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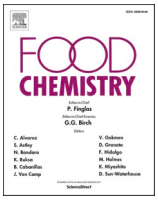

Bioaccessibility of bioactive amines in dark chocolates made with different proportions of under-fermented and fermented cocoa beans

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ABSTRACT

The influence of under-fermented (UF) cocoa (0 to 65 %) on bioactive amines in chocolate and their *in vitro* bioaccessibility was investigated. The same amines were found in all treatments; however, treatments were divided into two groups regarding total amines [0 & 20 % UF (34 mg/kg) and 35 to 65 % UF (17 mg/kg)] and phenolic levels [lower and higher, respectively]. Serotonin, tyramine, putrescine, cadaverine, agmatine and phenylethylamine were higher in chocolate with ≤ 20 % UF cocoa. Histamine and spermidine were not affected. Digestibility studies indicated that low levels of amines were present in the oral phase. Gastric digestion was effective in releasing tyramine, spermidine and phenylethylamine from conjugates. Serotonin and agmatine were not detected after *in vitro* digestion of chocolate with ≥ 35 % UF cocoa. Histamine was released during *in vitro* intestinal digestion. By adding different proportions of UF cocoa during chocolate production, the levels and bioaccessibility of amines can be modulated.

1. Introduction

Chocolate, as well as other products derived from the processing of cocoa (*Theobroma cacao* L.), are widely consumed and enjoyed worldwide due to their sensory characteristics (Muñoz; Cortina; Vaillant, & Parra, 2020). In addition, cocoa beans can contribute to human nutrition and health by providing energy, nutrients (lipids, polysaccharides, amino acids, fibers and minerals) (Oracz, Nebesny, Żyżelewicz, Budryn, & Luzak, 2019) and health promoting polyphenolic and nitrogenous compounds (Yılmaz & Gökmen, 2020). Several studies are available regarding the benefits associated with polyphenolic compounds, including the positive effects on cardiovascular health, antioxidant protection, and cholesterol (Ebaditabar, Djafarian, Saedifard, & Shab-Bidar, 2020; Darand, Oghaz, Hadi, Atefi, & Amani, 2021). There is also information on the anti-inflammatory and hypoglycemic properties of methylxanthine from cocoa products (Sarriá, Gomez-Juaristi, López, Cordero, Bravo, & Briz, 2020). The bioaccessibility and bioavailability of these compounds have also been a matter of studies (Gültekin-Özgüven, Berktas, & Özçelik, 2016; Oracz et al., 2019; Sarriá et al., 2020).

Bioactive amines like spermidine, phenylethylamine, serotonin, tryptamine, histamine and tyramine are also present in cocoa and chocolate (Dala-Paula, Starling, & Gloria, 2021b; Deus, Bispo, Franca, & Gloria, 2020, 2021). These compounds can contribute to human health by providing cardiovascular protection, antioxidant and anti-aging activities (Eisenberg et al., 2016; Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, Toro-Funes, Veciana-Nogués, & Vidal-Carou, 2019; Liang et al., 2021) and mood modulation (Yılmaz and Gökmen, 2020). It is important to observe that some of the health promoting properties attributed to amines are the same as those reported for the polyphenolic compounds; thereby, it is possible that amines are responsible or contribute with important health promoting roles attributed to chocolate. Recent studies have described the occurrence, formation and relevance of bioactive amines in cocoa and chocolate (Dala-Paula, Starling, & Gloria, 2021b; Delgado-Ospina et al., 2020, 2021, Deus, Bispo, Franca, & Gloria, 2020, 2021; Restuccia, Spizzirri, Luca, Parisi, & Picci, 2016; Restuccia, Spizzirri, Puoci, & Picci, 2015; Spizzirri et al., 2019). In addition, it has been reported that, during the *in vitro* digestion of chocolate, there can be additional release of amines from conjugated forms, with other cocoa components, such as nucleic acids,

Abbreviations: UF, under-fermented; Epi, Epicatechin equivalent; AGM, Agmatine; CAD, Cadaverine; HIM, Histamine; PHM, Phenylethylamine; PUT, Putrescine; SPD, Spermidine; SRT, Serotonin; TYM, Tyramine.

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phospholipids, phenolic acid, lignin, protein and polysaccharides due to enzyme activity, changes in pH and ionic strength (Dala-Paula et al., 2021a).

