín, Table 1); this behavior, demonstrates that the first days of fermentation is characterized by a low concentration due to the anaerobic phase (Deus et al., 2021; Spizzirri et al., 2019), this content considerable increased from the third day of the process coinciding with what was reported by de

Table 1
Average values* of amino acids during SF and SC in cocoa beans from Copallín.

Aminoacids	Spontaneous Fermentation								Starter Culture							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Asp	0.00 ± 0.00f	0.00 ± 0.00f	1.10 ± 0.08b	0.64 ± 0.01d	1.43 ± 0.005a	0.40 ± 0.05e	0.98 ± 0.001c	1.11 ± 0.003b	1.25 ± 0.008a	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b
Gly	1.777 ± 0.04 g	1.88 ± 0.01 g	3.60 ± 0.008d	5.55 ± 0.03a	4.12 ± 0.02b	3.82 ± 0.07c	3.05 ± 0.002e	2.88 ± 0.08f	1.72 ± 0.006e	1.74 ± 0.02e	3.07 ± 0.03b	1.58 ± 0.01f	1.55 ± 0.009f	6.04 ± 0.03a	2.28 ± 0.02d	2.54 ± 0.02c
Ala	9.72 ± 0.02f	7.89 ± 0.01 g	17.05 ± 0.09c	23.04 ± 0.11a	15.89 ± 0.12d	19.31 ± 0.46b	12.08 ± 0.06e	15.72 ± 0.002d	11.1 ± 0.09e	5.45 ± 0.14f	13.1 ± 0.23d	11.5 ± 0.01e	11.1 ± 0.09e	22.4 ± 0.26a	14.1 ± 0.54c	19.9 ± 0.47b
Val	4.13 ± 0.01e	3.55 ± 0.008f	10.73 ± 0.06c	15.43 ± 0.12a	11.18 ± 0.11c	14.56 ± 0.41b	8.30 ± 0.04d	10.69 ± 0.17c	5.83 ± 0.02e	3.31 ± 0.02f	7.58 ± 0.03d	7.62 ± 0.03d	5.88 ± 0.04e	14.6 ± 0.29a	9.68 ± 0.47c	12.9 ± 0.23b
Lys	0.00 ± 0.00 g	0.00 ± 0.00 g	4.82 ± 0.26d	25.21 ± 0.19a	3.67 ± 0.05e	18.34 ± 0.26b	2.37 ± 0.02f	13.33 ± 0.02c	0.00 ± 0.00f	0.00 ± 0.00f	1.69 ± 0.06e	5.38 ± 0.01d	6.00 ± 0.06d	10.05 ± 0.55c	25.78 ± 0.66a	16.07 ± 0.73b
Iso	1.11 ± 0.08f	0.75 ± 0.002 g	3.91 ± 0.03c	6.90 ± 0.05a	3.81 ± 0.02 cd	5.49 ± 0.08b	3.03 ± 0.04e	3.68 ± 0.07d	1.68 ± 0.01e	0.98 ± 0.0006f	2.33 ± 0.07d	2.91 ± 0.04c	2.46 ± 0.08d	6.36 ± 0.01a	2.91 ± 0.17c	5.10 ± 0.14b
Phe	3.12 ± 0.04 g	2.56 ± 0.01 h	16.72 ± 0.09d	34.60 ± 0.07a	12.41 ± 0.04e	27.79 ± 0.52b	10.59 ± 0.07f	22.64 ± 0.10c	3.51 ± 0.05f	2.13 ± 0.007 g	8.22 ± 0.17e	13.02 ± 0.05d	13.09 ± 0.10d	34.78 ± 0.32a	16.36 ± 0.72c	24.01 ± 0.48b
Tyr	3.49 ± 0.03 g	2.60 ± 0.008 h	8.65 ± 0.03d	16.59 ± 0.18a	7.63 ± 0.07e	13.31 ± 0.30b	6.40 ± 0.08f	9.18 ± 0.02c	3.57 ± 0.10f	2.24 ± 0.05 g	4.70 ± 0.08e	5.80 ± 0.02d	5.68 ± 0.06d	15.10 ± 0.29a	7.56 ± 0.19c	11.61 ± 0.17b
Thr	0.93 ± 0.05d	0.81 ± 0.02e	0.33 ± 0.007f	1.40 ± 0.0004c	0.32 ± 0.008f	2.59 ± 0.04b	2.71 ± 0.005a	0.00 ± 0.00 g	1.03 ± 0.01c	0.77 ± 0.15c	2.27 ± 0.05b	0.71 ± 0.01c	0.65 ± 0.01c	7.30 ± 0.37a	1.07 ± 0.11c	2.15 ± 0.13b
Cys	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.39 ± 0.00b	0.00 ± 0.00d	0.84 ± 0.17a	0.16 ± 0.007 cd	0.30 ± 0.01bc	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.22 ± 0.01b	0.37 ± 0.007a	0.35 ± 0.01a	0.35 ± 0.01a	0.19 ± 0.003b
Met	0.00 ± 0.00f	0.00 ± 0.00f	1.58 ± 0.01c	5.14 ± 0.00a	0.08 ± 0.01ef	0.16 ± 0.13e	0.48 ± 0.02d	4.23 ± 0.05b	0.00 ± 0.00f	0.00 ± 0.00f	0.430 ± 0.01d	0.00 ± 0.00f	1.18 ± 0.009b	6.72 ± 0.07a	1.01 ± 0.00c	0.27 ± 0.007e
Ser	2.49 ± 0.03f	1.82 ± 0.01 g	4.23 ± 0.01d	8.64 ± 0.05a	4.22 ± 0.02d	7.97 ± 0.18b	3.18 ± 0.02e	5.63 ± 0.005c	2.30 ± 0.01f	1.33 ± 0.006 g	2.88 ± 0.05e	3.29 ± 0.009d	3.09 ± 0.01de	8.91 ± 0.11a	4.85 ± 0.17c	7.27 ± 0.18b
Leu	2.19 ± 0.01f	2.06 ± 0.03f	16.70 ± 0.08d	36.13 ± 3.03a	12.32 ± 0.04e	32.89 ± 0.60b	10.14 ± 0.06e	24.74 ± 0.09c	2.56 ± 0.05f	1.80 ± 0.008f	7.64 ± 0.15e	14.47 ± 0.009d	14.61 ± 0.09d	41.28 ± 0.52a	19.79 ± 0.97c	29.08 ± 0.90b
Pro	0.37 ± 0.13bc	0.00 ± 0.00c	0.52 ± 0.21b	1.38 ± 0.23a	0.74 ± 0.20b	0.52 ± 0.08b	1.59 ± 0.12a	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.93 ± 0.13b	0.00 ± 0.00c	1.34 ± 0.16a
Glu	4.89 ± 0.11a	3.77 ± 0.98b	5.32 ± 0.00a	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00f	1.19 ± 0.02e	2.22 ± 0.08d	2.53 ± 0.07c	2.87 ± 0.04b	6.73 ± 0.10a	2.27 ± 0.09d	3.05 ± 0.04b
Arg	7.89 ± 0.05f	7.25 ± 0.04 g	13.70 ± 0.05d	32.71 ± 0.27a	14.03 ± 0.13d	26.84 ± 0.48b	10.78 ± 0.11e	18.44 ± 0.07c	8.41 ± 0.05e	5.75 ± 0.58f	9.43 ± 0.14d	9.71 ± 0.04d	10.06 ± 0.09d	32.61 ± 0.21a	15.75 ± 0.30c	24.15 ± 0.09b
Hist	1.38 ± 0.07e	0.90 ± 0.00f	2.42 ± 0.02b	3.66 ± 0.03a	2.35 ± 0.03bc	2.23 ± 0.10c	1.528 ± 0.007e	1.92 ± 0.03d	2.55 ± 0.01b	0.40 ± 0.01 h	0.98 ± 0.01e	0.58 ± 0.01 g	0.81 ± 0.01f	3.35 ± 0.03a	1.07 ± 0.03d	1.49 ± 0.02c

* Means and standard deviation (expressed in mg of the amino acid/100 g sample), with different lowercase letters in the same columns (time) indicating statistical differences (Tukey test, $p \leq 0.05$). Asp – Aspartic acid, Gly – glycine, Ala – alanine, Val – valine, Lys – lysine, Iso – isoleucine, Phe – phenylalanine, Tyr – tyrosine, Thr – threonine, Cys – cysteine, Met – methionine, Ser – serine, Leu – leucine, Pro – proline, Glu – glutamic acid, Arg – arginine, His – histidine.

Table 2
Average values* of amino acids during SF and SC in cocoa beans from Guadalupe.