The levels of bioactive compounds in cocoa and chocolate can be affected by several factors, including variety, geographical origin, edaphoclimatic conditions, agricultural practices (conventional or organic), pre-processing (harvest of the fruits, removal of pulp and seeds, fermentation and drying) and processing (roasting, refining, conching, tempering, and crystallizing) steps (Restuccia et al., 2015; Spizzirri et al., 2019; De Andrade et al., 2021; Delgado-Ospina et al., 2021; Deus et al., 2021). Fermentation is one of the most critical steps determining the quality of cocoa (Ouattara, Ouattara, Droux, Reverchon, Nasser, & Niamke, 2017; Muñoz et al., 2020; De Andrade et al., 2021). During fermentation, there is a succession of microbial and enzymatic reactions resulting in free amino acids, peptides, and reducing sugars which, upon Maillard reaction during roasting, are essential for the generation of the desirable aroma, flavor and color (Muñoz et al., 2020; Chagas Junior, Ferreira, & Lopes, 2021a; De Andrade et al., 2021). There is production of lactic and acetic acids increasing acidity and, concomitantly, decrease in pH (Melo, Pires, Engelmann, Monteiro, Maciel, & Bispo, 2021). In addition, there is reduction in phenolic compounds resulting in lower bitterness and astringency – in the first 48 h fermentation, there is 30 % loss of polyphenolic compounds reaching 90 % reduction at the end of the process (144 h) (De Andrade et al., 2021). Furthermore, there is a change in the profile and levels of some bioactive amines, the levels of phenylethylamine increase whereas the levels of spermidine, serotonin, tryptamine, and tyramine decrease (Deus et al., 2021).

Despite its relevance to cocoa quality, fermentation is still an artisanal process undertaken naturally in cocoa farms using wooden boxes covered with banana leaves and burlap, in a lengthy process (ca. 6 days), which can be a burden to cocoa production. Furthermore, the changes brought about during fermentation, although advantageous regarding some aspects (color, flavor and some bioactive compounds) can be detrimental to others, such as decreased levels of phenolic compounds and lower antioxidant activity (Melo et al., 2021). In this context, it would be interesting if one could optimize the final composition and characteristics of the chocolate by using different proportions of partially (under-fermented) and fully fermented cocoa. According to De Andrade et al. (2021), by using increased proportions (0 to 80 %) of under-fermented (48 h fermentation) with fermented (144 h) cocoa for chocolate, it was possible to increase phenolic compounds and bitterness and to improve the antioxidant activity of the chocolate. However, sensory evaluation studies indicated that the use of under-fermented cocoa was limited to 65 % due to consumers acceptance (De Andrade et al., 2021). No information is available regarding the influence of these changes on the levels and bioaccessibility of bioactive amines in chocolate.

In this context, the objective of this study was to investigate the influence of the use of different proportions of under-fermented in fermented cocoa on the profile and levels of bioactive amines in the chocolate. In addition, the influence of the composition of the chocolates on the *in vitro* oral, gastric and intestinal bioaccessibility of amines was also investigated.

2. Materials and methods

2.1. Reagents and samples

Analytical grade reagents were used, except HPLC solvents which were LC grade. Ultra-pure water was from Milli-Q Plus (Millipore Corp., Milford, MA, USA). Organic and aqueous solvents for HPLC analysis were filtered using HAWP and HVWP membranes, respectively (0.45 μm, Millipore Corp., Milford, MA, USA). Alpha-amylase (Sigma A-3176); bile salts (Sigma B-8756); pancreatin and pepsin from porcine gastric mucosa (Sigma P-3292 and P-7012, respectively); and bioactive amine standards (spermidine trihydrochloride, agmatine sulfate, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, serotonin hydrochloride, tryptamine hydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

The cocoa used as raw material was obtained from a farm located in the South of Bahia, Brazil (14°41′96 "S and 39°12′109″W). The fermentation, drying and roasting steps were also undertaken at the same farm under traditional commercial conditions.

2.2. Cocoa fermentation

Spontaneous commercial cocoa fermentations were performed in the same farm (South of Bahia, Brazil), following standard protocols. Immediately after harvest, the fruits of multivarietal cocoa were cut open (stainless steel knives), and the beans and pulp (~40 kg), without the peel and placenta, were placed in 50 × 40 × 70 cm wooden boxes, with 1 cm diameter holes at each 5 cm. The boxes were covered with banana leaves and burlap. Fermentation was carried out at two different periods of time: partial fermentation (48 h) – under-fermented and full fermentation (144 h) – fully fermented. The average local daily temperature during fermentation varied from 22.3 to 29.6 °C. From 48 h fermentation on, the seeds were mixed at 24 h intervals to allow aeration and to homogenize fermentation of the mass. The fermented cocoa from both treatments were sun dried separately on stainless steel trays for 5 to 7 days until moisture content reached ~ 8 g/100 g (Melo et al., 2021). The fermented and dried cocoa were cleaned and roasted in forced air circulation ovens using perforated trays (DeLeo drying oven, model A35EAF8, Porto Alegre, RS, Brazil) at 120 °C for 25 min. Then, the roasted beans were pressed through a manual press with wooden roll to remove the peel and germ and obtain the cocoa nibs for chocolate making. The samples were frozen and stored under vacuum at −18 °C. The experiment was performed in triplicate.

2.3. Chocolate processing

Five different chocolates were made using two independent variables, corresponding to the percentages of under-fermented (48 h fermentation) and fully fermented (144 h fermentation) cocoa. The proportions of under-fermented cocoa used to make the dark chocolate ranged from 0 % to 65 % (Table 1). These proportions were chosen based on consumer acceptance by sensory analysis (De Andrade et al., 2021).

The chocolates were produced at the Chocolate Technology and Analysis Laboratories from Faculdade de Farmácia, UFBA. Dark chocolates (66.3 % cocoa) were formulated using the following ingredients: cocoa mass (61.0 g/100 g), commercial refined sugar (33.35 g/100 g), cocoa butter (5.3 g/100 g) and soy lecithin (0.35 g/100 g). The cocoa was mixed and ground in a processor (BL480BR30 NutriNinja, HAI XIN Technology, Shezhen, China) to produce the cocoa mass. Then, the masses were transferred to a stone mill (Melanger Spectra 11, Hillsborough Township, NJ, USA), along with the other ingredients (sugar, lecithin, cocoa butter) for conching and refining for 24 h. Afterwards, the chocolate was tempered on marble surface (~32 °C) for the formation of stable fat crystals, and it was immediately molded, using polyethylene molds, into 7 g bars. The chocolates were refrigerated,

Table 1
Chocolates made with different proportions of under-fermented (48 h) and fully fermented (144 h) cocoa beans.

Chocolates	Cocoa nibs (%) from fermentations	
	Under-fermented (48 h)	Fermented (144 h)
C1	0	100
C2	20	80
C3	35	65
C4	50	50
C5	65	35

wrapped, and stored at −18 °C until analysis. The samples were analyzed for free bioactive amines before and after *in vitro* bioaccessibility studies.

2.4. Determination of free bioactive amines by HPLC

Free bioactive amines were extracted from 5 g ground chocolate samples by three successive extractions with 7 mL 5 % trichloroacetic acid (TCA) followed by stirring for 5 min, and centrifugation at 11,180 × *g* at 4 °C/10 min. The supernatants were collected into a 25 mL volumetric flask (Deus et al., 2020). The fractions resulting from the *in vitro* digestions were analyzed directly. The separation and quantification of nine free bioactive amines (tyramine, putrescine, cadaverine, histamine, serotonin, agmatine, spermidine, tryptamine and phenylethylamine) were performed by HPLC using a LC-10 AD system connected to a RF-551 spectrofluorimetric detector operating at 340 and 445 nm of excitation and emission, respectively, and a CBM-10 AD controller (Shimadzu Corp., Kyoto, Japan). A μBondapack C18 column (300 × 3.9 mm i.d., 10 μm) was used. The mobile phases used were: (i) 0.2 M sodium acetate buffer and 10 mM sodium salt of 1-octane sulfonic acid, pH adjusted to 4.9 with glacial acetic acid; and (ii) acetonitrile (flow rate of 0.8 mL/min) and the gradient used for phase (ii) was 13 min at 11 %, 19 min at 30 %, 24 min at 11 %, and 45 min at 11 %. Detection was performed after post-column derivatization with *o*-phthalaldehyde (OPA). The derivatizing solution consisted of 1.5 mL Brij-35, 1.5 mL mercaptoethanol and 0.32 g OPA in 500 mL solution of 25 g boric acid and 22 g KOH (pH 10.5) and was delivered at 0.4 mL/min. The column was kept at 23 ± 1 °C. The amines were identified based on retention times and the identity was confirmed by addition of the suspected amine to the sample. Quantification was possible by area interpolation in an external standard curve constructed with the nine investigated amines at nine different concentrations, three repetitions each ($r^2 \geq 0.998$) (Chagas Junior, Ferreira, Gloria, Martins, & Lopes, 2021b).