Aminoacids	Spontaneous Fermentation								Starter Culture							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Asp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	12.85 ± 0.05a	1.34 ± 0.03d	2.97 ± 0.11c	3.99 ± 0.09b	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00e
Gly	1.85 ± 0.06c	1.05 ± 0.01e	3.39 ± 0.03a	0.00 ± 0.00f	2.62 ± 0.04b	1.84 ± 0.01c	3.34 ± 0.33a	1.43 ± 0.007d	2.94 ± 0.01f	1.06 ± 0.01 h	1.40 ± 0.01 g	3.93 ± 0.02c	3.24 ± 0.02e	3.62 ± 0.02d	5.44 ± 0.06b	5.81 ± 0.09a
Ala	43.92 ± 0.07d	11.30 ± 0.22e	49.74 ± 0.89c	0.00 ± 0.00f	64.23 ± 2.46a	49.42 ± 1.92c	45.24 ± 0.48d	59.63 ± 0.05b	16.86 ± 0.05d	13.97 ± 0.07e	22.28 ± 0.57c	30.56 ± 0.39a	22.92 ± 1.13c	27.43 ± 0.13b	22.90 ± 0.06c	29.45 ± 0.47a
Val	2.05 ± 0.01f	0.93 ± 0.02 g	9.49 ± 0.10d	0.00 ± 0.00 h	12.26 ± 0.27a	10.68 ± 0.62c	8.71 ± 0.09e	11.43 ± 0.04b	3.31 ± 0.00e	2.68 ± 0.01e	12.10 ± 0.18d	15.60 ± 0.27b	14.12 ± 0.61c	16.67 ± 0.10a	12.89 ± 0.04d	14.40 ± 0.39c
Lys	0.00 ± 0.00d	0.00 ± 0.00d	1.09 ± 0.03c	0.00 ± 0.00d	9.59 ± 0.39a	9.17 ± 0.71a	9.41 ± 0.09a	7.44 ± 0.02b	0.00 ± 0.00e	0.00 ± 0.00e	2.58 ± 0.15d	10.97 ± 0.25c	12.31 ± 1.17c	16.75 ± 0.15b	16.02 ± 0.39b	21.59 ± 0.56a
Iso	0.87 ± 0.01d	0.0004 ± 0.00e	3.42 ± 0.10c	0.00 ± 0.00e	4.51 ± 0.19a	3.97 ± 0.19b	3.89 ± 0.05b	4.16 ± 0.03b	0.38 ± 0.00e	0.62 ± 0.02e	4.81 ± 0.22d	5.60 ± 0.08c	6.08 ± 0.41bc	7.08 ± 0.09a	5.77 ± 0.05bc	6.20 ± 0.14b
Phe	1.37 ± 0.01c	6.31 ± 4.84b	9.31 ± 0.17b	0.00 ± 0.00c	21.69 ± 0.64a	19.37 ± 0.60a	21.35 ± 0.16a	18.01 ± 0.01a	0.97 ± 0.01f	1.55 ± 0.00f	13.98 ± 0.27e	25.72 ± 0.29d	27.51 ± 1.20c	31.31 ± 0.24b	28.62 ± 0.13c	33.20 ± 0.57a
Tyr	1.43 ± 0.008d	0.75 ± 0.07d	6.63 ± 0.17c	0.00 ± 0.00e	9.77 ± 0.37b	9.73 ± 0.26b	10.38 ± 0.53ab	10.62 ± 0.14a	0.17 ± 0.00f	0.08 ± 0.00f	0.75 ± 0.02e	1.02 ± 0.01d	1.29 ± 0.06b	1.51 ± 0.02a	1.13 ± 0.07c	1.57 ± 0.02a
Thr	0.72 ± 0.20d	0.016 ± 0.00e	2.57 ± 0.08a	0.00 ± 0.00e	1.84 ± 0.10bc	1.51 ± 0.12c	2.49 ± 0.48a	2.22 ± 0.03ab	1.27 ± 0.01d	0.78 ± 0.01d	1.80 ± 0.09c	2.23 ± 0.08c	2.00 ± 0.24c	3.10 ± 0.05b	6.84 ± 0.40a	3.51 ± 0.09b
Cys	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.55 ± 0.003a	0.519 ± 0.02b	0.53 ± 0.004ab	0.515 ± 0.005b	0.00 ± 0.00d	0.00 ± 0.00d	0.28 ± 0.04bc	0.39 ± 0.11ab	0.49 ± 0.03a	0.38 ± 0.02ab	0.22 ± 0.01c	0.27 ± 0.01bc
Met	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	2.42 ± 0.04a	0.00 ± 0.00b	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	3.96 ± 0.03b	4.22 ± 0.12a
Ser	3.96 ± 0.14d	1.09 ± 0.01e	4.06 ± 0.07d	0.00 ± 0.00f	7.31 ± 0.25a	6.63 ± 0.23b	5.53 ± 0.05c	7.25 ± 0.01a	3.93 ± 0.07e	2.29 ± 0.01f	5.86 ± 0.17d	7.49 ± 0.08b	7.05 ± 0.32c	8.69 ± 0.05a	7.11 ± 0.02bc	8.53 ± 0.11a
Leu	0.95 ± 0.0007d	0.59 ± 0.02d	8.97 ± 0.23c	0.00 ± 0.00d	24.81 ± 0.91a	22.93 ± 0.92b	22.03 ± 0.23b	21.92 ± 0.02b	0.81 ± 0.00e	1.86 ± 0.01e	13.82 ± 0.32d	27.80 ± 0.33c	29.88 ± 1.44b	33.61 ± 0.23a	29.02 ± 0.12bc	33.88 ± 0.53a
Pro	0.067 ± 0.09bc	0.00 ± 0.00c	0.28 ± 0.10b	0.00 ± 0.00c	0.74 ± 0.16a	0.26 ± 0.07bc	0.088 ± 0.05bc	0.78 ± 0.12a	0.00 ± 0.00d	0.00 ± 0.00d	0.07 ± 0.00 cd	1.73 ± 0.20a	0.88 ± 0.29b	1.40 ± 0.26a	0.59 ± 0.27bc	0.85 ± 0.12b
Glu	10.16 ± 0.32a	2.28 ± 0.04de	5.50 ± 0.21c	0.00 ± 0.00f	6.08 ± 0.22b	2.63 ± 0.039d	1.93 ± 0.046e	2.55 ± 0.046d	14.51 ± 0.06a	3.56 ± 0.01d	7.80 ± 0.29bc	8.71 ± 0.14b	6.17 ± 0.31c	6.60 ± 1.85c	3.36 ± 0.09d	4.16 ± 0.07d
Arg	16.62 ± 8.33c	15.86 ± 0.08c	27.47 ± 0.11b	0.00 ± 0.00d	54.20 ± 0.94a	51.80 ± 1.39a	48.71 ± 0.27a	46.01 ± 0.11a	6.89 ± 0.04 g	3.16 ± 0.03 h	9.76 ± 0.10f	17.97 ± 0.12e	19.66 ± 0.37d	23.99 ± 0.13b	20.89 ± 0.26c	27.17 ± 0.32a
Hist	0.68 ± 0.07d	0.00 ± 0.00f	0.97 ± 0.008c	0.00 ± 0.00f	1.16 ± 0.05b	1.00 ± 0.01c	1.39 ± 0.03a	0.28 ± 0.009e	1.31 ± 0.06d	0.00 ± 0.00f	0.59 ± 0.00e	2.04 ± 0.05c	2.02 ± 0.03c	2.22 ± 0.05b	2.28 ± 0.01b	3.30 ± 0.08a

* Means and standard deviation (expressed in mg of the amino acid/100 g sample), with different lowercase letters in the same columns (time) indicating statistical differences (Tukey test, $p \leq 0.05$). Asp – Aspartic acid, Gly – glycine, Ala – alanine, Val – valine, Lys – lysine, Iso – isoleucine, Phe – phenylalanine, Tyr – tyrosine, Thr – threonine, Cys – cysteine, Met – methionine, Ser – serine, Leu – leucine, Pro – proline, Glu – glutamic acid, Arg – arginine, His – histidine.

Table 3
Average values* of amino acids during SF and SC in cocoa beans from Tolopampa.

Aminoacids	Spontaneous Fermentation								Starter Culture							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Asp	0.00 ± 0.00e	0.00 ± 0.00e	0.00 ± 0.00e	1.06 ± 0.01d	1.17 ± 0.01c	0.98 ± 0.009d	1.47 ± 0.07b	2.12 ± 0.03a	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	1.82 ± 0.04a	0.89 ± 0.01c	1.44 ± 0.02b
Gly	2.65 ± 0.008c	2.31 ± 0.01c	3.52 ± 0.02b	3.33 ± 0.02b	4.53 ± 0.02a	4.15 ± 0.01a	3.64 ± 0.02b	3.61 ± 0.46b	1.79 ± 0.01f	4.61 ± 0.02b	5.45 ± 0.01a	4.42 ± 0.005c	4.66 ± 0.05b	2.08 ± 0.05e	1.70 ± 0.01f	2.38 ± 0.09d
Ala	14.68 ± 0.03e	12.82 ± 0.02f	17.00 ± 0.16d	19.03 ± 0.14c	20.72 ± 0.35b	20.45 ± 0.18b	19.30 ± 0.20c	21.98 ± 0.56a	11.72 ± 0.26e	18.03 ± 0.08d	20.25 ± 0.27c	25.70 ± 0.96a	21.89 ± 0.73b	22.52 ± 0.23b	19.66 ± 0.07c	25.23 ± 0.78a
Val	10.16 ± 0.03d	8.09 ± 0.02e	11.50 ± 0.48c	13.12 ± 0.05b	15.73 ± 0.26a	15.22 ± 0.05a	4.17 ± 0.05f	15.74 ± 0.30a	2.89 ± 0.03 g	8.73 ± 0.03f	12.53 ± 0.13e	16.75 ± 0.46b	12.99 ± 0.36de	15.92 ± 0.07c	13.67 ± 0.22d	18.63 ± 0.45a
Lys	4.28 ± 0.04f	7.41 ± 0.01e	14.28 ± 0.14d	15.59 ± 0.21c	21.29 ± 0.33a	19.06 ± 0.38b	0.00 ± 0.00 g	20.87 ± 0.42a	0.00 ± 0.00 g	1.98 ± 0.02f	10.25 ± 0.08e	18.73 ± 0.54c	20.51 ± 0.56b	19.86 ± 0.19bc	17.32 ± 0.02d	25.49 ± 1.11a
Iso	3.84 ± 0.008e	3.48 ± 0.07f	5.25 ± 0.13c	4.90 ± 0.08d	6.07 ± 0.09a	5.83 ± 0.13ab	1.23 ± 0.02 g	5.69 ± 0.18b	1.21 ± 0.02 g	3.39 ± 0.02f	4.91 ± 0.06e	7.15 ± 0.23b	6.42 ± 0.10c	6.82 ± 0.08bc	5.71 ± 0.04d	7.57 ± 0.29a
Phe	14.94 ± 0.03d	16.76 ± 0.08d	24.10 ± 1.58c	27.37 ± 0.18b	33.34 ± 0.58a	31.63 ± 0.14a	3.94 ± 0.05e	32.15 ± 0.70a	2.39 ± 0.03 g	6.36 ± 0.009f	23.65 ± 0.26e	31.08 ± 1.48 cd	33.06 ± 0.85bc	34.59 ± 0.27b	29.77 ± 0.08d	38.61 ± 1.58a
Tyr	7.56 ± 0.02c	7.85 ± 0.02c	10.78 ± 0.69b	12.45 ± 0.36b	16.22 ± 1.72a	14.93 ± 0.17a	5.28 ± 0.10d	14.50 ± 0.22a	2.43 ± 0.03f	7.09 ± 0.23e	11.66 ± 0.20d	13.98 ± 0.57bc	14.45 ± 0.28bc	15.69 ± 1.44ab	13.63 ± 0.57c	17.38 ± 0.23a
Thr	1.22 ± 0.01a	1.12 ± 0.01a	1.74 ± 0.05a	3.61 ± 0.22a	4.51 ± 0.22a	4.18 ± 0.01a	1.93 ± 0.01a	2.71 ± 4.11a	1.01 ± 0.01f	2.95 ± 0.0d	4.02 ± 0.06bc	1.89 ± 0.21e	1.74 ± 0.15e	4.22 ± 0.05b	3.71 ± 0.01c	5.13 ± 0.15a
Cys	0.38 ± 0.005c	0.28 ± 0.007d	0.39 ± 0.009c	0.25 ± 0.04d	0.68 ± 0.01a	0.71 ± 0.01a	0.00 ± 0.00e	0.59 ± 0.01b	0.00 ± 0.00e	0.00 ± 0.00e	0.34 ± 0.01d	0.39 ± 0.01d	0.37 ± 0.01d	0.45 ± 0.02c	0.50 ± 0.008b	0.78 ± 0.02a
Met	0.00 ± 0.00f	1.40 ± 0.02c	2.54 ± 0.07b	0.21 ± 0.009e	0.44 ± 0.007d	0.45 ± 0.02d	0.00 ± 0.00f	6.59 ± 0.07a	0.00 ± 0.00d	0.00 ± 0.00d	0.73 ± 0.008b	0.00 ± 0.00d	2.51 ± 0.10a	0.00 ± 0.00d	0.09 ± 0.01d	0.44 ± 0.02c
Ser	4.56 ± 0.01c	4.34 ± 0.002c	6.34 ± 0.04b	0.343 ± 0.10e	0.31 ± 0.01e	0.342 ± 0.07e	4.03 ± 0.04d	7.69 ± 0.22a	3.07 ± 0.03e	5.69 ± 0.01d	7.16 ± 0.07c	8.73 ± 0.27a	8.15 ± 0.09b	8.36 ± 0.13b	0.31 ± 0.005f	0.37 ± 0.06f
Leu	15.39 ± 0.02 g	17.77 ± 0.03f	27.98 ± 0.23e	30.85 ± 0.39d	37.35 ± 0.41a	34.95 ± 0.32c	2.48 ± 0.05 h	36.18 ± 0.65b	1.84 ± 0.027f	6.58 ± 0.029e	24.16 ± 0.30d	36.83 ± 1.30b	37.01 ± 0.77b	39.51 ± 0.49b	33.00 ± 1.40c	43.32 ± 1.67a
Pro	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00c	0.14 ± 0.05c	0.95 ± 0.09b	0.95 ± 0.03b	0.80 ± 0.25b	1.29 ± 0.00a	0.00 ± 0.00b	0.41 ± 0.20a	0.00 ± 0.00b	0.066 ± 0.00b	0.049 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.42 ± 0.26a
Glu	2.78 ± 0.007b	1.81 ± 0.01c	3.02 ± 0.05a	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d	5.78 ± 0.08b	7.84 ± 0.22a	4.50 ± 1.45bc	4.32 ± 0.25bc	3.51 ± 0.05c	0.00 ± 0.00d	0.00 ± 0.00d	0.00 ± 0.00d
Arg	11.56 ± 0.02f	14.03 ± 0.04e	20.83 ± 0.22d	24.36 ± 0.12c	30.71 ± 0.26a	28.94 ± 0.16b	9.96 ± 0.05 g	29.34 ± 0.18b	8.42 ± 0.05 g	14.40 ± 0.02f	17.86 ± 0.05e	24.10 ± 0.32d	28.69 ± 0.18b	27.41 ± 0.35c	24.24 ± 0.23d	34.18 ± 0.82a
Hist	1.84 ± 0.01f	1.94 ± 0.01e	3.00 ± 0.03b	2.33 ± 0.03d	2.89 ± 0.03c	3.01 ± 0.01b	2.32 ± 0.04d	4.00 ± 0.03a	0.91 ± 0.02e	2.24 ± 0.02d	3.37 ± 0.01a	3.13 ± 0.003b	3.40 ± 0.03a	2.56 ± 0.04c	2.17 ± 0.02d	3.39 ± 0.04a

* Means and standard deviation (expressed in mg of the amino acid/100 g sample), with different lowercase letters in the same columns (time) indicating statistical differences (Tukey test, $p \leq 0.05$). Asp – Aspartic acid, Gly – glycine, Ala – alanine, Val – valine, Lys – lysine, Iso – isoleucine, Phe – phenylalanine, Tyr – tyrosine, Thr – threonine, Cys – cysteine, Met – methionine, Ser – serine, Leu – leucine, Pro – proline, Glu – glutamic acid, Arg – arginine, His – histidine.

Brito et al. (2001 and Deus et al. (2021), demonstrating the importance of the fermentation time.

Threonine (Thr), methionine (Met), and histidine (His) are amino acids in low concentrations during fermentations in cocoa beans, as can be seen in Tables 1, 2, and 3, which coincides with the studies reported by Balcázar-Zumaeta, Castro-Alayo, et al. (2023). At the beginning of the fermentation process, Thr showed a low content similar to that reported by Dala-Paula et al. (2021) and Spizzirri et al. (2019) due to an increase in the temperature during fermentation (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023), which affects protein digestibility resulting in a low concentration (Dala-Paula et al., 2021); in addition, a high concentration was achieved in the SC (7.30 mg/100 g, Table 1) compared to SF (4.51 mg/100 g, Table 3), in both cases this content was obtained between the fourth and fifth day of bean fermentation.

On the other hand, in our study, during SF and SC, the concentration of Met was high from the third day to final fermentation process (6.59 and 6.72 mg/100 g, respectively), different from what was observed in Sari et al. (2023), this finding can be explained due to the previous separation of the pulp from the bean; demonstrating the contribution of the pulp to the presence of amino acids (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023). Regarding His, its concentration during fermentation was relatively low compared to the study of Deus et al. (2021); it barely exceeded 4.0 mg/100 g (Table 3) in the SF and 3.40 mg/100 g in the SC (Table 3), the low concentration in the SC agrees with that reported by Korcari et al. (2023) when using starter cultures, which is explained by a low capacity of this yeast to produce His by decarboxylation (Delgado-Ospina et al., 2021).

Balcázar-Zumaeta, Castro-Alayo, et al. (2023) mentioned that cystine (Cys) is a hydrophobic amino acid reported in Trinitario, Forastero, and clones (Deus et al., 2020, 2021). In our study, the Criollo cocoa contained Cys in the SF (0.28 to 0.84 mg/100 g) and in the SC (0.19 to 0.77 mg/100 g); these low concentrations favor the consumption of chocolate that can be obtained from this cocoa since Cys is a sulfur amino acid (Lasta, da Silva Pereira Ronning, Dekker, & da Cunha, 2021). Finally, the presence of serine (Ser) has been detected in the cocoa bean, an amino acid characteristic of cocoas (Dala-Paula et al., 2021); a high concentration was achieved between the third and fifth day (except for SF in Tolopampa cocoa, Table 3), confirming the findings of Balcázar-Zumaeta, Castro-Alayo, et al. (2023), who found high concentrations of this amino acid after day 2 of the process. In addition, the concentration of Ser in the SC reached 8.91 mg/100 g, slightly higher than SF, which was 8.64 mg/100 g. The study allowed us to observe how the type of fermentation and time can affect the concentration of amino acids; however, the origin of the cocoa is a factor that can affect the concentration of amino acids (Calvo et al., 2021). Additional studies are required in this regard.

Fig. 2 reports the content of free amino acids (FAA); at the beginning of fermentation, it was 43.5 mg/100 g (Copallín, Fig. 2a), and from day 3 until the end of fermentation, an increase of >150 mg/100 g of FAA was observed (except for cocoa from Guadalupe, day 3), where a maximum content (221.3 mg/100 g) for FAA was registered on day 4; thus, there was a 5-fold increase in FAA levels compared to what was reported at the beginning of the fermentation. This finding is higher than the report of Deus et al. (2021), where the increase was up to 2.6 times. On the other hand, in Fig. 2a, the cocoa from Copallín registered a low content during almost the entire fermentation process. The FAA content was below the value mentioned by Tchouatcheu et al. (2019), where a fermented bean contained around 800 to 1400 mg/100 g; this may be because the study was performed in an aerobic process (with turnovers) (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023), producing the oxidation of flavan-3-ols, which reacted with nucleophilic groups of amino acids to form insoluble complexes (Schlüter et al., 2022), and the presence of microorganisms which limited the production of FAA content (Rahardjo et al., 2022).

Fig. 2b indicates that the FAA level at the beginning of fermentation in the SC was 43.4 mg/100 g and started to increase from day 2 until the end of the process, where FAA levels were higher than 150 mg/100 g; also, high content was obtained on day 5 (233.3 mg/100 g, Copallín), 5 times higher compared to the beginning of the fermentation, and above to that reported by Deus et al. (2021). A more illustrated behavior can be observed in the increase of FAA during fermentation (SC) with starter culture, where the cocoa from Copallín showed again lower content compared to the bean from the other two places; these differences could be due to the variety, origin, maturity, and harvesting time (Perez et al., 2022; Rottiers et al., 2019). It could be evidenced that in the SC, there is a slight increase in FAA content during the fermentation process; this increase in FAA has been shown to generate an increased content of desirable volatile compounds in cocoa (Purbaningrum et al., 2023).

In both fermentations, there was a growth in the level of FAA as fermentation elapsed, particularly the hydrophobic ones (while Asp and Glu decreased) behavior similar to that reported by Rottiers et al. (2019). This increase in the FAA content was previously reported Balcázar-Zumaeta, Castro-Alayo, et al. (2023) and Delgado-Ospina et al. (2021), and it was associated with a decrease in the pH of the cocoa bean, agreeing with (Afoakwa et al., 2008; John et al., 2019).

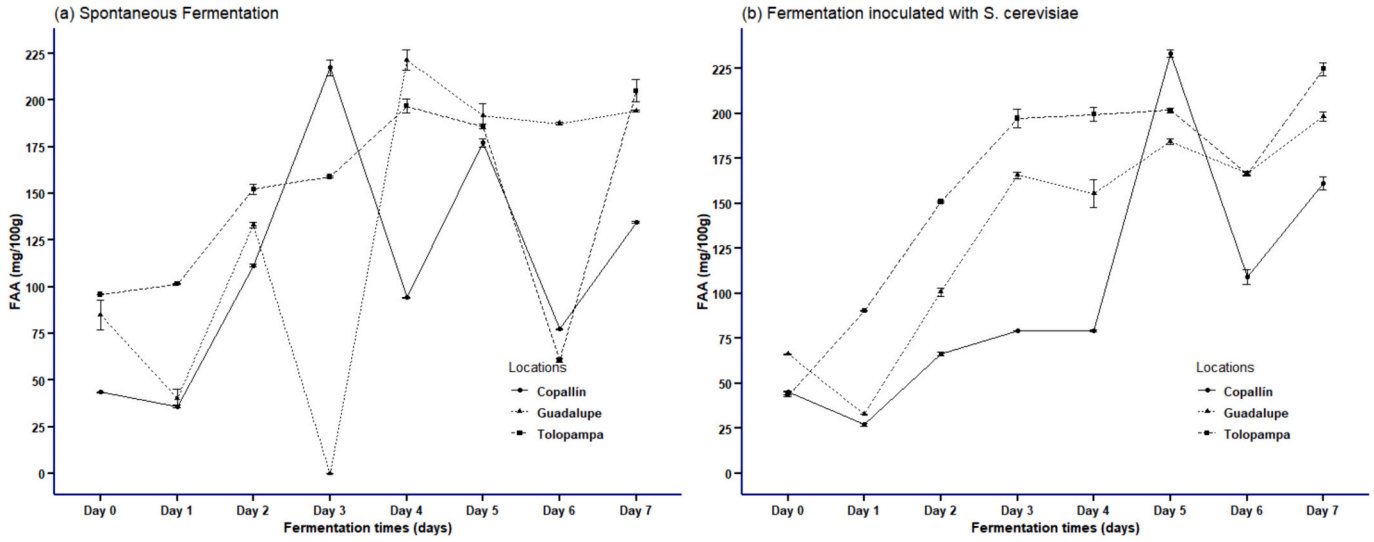


Fig. 2. Free amino acid (FAA) levels during spontaneous fermentation (SF) and fermentation with starter culture (SC) of cocoa bea.

3.2. Amino acid percentage (%) based on human nutritional requirements

The percentage contribution of amino acids classified according to their nutritional contribution to human health; their structural conformation have unique properties due to their amides and carboxylic chemical composition (Salaria et al., 2022) that conforms the protein fundamental units (Niemenak et al., 2020). The percentage of essential amino acids in the SF and SC increased considerably on day 2 (Fig. 3), representing 60% in the SF on day 3 (Tolopampa) and 30% on day 4 (Guadalupe). In the case of fermentation with *S. cerevisiae* inoculum (SC), it was observed that from day 2, the percentage of essential amino acids was high and remained like that until the end of fermentation (Fig. 4). It is evident that the yeast contributes to modulate the percentage of essential amino acids in cocoa (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Deus et al., 2021).

As for conditionally essential amino acids, although they can be produced by the human body, a precursor amino acid or a nitrogen donation from other amino acids is required (Hasan & Rima, 2021). Five amino acids contributed to the group, where they represented in the SF a percentage between 20% and 44% of the total during the whole process (Fig. 3), while in the SC, it was between 12% and 35% (in cocoa from Guadalupe it does not exceed 20%, Fig. 4B); it is essential to note that this group of amino acids under certain conditions of physiological stress cannot be produced in the organism (Hasan & Rima, 2021; Lopez & Mohiuddin, 2022), so the presence of this group in Criollo cocoa guarantees the disponibility of these amino acids, since their abundance may decrease conditionally (Salaria et al., 2022).

Finally, four amino acids contribute to the group of non-essential amino acids the human body can synthesize, hence their dispensable status (Lopez & Mohiuddin, 2022; Salaria et al., 2022). According to our study, in both types of fermentation, they decreased considerably, around 11% in the SF (Fig. 3C) and 12% in the SC (Fig. 4C). Their high percentage at the beginning of the fermentation process may be due to acidic amino acids amount, because they will start fermenting (Marseglia et al., 2014).

3.3. Percentage variation of amino acids grouped according to R group nature

Carenzi, Sacchi, Abbondi, and Pollegioni (2020) mention that amino acids are structured from a central carbon atom (α-carbon) attached to amino and carboxyl groups. From this, the amino acids identified in the study, depending on the incorporation of R groups to proteins, confer different properties to each of these groups (Dewangan, Berdimurodov, & Verma, 2023; Emery, 2013).