2.5. In vitro bioaccessibility of amines in chocolates

Gastrointestinal digestion was performed as described by Dala-Paula et al. (2021a). The protocol simulated three stages of the digestive process: oral, oral + gastric, and oral + gastric + intestinal, as described in Fig. 1. Briefly, in the oral phase, the chocolate was grated to decrease particles' size simulating chewing. Chocolate (2.0 g) was mixed in a centrifuge tube with 8 mL of saliva solution [(phosphate buffer solution (0.04 % NaCl and 0.004 % $CaCl_2$, pH 6.9) prepared with 0.07 mg α-amylase (30 U/mg)]. The mixture was shaken (45 rpm) in an incubator at 37 °C for 5 min. For the gastric phase (oral + gastric digestion), the solution from the oral phase was adjusted to pH 2.0 (100 μL 6 M HCl) and mixed with 0.17 mg pepsin (2188 U/mg) in 1.0 mL 0.01 N HCl. The mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h. For the intestinal phase (oral + gastric + intestinal digestions), the solution from the gastric digestion was adjusted to pH 6.5 with 900 μL saturated $NaHCO_3$ solution and mixed with 5 mL duodenal juice (2.5 mL bile salt solution, 50 mg/mL) and 2.5 mL pancreatin solution (8 mg/mL). This mixture was shaken (45 rpm) in an incubator at 37 °C for 2 h. Prior to the removal of samples for the oral and the oral + gastric phases, the enzymatic reactions were interrupted by adjusting the pH to 2.0 and 6.5, respectively. The interruption of the enzymatic reaction at the end of the oral + gastric + intestinal phase was performed by immersion of the test tube in an ice bath. The resulting mixtures from each phase were centrifuged at 7,000 × g at 4 °C (MOD 280R, FANEN Excelsa 4, São Paulo, SP, Brazil) for 10 min. Then the supernatants were collected, filtered, and stored in Eppendorf type tubes at −80 °C until the analyses for bioactive amines. The digestion protocol was performed in duplicate for each phase and the samples were filtered through 0.45 μm membrane, prior to HPLC analysis.

2.6. Statistical analysis

The results were submitted to analyses of variance and the means were compared by the Tukey test at 5 % significance (Minitab® 16.2.3). Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for characterization of the *in vitro* digestion phases and the respective original chocolates (Minitab® 16.2.3).

3. Results and discussion

3.1. Free bioactive amines in chocolate made with fully fermented cocoa

Eight of the nine amines investigated were detected in the 66.3 % cocoa chocolate produced with fully fermented cocoa, as indicated in Fig. 2. Tryptamine was not detected in any sample. All these amines have been reported previously in 70 % cocoa chocolates made with different cocoa cultivars grown at the same farm in Bahia, Brazil (Deus et al., 2020). But only five of them, cadaverine, phenylethylamine, putrescine, spermidine and tyramine were detected in commercial Brazilian 70 % cocoa chocolate (Dala-Paula et al., 2021a).

The total level of amines found in the chocolate made with fully fermented cocoa – control treatment (C1) – was 33.43 mg/kg, which is lower than values reported for Brazilian commercial 70 % cocoa chocolate with 86.7 mg/kg (Dala-Paula et al., 2021a) and for commercial 60 and 70 % dark chocolates – 46.3 to 62.0 mg/kg (Restuccia et al., 2016). However, the total levels were similar or higher compared to Brazilian monoclonal chocolates from cocoa clones cultivated at the same farm –

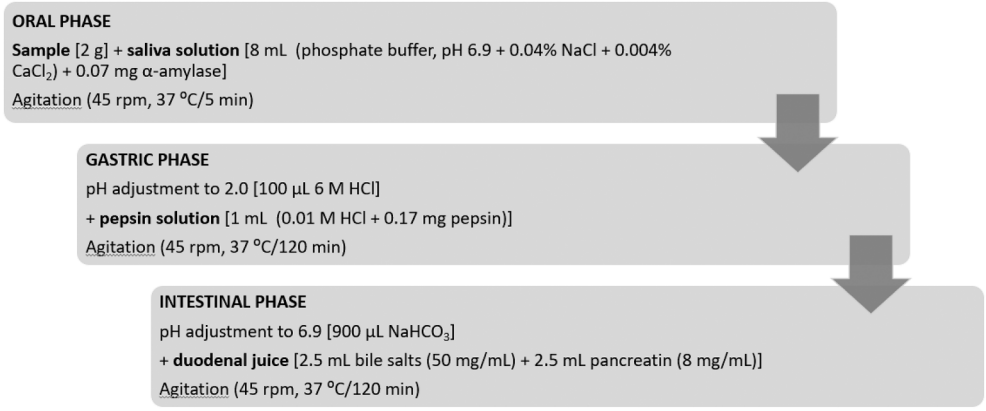

Fig. 1. Schematic representation of *in vitro* gastrointestinal digestion phases: oral, gastric and intestinal of chocolates made with different proportions of under-fermented (48 h) and fermented (144 h) cocoa beans. Source: Dala-Paula et al. (2021a).