The small neutral group consisted of Gly and Ala, which participate as metabolic intermediates because they have lateral small side chains, hydrogen atoms, and a methyl group (Dewangan et al., 2023; Emery, 2013). In our study, Gly and Ala were in high percentages concerning the total amount in the cocoa bean during fermentation (Fig. 5), especially at the beginning (54%, Fig. 5b), except on the third day when no amino acid content was reported (possibly due to meager amounts to be detected by the chromatograph). A tendency to decrease (between 12% and 13%) was observed as the fermentation process progressed (Fig. 5a and c). Regarding SC, there was a high percentage (45.6%) at the beginning of the process (day 1, Fig. 6b). However, as fermentation progressed, it decreased to 12% in cocoa from the three origins, this group with other amino acids were crucial because they contributed to the formation of pyrazines by Maillard reaction (Purbaningrum et al., 2023).

In the branched-chain group represented by Val, Iso, and Leu in cocoa, amino acids of voluminous and non-polar side chains (Yoo, Shanmugalingam, & Smith, 2022) increased from the second day of fermentation, and the highest contribution (28 to 30%) was observed between day 3 and day 5 (Fig. 5a and c). The cocoa from Guadalupe recorded a maximum of 19.6% (day 5, Fig. 5b). Maximum concentrations of 31.5% (day 3, Fig. 6a), 32.1% (day 5, Fig. 6b) and 31.4% (day 6, Fig. 6c) were recorded in the SC; likewise, this group of amino acids is characterized by producing sweet aromatic notes, characteristic of chocolate (Castro-Alayo et al., 2019).

Within the aromatic group, we find Phe and Tyr, which are precursors of tyramine and phenylethylamine (Deus et al., 2021), and are responsible of chocolate aroma due to their aromatic ring (Gutiérrez-Ríos et al., 2022; Tamimi et al., 2023). According to Dewangan et al. (2023) these compounds are bulky non-polar-amino acids capable of interacting with other hydrophobic molecules. In the SF of the cocoa from Copallín (Fig. 5a) and Tolopampa (Fig. 5c), Phe and Tyr remained relatively stable between 15% and 25%. Meanwhile, in the cocoa from Guadalupe, the lowest percentage of this group was recorded on day 0 and day 3 (Fig. 5b). In the same way, low percentages (< 5%) were recorded in the SC of cocoa from Guadalupe between day 0 and day 1 (Fig. 6b). In addition, the highest rates of aromatics were obtained between days 4 and 6 of fermentation; these values were slightly higher than SF may be due to the yeast contributing to increase these aromatic

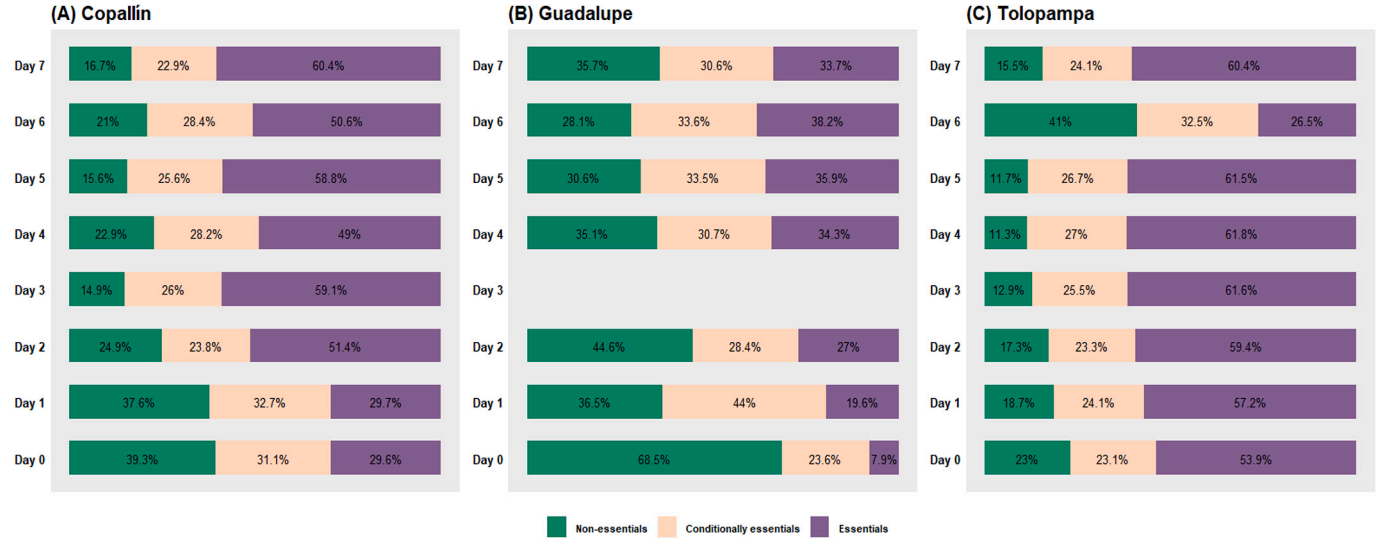


Fig. 3. Percentage contribution of amino acids during spontaneous fermentation (SF) in cocoa beans grouped according to human nutritional requirement: Essentials (Val, Lys, Iso, Phe, Thr, Met, Leu, and His), Conditionally essentials (Gly, Tyr, Cys, Pro and Arg), and Non-essentials (Asp, Ala, Ser, and Glu).

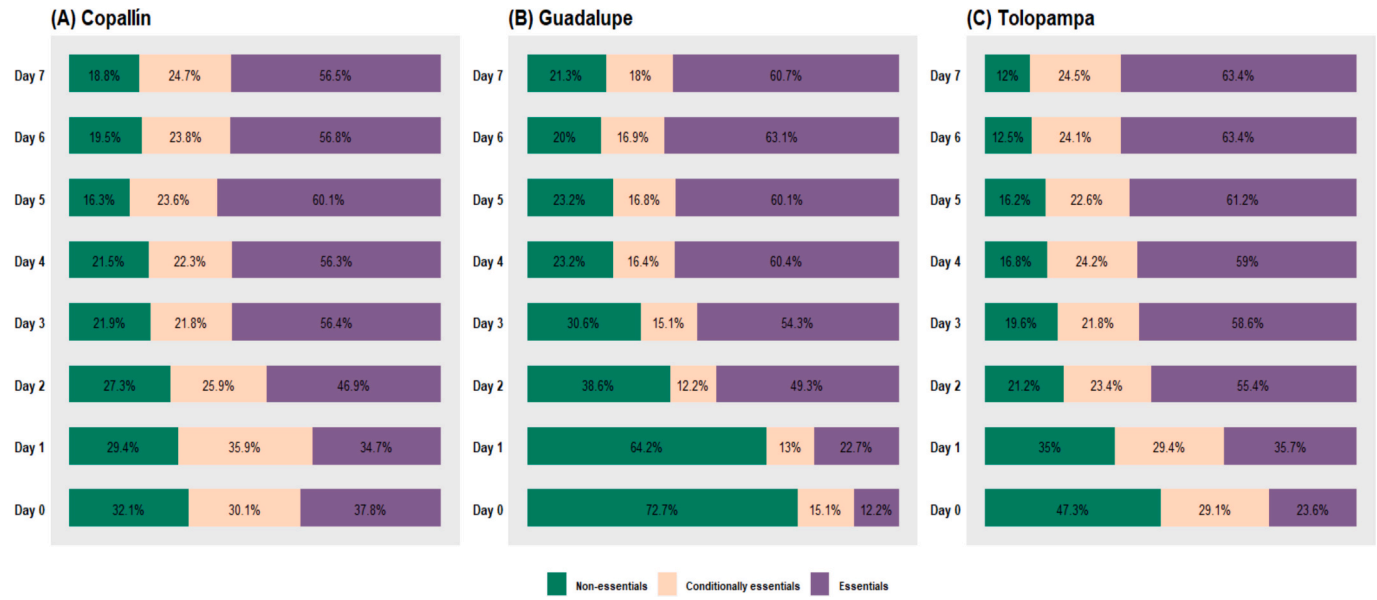


Fig. 4. Percentage contribution of amino acids during starter culture (SC) fermentation in cocoa beans grouped according to human nutritional requirement: Essentials (Val, Lys, Iso, Phe, Thr, Met, Leu, and His), Conditionally essentials (Gly, Tyr, Cys, Pro and Arg), and Non-essentials (Asp, Ala, Ser, and Glu).

amino acids precursor such as aldehydes (Purbaningrum et al., 2023).

The hydroxyl-containing group comprises Thr and Ser amino acids, which are weak and soluble acidic polar molecules capable of forming hydrogen bonds (Emery, 2013; Salter, Wierzbicki, & Honkanen, 2020). Thr contributes to the flavor profile (savory, pleasant), and Ser to the fats' and fatty acids' metabolism. According to Fig. 5, the percentage of this group in the SF remained below 8%, with the highest value (7.8%) recorded in the first day of fermentation (Copallín, Fig. 5a), while at the end was 5.0% (Tolopampa, Fig. 5c). On the other hand, the percentage in the SC was <10% (Fig. 6), being higher (9.5%) 24 h later (Tolopampa, Fig. 6c), after that decreased to 6.0% (Guadalupe, Fig. 6b). The sulfur-containing group, such as Cys (polar) and Met (non-polar), form weak hydrogen bonds with oxygen and nitrogen, and is barely found in the active site of enzymes (Dewangan et al., 2023). During SF, it was <2%. However, at the end of the process, it registered 3.3% in the cocoa from Copallín (Fig. 5a) and Tolopampa, which reported 3.5% (Fig. 5c). On the other hand, this group was not identified in the SC on day 1 (Fig. 6) but increased gradually to 3.0% on day 5 (Copallín, Fig. 6a). Since this group contains sulfides, it is recommendable to be kept at low levels or null, as corroborated by the study of Calvo et al. (2021).

The imino acid group is conformed by Pro, which contains a secondary amine instead of a primary one, can form peptide bonds, and is incorporated into proteins as an amino acid (Patriarca et al., 2021). During the spontaneous fermentative process, the percentage was below 1% until day 5; but, the maximum percentage (2% and 1.3%) was recorded on the sixth day (Copallín and Tolopampa, respectively). In the cacao from Guadalupe, its percentage was <0.5% (Fig. 5b), whereas, in the SC, a higher percentage (1.05%) was obtained on the third day of fermentation (Guadalupe, Fig. 6b) and 0.8% on the end of fermentation (Copallín, Fig. 6a). We can observe that in the SC, this group shows low participation. Another group present was acidic side chains formed by Glu and Asp, amino acids located on the protein surface that participate in transamination reactions and equilibration with oxaloacetate and 2-oxoglutarateketoacids, respectively (Emery, 2013). At the beginning of the SF, this group content was high (12.0%) in cocoa from Guadalupe (Fig. 5b), followed by Copallín (11.2%; Fig. 5a). During fermentation, they tended to decrease below 1% for the three places; likewise, it was observed in the SC that this group was high (41.3% and 13.3%) on Day 1 (Guadalupe, Fig. 6b and Tolopampa, Fig. 6c; respectively), but after the second day of fermentation it dropped below 1% as in the case of Tolopampa at the end of fermentation (Fig. 6c). 6c). In the case of cocoa from Copallín, its percentage was <5.0% (Fig. 6a). The low participation of this group is because, during fermentation, these acid amino acids concentration is reduced to generate the increase of hydrophobic amino acids (Balcázar-Zumaeta, Castro-Alayo, et al., 2023; Brunetto et al., 2020b).

Finally, the basic side chains group (Lys, Arg, and His) varied during cocoa fermentation; they are hydrophilic amino acids positively charged at neutral pH (Emery, 2013) and are one of the principal groups in cocoa (Zong et al., 2023). We observed that in the SF, the percentage was higher than 20% in all three locations. Higher rates were recorded between the second and fifth day of fermentation, reaching a maximum of 39.4% (day 2), followed by 28.3% (day 3) and 27.8% (day 4) for cocoa from Guadalupe, Copallín, and Tolopampa, respectively. For SC, this group increased as fermentation progressed, reaching high percentages from day 4, registering a maximum content of 28.0% at the end of fermentation (Fig. 6c). This trend has been reported previously in the study of Rottiers et al. (2019), which is due to the proteolytic activity of aspartic endoprotease and carboxypeptidase, which releases this group of amino acids from the proteins contained in the cocoa beans.

3.4. Multivariate analysis of amino acid content changes during fermentation (SF and SC) of cocoa beans

To group the fermentation days according to the amino acid content in the cocoa bean, a multivariate analysis was performed (Figs. 7 and 8) using k-means analysis. Accordingly, the ratio between the sum of squares between groups and the total sum of squares in the SC was 80.9% higher than SF, which was 77.9%. From this, Figs. 7a and 8a suggest that the grouping according to the fermentation days is considerably reliable and similar to previous studies reported by Balcázar-Zumaeta, Pajuelo-Muñoz, et al. (2023), Chagas et al. (2021), and Deus et al. (2021).

In the SF, the grouping in three clusters was determined (Fig. 7a and b); cluster 1 grouped day 0, day 1, and day 2 and included day 3 in the cacao from Guadalupe, where no amino acids were reported. This cluster was characterized by presenting lower mean values of the amino acids studied with a Glu content higher than cluster 2 (Fig. 6b). The low values of Asp and Glu in the first days of fermentation were because they are degraded to promote the progressive increase of hydrophobic amino

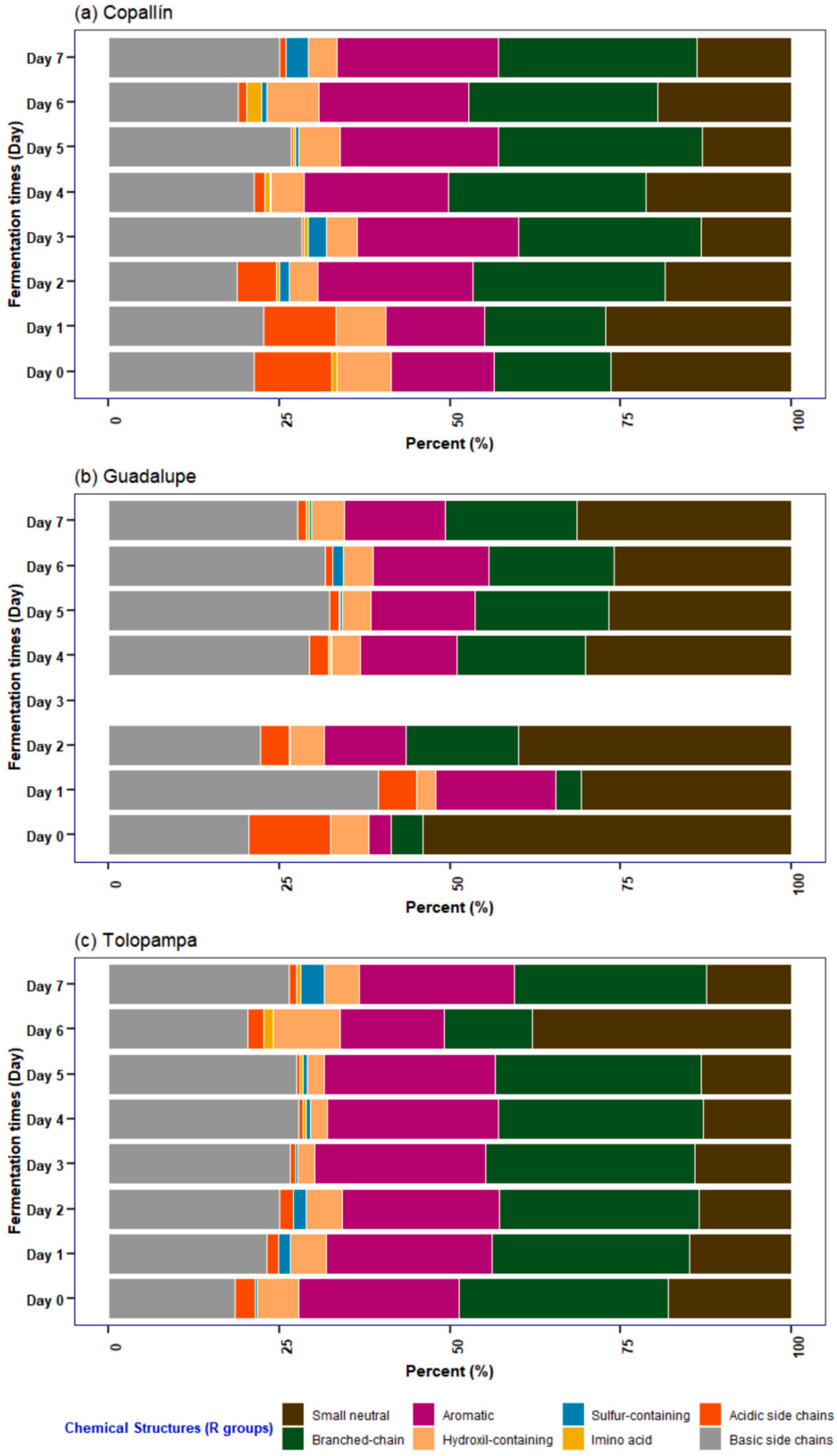


Fig. 5. Percentage contribution of amino acids during spontaneous fermentation (SF) in cocoa beans according to R groups: Small neutral (Gly and Ala), Branched-chain (Val, Iso and Leu), Aromatic (Phe and Tyr), Hydroxyl-containing (Thr and Ser), Sulfur-containing (Cys and Met), Imino acid (Pro), Acidic side chains (Glu and Asp), and Basic side chains (Lys, Arg and His).

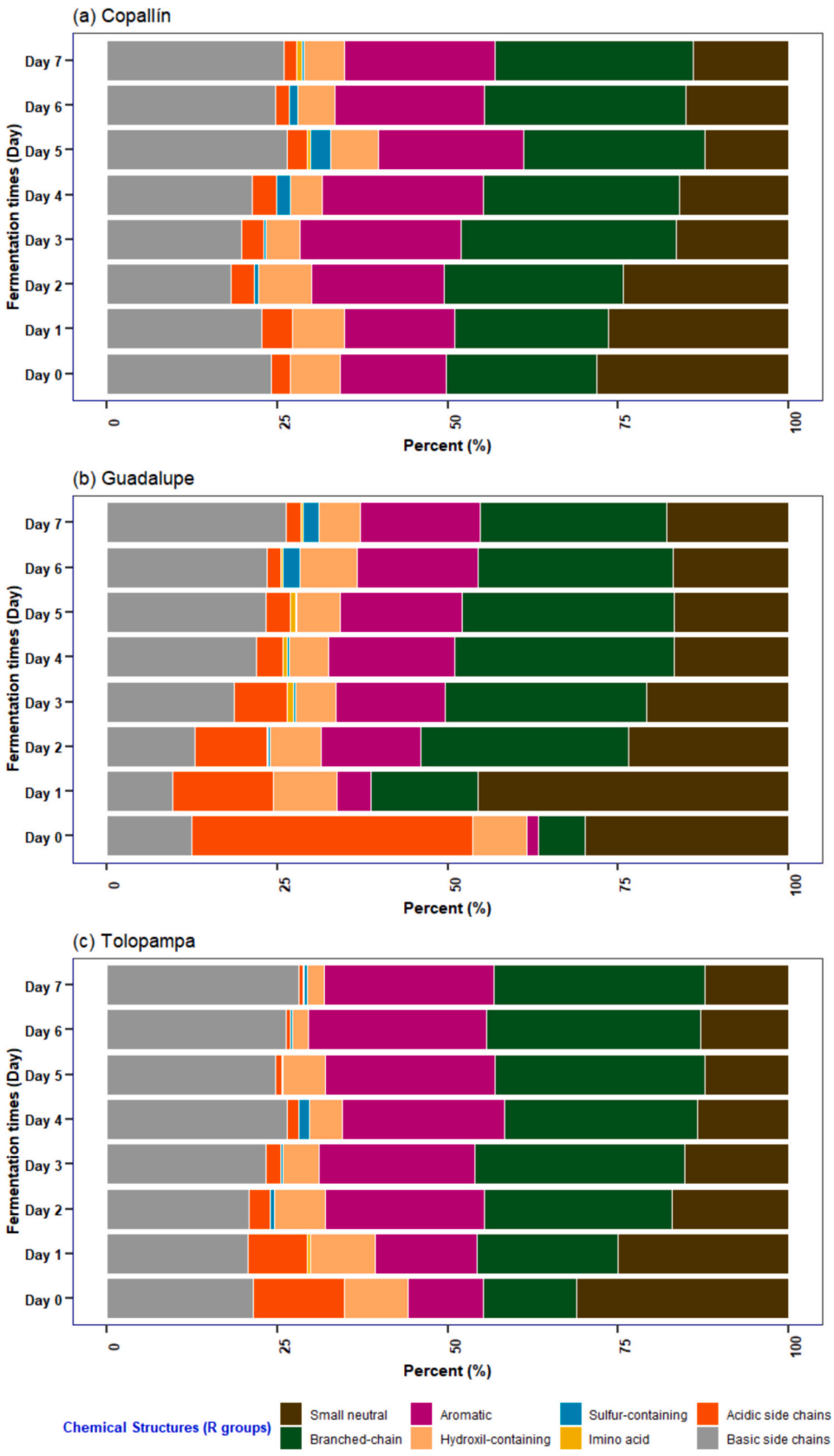


Fig. 6. Percentage contribution of amino acids during starter culture (SF) fermentation in cocoa beans according to R groups: Small neutral (Gly and Ala), Branched-chain (Val, Iso and Leu), Aromatic (Phe and Tyr), Hydroxyl-containing (Thr and Ser), Sulfur-containing (Cys and Met), Imino acid (Pro), Acidic side chains (Glu and Asp), and Basic side chains (Lys, Arg and His).