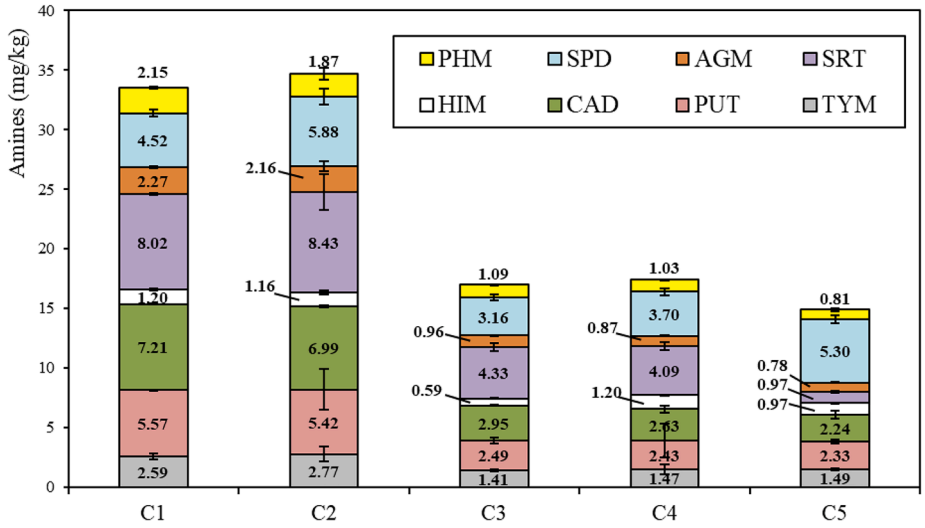

Fig. 2. Levels of free bioactive amines in chocolates made with different proportions of under-fermented (48 h) and fermented (144 h) cocoa beans. TYM-tyramine; PUT-putrescine; CAD-cadaverine; HIM-histamine; SRT-serotonin; AGM-agmatine; SPD-spermidine; PHM-phenylethylamine. Proportion of nibs fermented for 48 h and 144 h: C1-0 % and 100 %; C2-20 % and 80 %; C3-35 % and 65 %; C4-50 % and 50 %; C5-65 % and 35 %, respectively.

9.10 to 32.47 mg/kg (Deus et al., 2020); and higher than organic-fair trade 70 % dark chocolates made with Dominican Republic and Ecuador cocoas – 0.4 to 14.7 mg/kg (Restuccia et al., 2016).

The chocolate from fully fermented cocoa was characterized by the predominance of serotonin (23.9 %) and cadaverine (21.5 %), followed by putrescine (16.6 %), spermidine (13.5 %), tyramine (7.7 %), agmatine (6.8 %), phenylethylamine (6.4 %) and histamine (3.6 %). This profile differs from that of Brazilian commercial 70 % cocoa chocolate, in which the polyamines (spermidine and spermine) were prevalent (22 %), followed by 2-phenylethylamine, cadaverine and putrescine (~15 %) (Dala-Paula et al., 2021a), and also from chocolates made with different Brazilian cocoa clones (Deus et al., 2020). Therefore, these results reinforce the influence of cocoa cultivar and processing on the levels and profile of amines in the chocolate (Oracz & Nebesny, 2014; Restuccia et al., 2016; Deus et al., 2020).