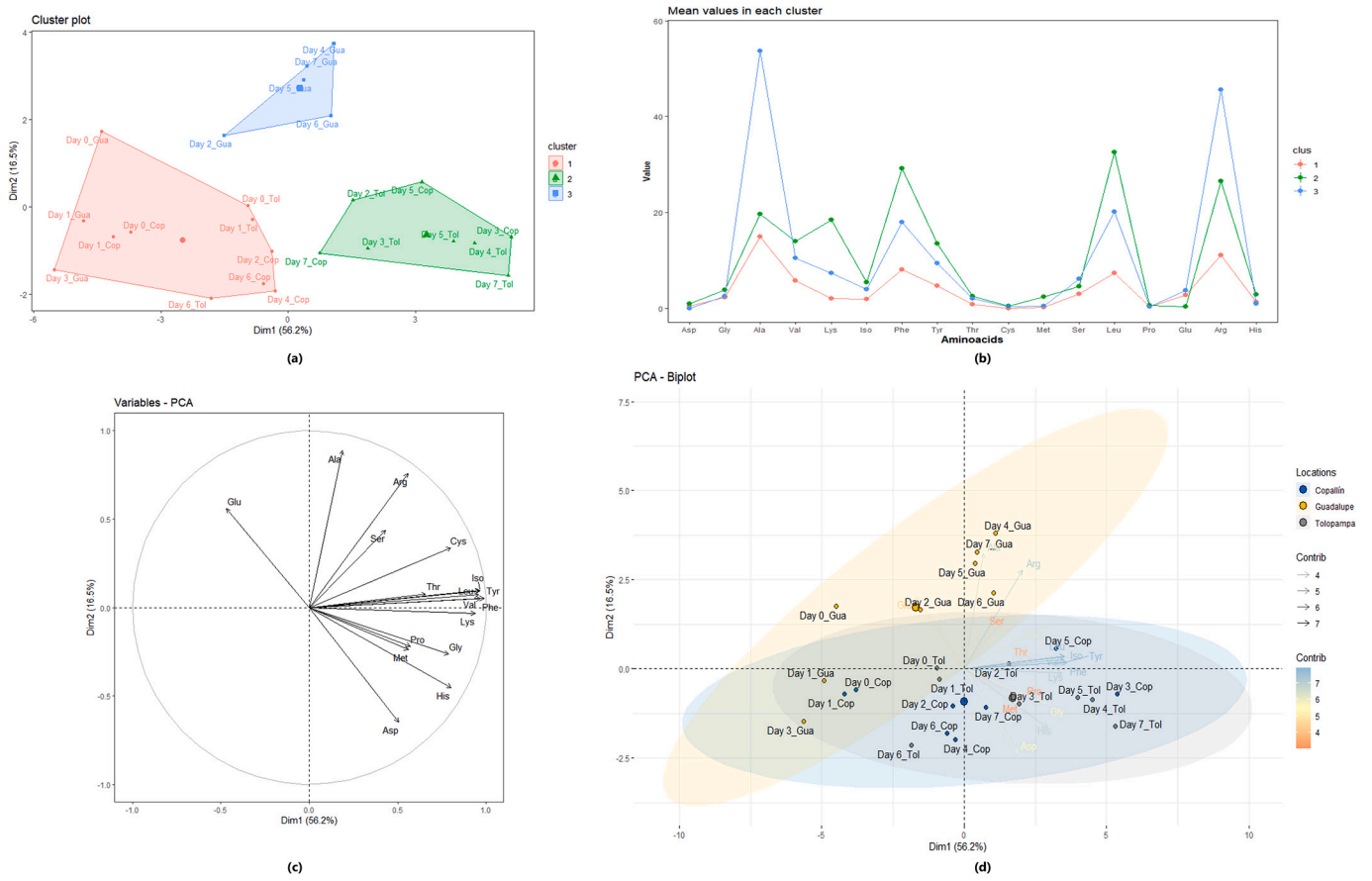


Fig. 7. K-means and principal component analysis (PCA) in cocoa bean SF: (a) K-means clustering shows three stages according to amino acid concentration; (b) Mean values of amino acid concentration in each group; (c) Biplot of principal component analysis of amino acid concentration; (d) Principal component analysis (PCA) of fermentation times as a function of the contribution of each observation. The data used are presented in Tables 1, 2, and 3.

acids (Brunetto et al., 2020b).

The second cluster of the SF was characterized by grouping mainly day 3, day 4, and day 5 of fermentation, which is highlighted by high mean values of Asp, Gly, Val, Lys, Iso, Phe, Tyr, Thr, Met, Leu and His, these increase as the fermentative process advances (Brunetto et al., 2020b), similarly Apriyanto, Sutardi, Supriyanto, and Harmayani (2017) and Deus et al. (2021) reported that in the case of Asp, Val, Lys, Iso, Phe, Thr and Leu remain high during this period (Tchouatcheu et al., 2019). Finally, cluster 3 grouped the last days of fermentation (day 6 and day 7), where high mean values of Ala, Ser, Glu, and Arg were recorded. The mean value of Cys was similar among the three clusters. The high content of Arg agrees with fermentation times of 6 days onwards, as reported by Deus et al. (2021). In addition, the principal component analysis (PCA) of the SF demonstrates that the first components (PC1 + PCA2) explained 72.7% of the data variance (Fig. 7c and d). In addition to this, considerably high eigenvalues (9.5 and 2.8, correspondingly) were determined. In the first component, PCA showed that amino acids such as **Tyr, Phe, Leu, Iso, Val, Lys**, His, Cys, and Gly were the variables with the highest contribution to the first component (Fig. 6c, the highlighted amino acids were those with the highest correlation, > 93%), similar to that obtained by Schlüter et al. (2022) where Gly, Tyr, and Phe contributed the highest and were correlated in the PCA correlation circle; while **Ala, Arg**, Asp, Glu, His and Ser were the amino acids with the highest contribution (Fig. 7c, the highlighted amino acids were those with the highest correlation, > 75%).

In the fermentation inoculated with *S. cerevisiae* (SC), the k-means analysis (Fig. 8a and b) can be distinguished as a group formed by the first days of fermentation (day 0, day 1 and day 2, cluster 3). This group is characterized by high mean values of Asp, Gly, and Cys, where the concentration of Asp decreased while the other amino acids increased (Brunetto et al., 2020b). The second cluster grouped day 2, day 3, and day 4 of the fermentation process, we can observe the highest mean values of Ser and Pro (cluster 2) and considerable mean values of Ala, Val, Lys, Iso, Phe, Tyr, Thr, Met, Leu, Arg and His. Finally, the third group was formed by the last days (day 5, day 6, and day 7, cluster 1) that presented high mean values of Ala, Val, Lys, Iso, Phe, Tyr, Thr, Met, Leu, Arg and His, these amino acids similarly were reported in the study of Deus et al. (2021) from the third day and maintained until day 6 (Phe, Lys, Leu, Ala and Thr) within the range determined in this study. Likewise, Arg and His (from day 6) are included; these results are similar to Apriyanto et al. (2017) in their fermentation study with inoculum. According to Deus et al. (2021), the period comprising clusters 2 and 3 is characterized by the presence of amino acids conferring sweetness (Ala, Gly, Pro, Ser, and Thr) and bitterness taste (Arg, His, Iso, Leu, Met, Phe, and Val), agreeing with Febrianto et al. (2022).

Likewise, PCA showed that the first components (PC1 + PC2) could explain 80.9% of the data variance (Fig. 7d); conversely, PC1 and PC2 had high eigenvalues of 9.8 and 2.5, respectively. According to the correlation circle (Fig. 8c), the first component contained **Phe, Leu, Arg, Lys, Iso, Val**, His, Ala, and Cys (highlighted amino acids were those with the highest correlation, >94%), similar to that reported by Schlüter et al. (2022); whereas, in the second component the amino acids with the highest contribution were Glu, Tyr, Ser, Pro, Gly and Asp (highlighted amino acid with the highest correlation, >89%); behavior similar to SF. In addition, multivariate analysis has shown that amino acids (primarily hydrophobic) prevailed at the end of the fermentation, similar to that reported by Deus et al. (2021), due to the ability of the inoculum to modulate the change in the concentration of aromatic precursors (Sande

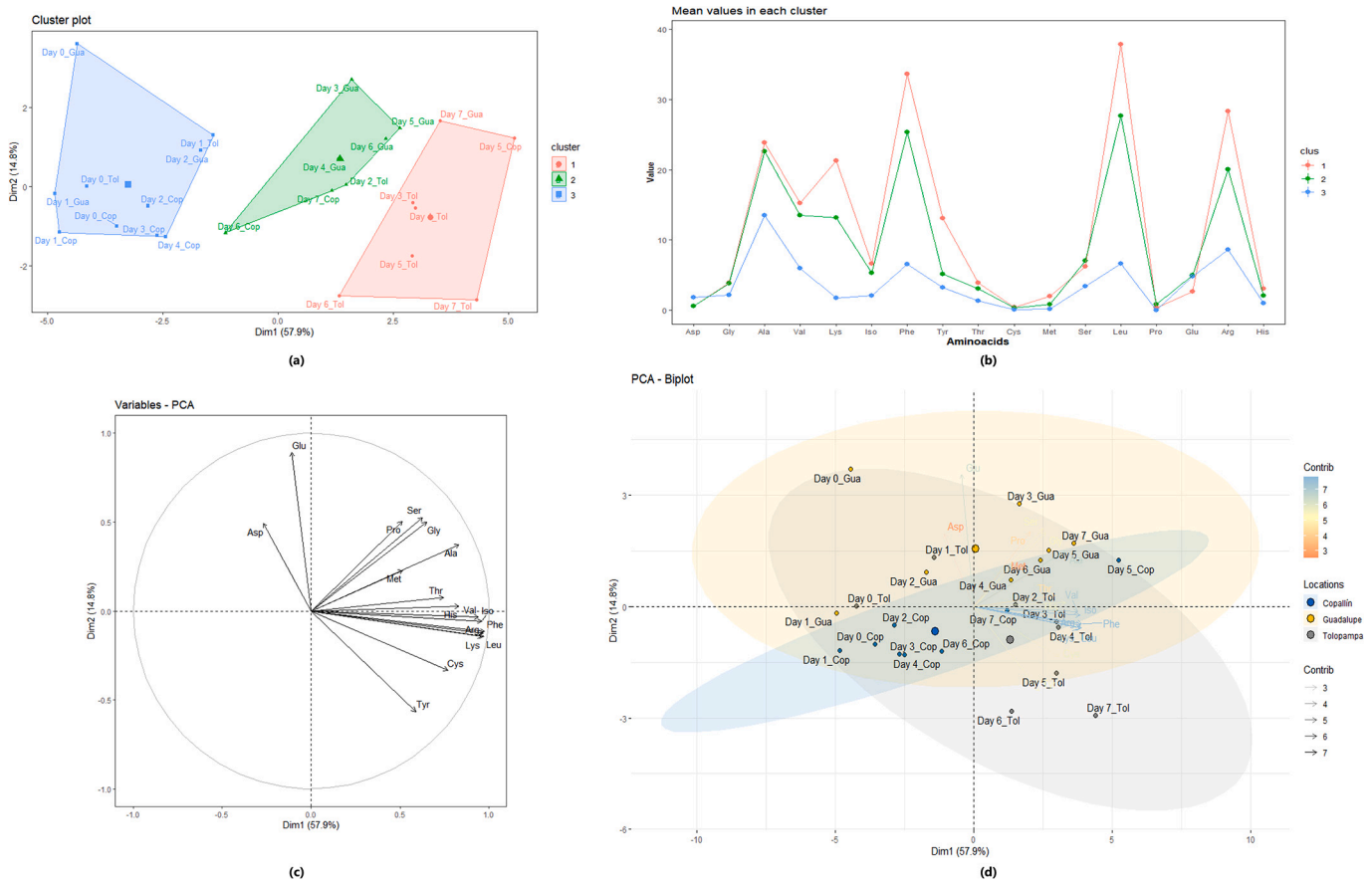


Fig. 8. K-means and principal component analysis (PCA) in cocoa bean SC: (a) K-means clustering shows three stages according to amino acid concentration; (b) Mean values of amino acid concentration in each group; (c) Biplot of principal component analysis of amino acid concentration; (d) Principal component analysis (PCA) of fermentation times as a function of the contribution of each observation. The data used are presented in Tables 1, 2 and 3.

et al., 2020), and influenced by the cacao origin, soil characteristics, climatic conditions, among others (Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023; Marseglia et al., 2014; Servent et al., 2018; Spizzirri et al., 2019), however further studies are warranted.

4. Conclusions

Our study allowed us to explore the variation of the amino acid profile; it was found that the essential amino acids are the predominant ones in the Criollo cocoa bean (where the origin of the cocoa may be an attribute to be studied later). The essential amino acids in the SF increased from the third day of fermentation. At the same time, in the SC, the starter culture (*S. cerevisiae*) accelerated the increase of these amino acids until the second day. Likewise, according to the structure of the R group, a low concentration of sulfur-containing amino acids was observed and could be associated with a better aroma in the fermented beans, as previously reported by Balcázar-Zumaeta, Castro-Alayo, et al. (2023), Balcázar-Zumaeta, Pajuelo-Muñoz, et al., 2023).

The study made it possible to determine three stages in the fermentation of cocoa beans. In the SF, the first stage (day 0 to day 2) was characterized by a low level of amino acids disappearing on day 3 in the Guadalupe cocoa bean. In the second stage (day 3 to day 5), high content of Asp, Gly, Val, Lys, Iso, Phe, Tyr, Thr, Met, Leu, and His was observed, while in the last stage, it was characterized by the presence of Ala, Ser, Glu and Arg.

Concerning SC, although the same number of stages were identified, they showed different features; in the first stage (day 0 to day 1), Asp was the predominant amino acid. The second stage (day 2 to day 4) was characterized by the presence of Ser and Pro, and in the third stage (day 5 to day 7) by the majority of amino acids (Ala, Val, Lys, Iso, Phe, Tyr, Thr, Met, Leu, Arg and His). The results suggest the need to reduce the duration of the fermentation process of Criollo cocoa to get a more significant aromatic profile.

This study demonstrates that the current fermentation time can be improved, primarily through a starter culture such as *S. cerevisiae*. Also, the farmers would optimize the time dedicated to obtaining higher-quality cocoa based on the amino acid profile. Our findings, along with what was previously reported by Balcázar-Zumaeta, Pajuelo-Muñoz, et al. (2023) and Castro-Alayo et al. (2022), are fundamental for the optimization of the Criollo cocoa fermentation. It is also recommended that further studies be carried out to learn about the various flavor-related compounds produced by the fermentation microbiome in cocoa.

Funding

The APC was financed by the Vicerrectorado de Investigación - Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas. This work was supported by the Proyecto CUI. No. 2343049: Creación de los Servicios de investigación en ingeniería de alimentos y post cosecha de la UNTRM. This work was subsidized by CONCYTEC through the PROCIENCIA Program in the framework of "Tesis de Pregrado y Postgrado en Ciencia, Tecnología e Innovación Tecnológica", according to Grant N° PE501085804–2023-PROCIENCIA.

CRediT authorship contribution statement

César R. Balcázar-Zumaeta: Writing – review & editing, Writing –

original draft, Project administration, Formal analysis, Conceptualization. **Editha Fernández-Romero:** Writing – original draft, Software, Investigation, Data curation. **Alessandra Santos Lopes:** Supervision. **Nelson Rosa Ferreira:** Validation, Data curation. **Gilson Celso Albuquerque Chagas-Júnior:** Writing – original draft, Visualization. **Ives Yoplac:** Software, Methodology, Formal analysis. **Heydi A. López-Trigoso:** Writing – original draft, Methodology. **Mery L. Tuesta-Occ:** Methodology. **Italo Maldonado-Ramirez:** Resources. **Jorge L. Maicelo-Quintana:** Funding acquisition. **Ilse S. Cayo-Colca:** Writing – review & editing. **Efrain M. Castro-Alayo:** Supervision, Software, Investigation, Data curation.

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.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank the Concytec (project: Estudio metabolómico del proceso de fermentación de cacao (Theobroma cacao L.) nativo orgánico fino de aroma, contract No. 56-2021-FONDECYT; and Project: Cristalización y polimorfismo de la manteca de cacao en granos de diferentes orígenes geográficos y su impacto en la calidad de chocolates finos, contract No. PE501077920-2022) of the Peruvian Government, and Cooperativa de Servicios Múltiples APROCAM for the facilities provided during the execution of this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101486.

References

Afoakwa, E. O., Paterson, A., Fowler, M., & Ryan, A. (2008). Flavor formation and character in cocoa and chocolate: A critical review. *Critical Reviews in Food Science and Nutrition, 48*(9), 840–857. https://doi.org/10.1080/10408390701719272

Apriyanto, M., Sutardi, S., Supriyanto, S., & Harmayani, E. (2017). Amino acid analysis of cocoa fermented by high performance liquid chromatography (HPLC). *Asian Journal of Dairy and Food Research, 36*(02). https://doi.org/10.18805/ajdfr.v36i02.7962

de Araujo, Q. R., de Abreu Loureiro, G. A. H., Póvoas, C. E. M., Steinmacher, D., de Almeida, S. S., Ahnert, D., & Baligar, V. C. (2021). Effect of different edaphic crop conditions on the free amino acid profile of PH-16 dry cacao beans. *Agronomy, 11*(8), Article 8. https://doi.org/10.3390/agronomy11081637

Balcázar-Zumaeta, C. R., Castro-Alayo, E. M., Cayo-Colca, I. S., Idrogo-Vásquez, G., & Muñoz-Astecker, L. D. (2023). Metabolomics during the spontaneous fermentation in cocoa (*Theobroma cacao* L.): An exploraty review. *Food Research International, 163*, Article 112190. https://doi.org/10.1016/j.foodres.2022.112190

Balcázar-Zumaeta, C. R., Pajuelo-Muñoz, A. J., Trigoso-Rojas, D. F., Iliquin-Chavez, A. F., Fernández-Romero, E., Yoplac, I., … Castro-Alayo, E. M. (2023). Reduction in the cocoa spontaneous and starter culture fermentation time based on the antioxidant profile characterization. *Foods, 12*(17), Article 17. https://doi.org/10.3390/foods12173291

de Brito, E. S., García, N. H. P., Gallão, M. I., Cortelazzo, A. L., Fevereiro, P. S., & Braga, M. R. (2001). Structural and chemical changes in cocoa (Theobroma cacao L) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture, 81*(2), 281–288. https://doi.org/10.1002/1097-0010(20010115)81:2<281::AID-JSFA808>3.0.CO;2-B

Brunetto, M.d. R., Gallignani, M., Orozco, W., Clavijo, S., Delgado, Y., Ayala, C., & Zambrano, A. (2020a). RP-HPLC-DAD determination of free amino acids in cocoa samples during fermentation and roasting. *Revista Colombiana de Química, 49*(1), 11–19. https://doi.org/10.15446/rev.colomb.quim.v1n49.77811

Brunetto, M.d. R., Gallignani, M., Orozco, W., Clavijo, S., Delgado, Y., Ayala, C., & Zambrano, A. (2020b). The effect of fermentation and roasting on free amino acids profile in Criollo cocoa (*Theobroma cacao* L.) grown in Venezuela. *Brazilian Journal of Food Technology, 23*, Article e2019150. https://doi.org/10.1590/1981-6723.15019

Cádiz-Gurrea, M.d.l. L., Fernández-Ochoa, Á., Leyva-Jiménez, F. J., Guerrero-Muñoz, N., Villegas-Aguilar, M.d. C., Pimentel-Moral, S., … Segura-Carretero, A. (2020). LC-MS and spectrophotometric approaches for evaluation of bioactive compounds from Peru cocoa by-products for commercial applications. *Molecules, 25*(14), 3177. https://doi.org/10.3390/molecules25143177

Calvo, A. M., Botina, B. L., García, M. C., Cardona, W. A., Montenegro, A. C., & Criollo, J. (2021). Dynamics of cocoa fermentation and its effect on quality. *Scientific Reports, 11*(1), Article 1. https://doi.org/10.1038/s41598-021-95703-2

Carenzi, G., Sacchi, S., Abbondi, M., & Pollegioni, L. (2020). Direct chromatographic methods for enantioresolution of amino acids: Recent developments. *Amino Acids, 52*(6), 849–862. https://doi.org/10.1007/s00726-020-02873-w

Castro-Alayo, E. M., Idrogo-Vásquez, G., Siche, R., & Cardenas-Toro, F. P. (2019). Formation of aromatic compounds precursors during fermentation of Criollo and Forastero cocoa. *Heliyon, 5*(1), Article e01157. https://doi.org/10.1016/j.heliyon.2019.e01157

Castro-Alayo, E. M., Torrejón-Valqui, L., Medina-Mendoza, M., Cayo-Colca, I. S., & Cárdenas-Toro, F. P. (2022). Kinetics crystallization and polymorphism of cocoa butter throughout the spontaneous fermentation process. *Foods, 11*(12), Article 12. https://doi.org/10.3390/foods11121769

Chagas, G. C. A., Ferreira, N. R., Gloria, M. B. A., Martins, L. H.d. S., & Lopes, A. S. (2021). Chemical implications and time reduction of on-farm cocoa fermentation by Saccharomyces cerevisiae and Pichia kudriavzevii. *Food Chemistry, 338*, Article 127834. https://doi.org/10.1016/j.foodchem.2020.127834

Chang, H., Gu, C., Wang, M., Chang, Z., Zhou, J., Yue, M., Chen, J., Qin, X., & Feng, Z. (2024). Integrating shotgun metagenomics and metabolomics to elucidate the dynamics of microbial communities and metabolites in fine flavor cocoa fermentation in Hainan. *Food Research International, 177*, Article 113849. https://doi.org/10.1016/j.foodres.2023.113849

Dala-Paula, B. M., Deus, V. L., Tavano, O. L., & Gloria, M. B. A. (2021). In vitro bioaccessibility of amino acids and bioactive amines in 70% cocoa dark chocolate: What you eat and what you get. *Food Chemistry, 343*, Article 128397. https://doi.org/10.1016/j.foodchem.2020.128397

De Vuyst, L., & Leroy, F. (2020). Functional role of yeasts, lactic acid bacteria and acetic acid bacteria in cocoa fermentation processes. *FEMS Microbiology Reviews, 44*(4), 432–453. https://doi.org/10.1093/femsre/fuaa014

Delgado-Ospina, J., Acquaticci, L., Molina-Hernandez, J. B., Rantsiou, K., Martuscelli, M., Kamgang-Nzekoue, A. F., … Chaves-López, C. (2021). Exploring the capability of yeasts isolated from colombian fermented cocoa beans to form and degrade biogenic amines in a lab-scale model system for cocoa fermentation. *Microorganisms, 9*(1), Article 1. https://doi.org/10.3390/microorganisms9010028

Deus, V. L., Bispo, E. S., Franca, A. S., & Gloria, M. B. A. (2020). Influence of cocoa clones on the quality and functional properties of chocolate – Nitrogenous compounds. *LWT, 134*, Article 110202. https://doi.org/10.1016/j.lwt.2020.110202

Deus, V. L., Bispo, E. S., Franca, A. S., & Gloria, M. B. A. (2021). Understanding amino acids and bioactive amines changes during on-farm cocoa fermentation. *Journal of Food Composition and Analysis, 97*, Article 103776. https://doi.org/10.1016/j.jfca.2020.103776

Dewangan, Y., Berdimurodov, E., & Verma, D. K. (2023). Chapter 1 - amino acids: Classification, synthesis methods, reactions, and determination. In C. Verma, & D. K. Verma (Eds.), *Handbook of biomolecules* (pp. 3–23). Elsevier. https://doi.org/10.1016/B978-0-323-91684-4.00015-3.

Domínguez-Pérez, L. A., Beltrán-Barrientos, L. M., González-Córdova, A. F., Hernández-Mendoza, A., & Vallejo-Cordoba, B. (2020). Artisanal cocoa bean fermentation: From cocoa bean proteins to bioactive peptides with potential health benefits. *Journal of Functional Foods, 73*, Article 104134. https://doi.org/10.1016/j.jff.2020.104134

Emery, P. W. (2013). Amino acids: Chemistry and classification. In *Encyclopedia of human nutrition* (pp. 64–71). Elsevier. https://doi.org/10.1016/B978-0-12-375083-9.00009-X.