3.2. Bioactive amines in chocolates made with different proportions of under-fermented cocoa

When comparing the profile and levels of amines in the chocolates made with different proportions of under-fermented and fermented cocoa, the same types of amines were found in all treatments (C1 – C5). However, the total levels of amine varied significantly ($p \leq 0.05$) among them (from 14.9 to 34.7 mg/kg). There were higher levels (greater than 30 mg/kg) for C1 and C2, which had 100 % and 80 % fully fermented cocoa (0 and 20 % under-fermented cocoa), respectively. Chocolate from the other treatments (C3 to C5 with 35 to 65 % under-fermented cocoa) had total levels of amines lower than 18 mg/kg. Based on these results, by using up to 20 % under-fermented cocoa, there was no effect on total amines levels. In a similar way, the use of 35 % up to 65 % under-fermented cocoa in chocolate resulted in similar total levels of amines among treatments. The impact of fermentation on the formation of amines in cocoa is expected as the conditions prevalent during fermentation, e.g., proteolysis with liberation of amino acids and the presence of microorganisms with amino acid decarboxylase activity, are conducive to amines production (Do Carmo Brito, Chisté, Da Silva Pena, Gloria, & Lopes, 2017; Delgado-Ospina et al., 2021; Chagas Junior et al., 2021b; Deus et al., 2021). In addition, the reduction in pH and increase in total titratable acidity brough about during fermentation stimulate the production and release of free amino acid decarboxylases from some microorganisms, enhancing amines formation (Chagas Junior et al., 2021b; Delgado-Ospina et al., 2021).

Differences were also observed in the prevalence of amines among treatments. In C1, C2, C3 and C4 there was predominance of serotonin (~24 %,), whereas in C5 the prevalent amine (35.6 %) was spermidine. Cadaverine was the second prevalent amine in C1 and C2 (~20 %), spermidine in C3 and C4 (18.6 and 21.2 %, respectively) and putrescine in C5 (15.6 %). These results indicate that by using different percentages of under-fermented cocoa, one can affect the levels and proportion of bioactive amines in chocolate. Considering that the presence of serotonin and spermidine are desirable in chocolate, all formulations except C5 can result in chocolates rich in both amines.

Serotonin contents ranged from 0.97 to 8.43 mg/kg, with the highest values in C2 and C1. Significant levels of serotonin in cocoa and chocolate are relevant as it is associated with the sensations of pleasure and well-being (Yılmaz & Gökmen, 2020). The changes observed for serotonin are different from those reported by Deus et al. (2021), who observed degradation of this amine during fermentation (144 h). However, it is well known that the profile of amines during cocoa fermentation can be affected by various factors, such as, cocoa variety, microbial flora, temperature, aeration (oxygen concentration) and pH. Phenylethylamine, another neuroactive amine, promotes the release of catecholamines, contributing to human health with cognitive, executive functions, and attention-related processes (Yılmaz & Gökmen, 2020). The highest phenylethylamine levels were found in C1 and C2, the chocolates with a higher proportion of fully fermented cocoa.

The highest putrescine and cadaverine contents were also found in the chocolates produced with a higher proportion of fully fermented cocoa. According to Deus et al. (2021) initial cocoa putrescine levels (~1.62 mg/kg) remained constant up to 144 h fermentation but did not detect cadaverine in any samples. Putrescine can be formed from the decarboxylation of ornithine or deimination of agmatine, whereas cadaverine is formed from the decarboxylation of lysine, which can be brought about by Enterococcus, Lactobacillus, Leuconostoc (Ouattara et al., 2017; Barbieri Montanari, Gardini, & Tabanelli, 2019), *Candida parapsilosis*, *Hydropichia burtonii*, *Pichia kudriavzevii*, *Pichia manshuric*a, *Saccharomyces cerevisiae*, *Trichosporon asahii* var. *asahii* and *Wickerharmomyces anomalus* (Delgado-Ospina et al., 2021). These microorganisms have been identified during cocoa fermentation or processing. High levels of putrescine and cadaverine are not desirable in food since these amines, at high concentrations, can impart a putrid flavor (Gloria, 2005; Dala-Paula, Starling, & Gloria, 2021b).

On the other hand, the addition of under-fermented cocoa did not affect the levels of spermidine in chocolate, considering that the highest

values were observed in C5 and C2. According to Do Carmo Brito et al. (2017) and Deus et al. (2021), the changes on spermidine levels during fermentation did not follow a standard pattern. However, the presence of this polyamine is relevant as it is involved in growth, renewal, and cellular metabolism (Gloria, 2005; Muñoz-Esparza et al., 2019). In addition, it has antioxidant activity, which could contribute to the stability of the chocolate, and also to human health (Muñoz-Esparza et al., 2019). Its intake has been associated with the prevention of cardiovascular disease, reduction in mortality rate and anti-aging properties (Eisenberg et al., 2016; Muñoz-Esparza et al., 2019; Liang et al., 2021).