Escobar, S., Santander, M., Zuluaga, M., Chacón, I., Rodríguez, J., & Vaillant, F. (2021). Fine cocoa beans production: Tracking aroma precursors through a comprehensive analysis of flavor attributes formation. *Food Chemistry, 365*, Article 130627. https://doi.org/10.1016/j.foodchem.2021.130627

Fang, Y., Li, R., Chu, Z., Zhu, K., Gu, F., & Zhang, Y. (2020). Chemical and flavor profile changes of cocoa beans (*Theobroma cacao* L.) during primary fermentation. *Food Science & Nutrition, 8*(8), 4121–4133. https://doi.org/10.1002/fsn3.1701

Febrianto, N. A., Wang, S., & Zhu, F. (2022). Chemical and biological properties of cocoa beans affected by processing: A review. *Critical Reviews in Food Science and Nutrition, 62*(30), 8403–8434. https://doi.org/10.1080/10408398.2021.1928597

Gutiérrez-Ríos, H. G., Suárez-Quiroz, M. L., Hernández-Estrada, Z. J., Castellanos-Onorio, O. P., Alonso-Villegas, R., Rayas-Duarte, P., … González-Rios, O. (2022). Yeasts as producers of flavor precursors during cocoa bean fermentation and their relevance as starter cultures: A review. *Fermentation, 8*(7), Article 7. https://doi.org/10.3390/fermentation8070331

Haruna, L., Abano, E. E., Teye, E., Adu, S., Agyei, K., Kuma, E., … Tukwarlba, I. (2024). *Effect of partial pulp removal and fermentation duration on drying behavior, nib acidification, fermentation quality, and flavor attributes of Ghanaian cocoa beans (SSRN Scholarly Paper 4698105)*. https://doi.org/10.2139/ssrn.4698105

Hasan, M. M., & Rima, R. (2021). Genetic engineering to improve essential and conditionally essential amino acids in maize: Transporter engineering as a reference. *Transgenic Research, 30*(2), 207–220. https://doi.org/10.1007/s11248-021-00235-0

Hernández-Hernández, C., Viera-Alcaide, I., Morales-Sillero, A. M., Fernández-Bolaños, J., & Rodríguez-Gutiérrez, G. (2018). Bioactive compounds in Mexican genotypes of cocoa cotyledon and husk. *Food Chemistry, 240*, 831–839. https://doi.org/10.1016/j.foodchem.2017.08.018

Hinneh, M., Semanhyia, E., Van de Walle, D., De Winne, A., Tzompa-Sosa, D. A., Scalone, G. L. L., … Dewettinck, K. (2018). Assessing the influence of pod storage on

sugar and free amino acid profiles and the implications on some Maillard reaction related flavor volatiles in Forastero cocoa beans. *Food Research International, 111*, 607–620. https://doi.org/10.1016/j.foodres.2018.05.064

John, W. A., Böttcher, N. L., Aßkamp, M., Bergounhou, A., Kumari, N., Ho, P.-W., … Ullrich, M. S. (2019). Forcing fermentation: Profiling proteins, peptides and polyphenols in lab-scale cocoa bean fermentation. *Food Chemistry, 278*, 786–794. https://doi.org/10.1016/j.foodchem.2018.11.108

Korcari, D., Fanton, A., Ricci, G., Rabitti, N. S., Laureati, M., Hogenboom, J., … Fortina, M. G. (2023). Fine cocoa fermentation with selected lactic acid bacteria: Fermentation performance and impact on chocolate composition and sensory properties. *Foods, 12*(2), Article 2. https://doi.org/10.3390/foods12020340

Lasta, E. L., da Silva Pereira Ronning, E., Dekker, R. F. H., & da Cunha, M. A. A. (2021). Encapsulation and dispersion of lactobacillus acidophilus in a chocolate coating as a strategy for maintaining cell viability in cereal bars. *Scientific Reports, 11*(1), Article 1. https://doi.org/10.1038/s41598-021-00077-0

Lopez, M. J., & Mohiuddin, S. S. (2022). *Biochemistry, essential amino acids*. Treasure Island (FL): StatPearls Publishing. http://europepmc.org/books/NBK557845.

Marseglia, A., Sforza, S., Faccini, A., Bencivenni, M., Palla, G., & Caligiani, A. (2014). Extraction, identification and semi-quantification of oligopeptides in cocoa beans. *Food Research International, 63*, 382–389. https://doi.org/10.1016/j.foodres.2014.03.046

Niemenak, N., Evina, V. J. E., Mouafi, A. S. D., Ngouambe, A. G. T., Bernhardt, C., Lieberei, R., & Bisping, B. (2020). Assessment of the profile of free amino acids and reducing sugars of cacao beans from local Cameroonian Trinitario (SNK varieties) and Forastero (TIKO varieties) using fermentation-like incubation. *Journal of Applied Botany and Food Quality, 93*, 321–329. https://doi.org/10.5073/JABFQ.2020.093.039

Olugosi, O. A., Agbede, J. O., Adebayo, I. A., Onibi, G. E., & Ayeni, O. A. (2019). Nutritional enhancement of cocoa pod husk meal through fermentation using Rhizopus stolonifer. *African Journal of Biotechnology, 18*(30), 901–908. https://doi.org/10.5897/AJB2019.16940

Ooi, T. S., Ting, A. S. Y., & Siow, L. F. (2020). Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study. *LWT, 122*, Article 108977. https://doi.org/10.1016/j.lwt.2019.108977

Patriarca, E. J., Cermola, F., D'Aniello, C., Fico, A., Guardiola, O., De Cesare, D., & Minchiotti, G. (2021). The multifaceted roles of proline in cell behavior. *Frontiers in Cell and Developmental Biology, 9*. https://doi.org/10.3389/fcell.2021.728576

Perez, M., Lopez-Yerena, A., & Vallverdú-Queralt, A. (2022). Traceability, authenticity and sustainability of cocoa and chocolate products: A challenge for the chocolate industry. *Critical Reviews in Food Science and Nutrition, 62*(2), 475–489. https://doi.org/10.1080/10408398.2020.1819769

Purbaningrum, K., Hidayat, C., Witasari, L. D., & Utami, T. (2023). Flavor precursors and volatile compounds improvement of unfermented cocoa beans by hydrolysis using bromelain. *Foods, 12*(4), Article 4. https://doi.org/10.3390/foods12040820

Quelal, O. M., Hurtado, D. P., Benavides, A. A., Alanes, P. V., & Alanes, N. V. (2023). Key aromatic volatile compounds from roasted cocoa beans, cocoa liquor, and chocolate. *Fermentation, 9*(2), Article 2. https://doi.org/10.3390/fermentation9020166

Rahardjo, Y. P., Rahardja, S., Samsudin, S., Dalapati, A., Amalia, A. F., Purwaningsih, H., & Syamsu, K. (2022). A literature review on cocoa fermentation techniques to shorten fermentation time. *IOP Conference Series: Earth and Environmental Science, 974*(1), 1–9. https://doi.org/10.1088/1755-1315/974/1/012111

Rawel, H., Huschek, G., Sagu, S., & Homann, T. (2019). Cocoa bean proteins—Characterization, changes and modifications due to ripening and post-Harvest processing. *Nutrients, 11*(2), 428. https://doi.org/10.3390/nu11020428

Rottiers, H., Tzompa Sosa, D. A., De Winne, A., Ruales, J., De Clippeleer, J., De Leersnyder, I., … Dewettinck, K. (2019). Dynamics of volatile compounds and flavor precursors during spontaneous fermentation of fine flavor Trinitario cocoa beans. *European Food Research and Technology, 245*(9), 1917–1937. https://doi.org/10.1007/s00217-019-03307-y

Salaria, S., Boatwright, J. L., Thavarajah, P., Kumar, S., & Thavarajah, D. (2022). Protein biofortification in lentils (*Lens culinaris* Medik.) toward human health. *Frontiers in Plant Science, 13*, 1–14.

Salter, E. A., Wierzbicki, A., & Honkanen, R. E. (2020). Quantum-based modeling of dephosphorylation in the catalytic site of serine/threonine protein phosphatase-5 (PPP5C). *Catalysts, 10*(6), Article 6. https://doi.org/10.3390/catal10060674

Sande, D., Passos, R., Ferreira, T., Silva, E. L., Reis, A. C., de Cerqueira, A. B., … Dias, J. (2020). Fermentation in fine cocoa type Scavina: Change in standard quality as the effect of use of starters yeast in fermentation. *Food Chemistry, 328*, Article 127110. https://doi.org/10.1016/j.foodchem.2020.127110

Sari, A. B. T., Fahrurrozi, U., Marwati, T., Djaafar, T. F., Hatmi, R. U., Purwaningsih, U., … Rahayu, E. S. (2023). Chemical composition and sensory profiles of fermented cocoa beans obtained from various regions of Indonesia. *International Journal of Food Science, 2023*, 1–13. https://doi.org/10.1155/2023/5639081

Schlüter, A., André, A., Hühn, T., Rohn, S., & Chetschik, I. (2022). Influence of aerobic and anaerobic moist incubation on selected nonvolatile constituents—comparison to traditionally fermented cocoa beans. *Journal of Agricultural and Food Chemistry, 70*(51), 16335–16346. https://doi.org/10.1021/acs.jafc.2c06493

Servent, A., Boulanger, R., Davrieux, F., Pinot, M.-N., Tardan, E., Forestier-Chiron, N., & Hue, C. (2018). Assessment of cocoa (*Theobroma cacao* L.) butter content and composition throughout fermentations. *Food Research International, 107*, 675–682. https://doi.org/10.1016/j.foodres.2018.02.070

Spizzirri, U. G., Ieri, F., Campo, M., Paolino, D., Restuccia, D., & Romani, A. (2019). Biogenic amines, phenolic, and aroma-related compounds of unroasted and roasted cocoa beans with different origin. *Foods, 8*(8), 306. https://doi.org/10.3390/foods8080306

Tamimi, K. A., Hidayat, C., Utami, T., & Witasari, L. D. (2023). Flavor precursor formation of non-fermented forastero cocoa beans after flavourzyme® and glucose treatment. *LWT, 184*, Article 114910. https://doi.org/10.1016/j.lwt.2023.114910

Tchouatcheu, G. A. N., Noah, A. M., Lieberei, R., & Niemenak, N. (2019). Effect of cacao bean quality grade on cacao quality evaluation by cut test and correlations with free amino acids and polyphenols profiles. *Journal of Food Science and Technology, 56*(5), 2621–2627. https://doi.org/10.1007/s13197-019-03749-y

Utrilla-Vázquez, M., Rodríguez-Campos, J., Avendaño-Arazate, C. H., Gschaedler, A., & Lugo-Cervantes, E. (2020). Analysis of volatile compounds of five varieties of Maya cocoa during fermentation and drying processes by Venn diagram and PCA. *Food Research International, 129*, Article 108834. https://doi.org/10.1016/j.foodres.2019.108834

Velásquez-Reyes, D., Rodríguez-Campos, J., Avendaño-Arrazate, C., Gschaedler, A., Alcázar-Valle, M., & Lugo-Cervantes, E. (2023). Forastero and Criollo cocoa beans, differences on the profile of volatile and non-volatile compounds in the process from fermentation to liquor. *Heliyon, 9*(4), Article e15129. https://doi.org/10.1016/j.heliyon.2023.e15129

Yoo, H.-S., Shanmugalingam, U., & Smith, P. D. (2022). Potential roles of branched-chain amino acids in neurodegeneration. *Nutrition, 103–104*, Article 111762. https://doi.org/10.1016/j.nut.2022.111762

Zong, P., Hu, W., Huang, Y., An, H., Zhang, Q., Chai, Z., … Cai, W. (2023). Effects of adding cocoa fermentation medium on cigar leaves in agricultural fermentation stage. *Frontiers in Bioengineering and Biotechnology, 11*, Article 1251413. https://doi.org/10.3389/fbioe.2023.1251413