Agmatine, which is seldom investigated in chocolate, was detected in every sample, with the highest levels in C1 and C2. Therefore, higher percentage of fully fermented beans contributed to its accumulation in the chocolates. Agmatine is synthesized from the decarboxylation of the amino acid L-arginine and has several physiological and pharmacological functions, such as: antidepressant effect, anti-inflammatory properties, positive relationship with memory health, improvement of anxiety symptoms, antioxidant action and neuroprotective properties (Aglawe, Kale, Rahangdale, Kotagale, Umekar, & Taksande, 2021).

However, the accumulation of some amines in food can cause adverse effects to human health. Histamine at high levels can cause headaches, low blood pressure, nausea, and heart palpitations; whereas tyramine can increase blood pressure and can also be associated with migraine episodes in susceptible individuals (EFSA, 2011; Delgado-Ospina et al., 2021). Tyramine at high levels can induce migraine in susceptible individuals. It can also initiate hypertension during treatment with monoamino oxidase inhibitor (MAOI) drugs (EFSA, 2011). The highest histamine levels were detected in C4 and C1; whereas the highest tyramine levels were found in C1 and C2. Therefore, higher proportions of fully fermented cocoa contributed to increased tyramine levels, whereas no specific pattern could be observed for histamine. Anyhow, the levels of tyramine and histamine found in the chocolates are below the no adverse effect level (NOAEL) established for healthy individuals (EFSA, 2011). However, individuals under treatment with monoaminoxidase inhibitor drugs can be at risk with the ingestion of tyramine. In addition, individuals susceptible to diet-induced migraines must avoid food containing tyramine (Ruiz-Capillas & Herrero, 2019). In a similar way, individuals with histamine sensitivity, must not be exposed to histamine.

Based on these results, the addition of under-fermented cocoa (0 to 65 %) during chocolate production did not affect the types of amines detected. It provided two distinct groups based on total amine levels – C1 and C2 with high levels (~30 mg/kg) and C3, C4 and C5 with lower levels (~16 mg/kg). By increasing the proportion of under-fermented cocoa (C3 – C4), there were significant decreases on the levels of agmatine, tyramine, putrescine, cadaverine and phenylethylamine. The addition of 65 % under-fermented cocoa caused a decrease on serotonin and spermidine levels in the chocolate. Based on these results, by adding under-fermented cocoa to fully fermented cocoa during chocolate making, one can modulate the levels of most amines.

The samples were also analyzed regarding total phenolic compounds, and C1 (100 % fully fermented cocoa) had lower levels – 9.17 ± 0.23 mg epicatechin equivalent (Epi)/g. When increased proportions of under-fermented cocoa were used, there were significant successive increases on total phenolic compounds reaching 20.27 ± 0.34 mg Epi/g when 65 % under-fermented cocoa was used (unpublished data). In fact, during cocoa fermentation, there is reduction on the levels of phenolic compounds, which is desirable as it decreases bitterness and astringency of the cocoa mass (Chagas Junior et al., 2021b; De Andrade et al., 2021). The reduction on phenolic compounds levels results mainly from the activity of polyphenol oxidases which accelerate oxidation and degradation of cocoa polyphenols and their derivatives (Toro-Uribe, Godoy-Chivatá, Villamizar-Jaimes, Perea-Flores, & López-Giraldo, 2020). The reaction is enhanced during the fermentation process, which leads to the development of ideal conditions for polyphenol oxidases, e.g., higher temperatures (42 and 45 °C) and oxygen availability from continuous aeration of the cocoa mass (Hernández-Hernández et al., 2016; Chagas Junior et al., 2021b).

3.3. *In vitro* bioaccessibility

Bioaccessibility refers to the amount of a food component that is released from the matrix in the gastrointestinal tract and is available for absorption, which can be affected by the composition of the matrix (Oracz et al., 2019). In this context, it is likely that when changing the composition of the chocolate by using different proportions of under-fermented cocoa along with the fully fermented, bioaccessibility can be affected. Therefore, the five different chocolates (C1 to C5) were submitted to oral, gastric and intestinal *in vitro* digestions and the final solutions were analyzed for free bioactive amines.

According to Fig. 3, the total levels of amines varied throughout digestion, with the highest levels observed after the intestinal phase. The total levels increased after intestinal digestion for formulations C4, C3 and C5, equivalent to 1.57, 1.50 and 1.44 times, respectively, when compared to the chocolates prior to digestion. On the other hand, the total amine content of C1 was similar to that before *in vitro* digestion (1.06 times) and C2, showed approximately 16 % reduction (0.84 times) in total amine content.

Based on these results, the free amines present in chocolates C1 and C2 (extracted with 5 % TCA) were totally available after intestinal digestion, with minor loss in C2. Whereas samples with higher proportion of under-fermented cocoa – C3, C4 and C5, which had 35, 50 and 65 % under-fermented cocoa, respectively, had, after intestinal digestion 50 % more free amines compared to 5 % TCA extracts from original chocolates. This result suggests that, by using 35 to 65 % under-fermented cocoa, other components with the ability to bind to amines could be present in the matrix and could hinder its extraction from the chocolate as free amines.

According to the literature, amines in foods can be free or conjugated with other compounds, including phenolic compounds (polyphenols, flavonoids, anthocyanins, lignin) and proteins (Casal, Mendes, Alves, Alves, Oliveira, & Ferreira, 2004; Barišić et al., 2020; Godočiková et al., 2020; Yılmaz & Gökmen, 2020). The levels of protein in the different chocolates (C1 – C5) are the same (unpublished data), which is expected since the same amount of cocoa and ingredients was used in all treatments. In addition, changes in proteins resulting from hydrolysis during fermentation, would not be detected by the methods used for analysis (Kjeldahl) as it quantifies total nitrogen).

The levels of total phenolic compounds were significantly different among treatments, varying from 9.17 mg Epi/g in C1 up to 20.27 mg Epi/g in C5. In fact, the higher the proportion of under-fermented cocoa, the higher would be the levels of phenolic compounds in the chocolate.

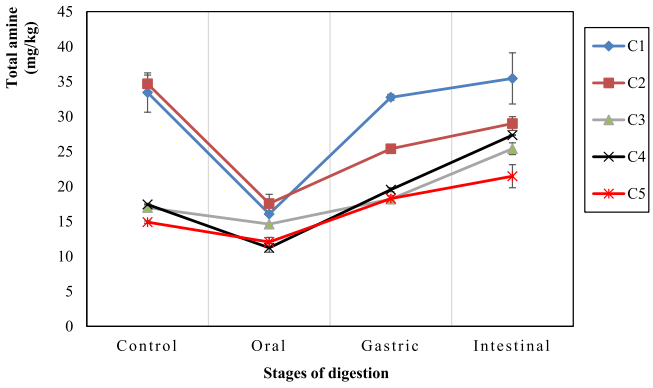

Fig. 3. Changes on the levels of total free bioactive amines during oral, gastric and intestinal stages of *in vitro* gastrointestinal digestion of chocolate made with different proportions of under-fermented (48 h) and fermented (144 h) cocoa beans.

This is so because throughout fermentation, there is a significant decrease in phenolic compounds, including flavonoids, anthocyanins, epicatechin, and catechin (Casal et al., 2004; Melo et al., 2021). When considering the ratio of bioactive amines per total phenolic compounds found in the samples – (amines/phenolics)x100, values of 3.7, 3.1, 1.2, 1.1 and 0.7 were found for C1, C2, C3, C4 and C5, respectively. Based on these values and bioaccessibility results, it is likely that when the ratio is similar or greater than 3.0, the amines would be in the free form (and, thereby, available for extraction by TCA), whereas, when the ratio is similar or lower than 1.2, some amines would be bound, and they would not be completely extracted by TCA, but they would be available for intestinal absorption.

When considering the influence of *in vitro* digestion on individual amines (Table 2), it can be observed that tyramine, putrescine, cadaverine, spermidine, and phenylethylamine were found in all samples throughout the three stages of the simulated digestion. Serotonin and agmatine were not detected after the oral (C—O) and the gastric (C-G) digestion phases for C3, C4, and C5, which had lower total levels of amines. On the other hand, histamine was only detected in the original chocolates (before digestion) and after the intestinal phase. Similar behavior was reported by Dala-Paula et al. (2021a) regarding histamine, which was only detected at the end of the intestinal digestion (C-I) of the chocolate. Because they did not detect histamine in the chocolate before simulated digestion, the authors hypothesized that the occurrence of this amine was related to the action of pancreatic enzymes to the sample.

3.3.1. Oral phase

In general, after oral digestion (Table 2), bioaccessibility was similar for tyramine, spermidine and phenylethylamines, for which, the levels were similar to those initially present in all chocolate formulations. Histamine was not detected in any sample, suggesting that this amine is present in chocolate in a conjugated form and that oral conditions (saliva: α-amylase and pH 6.9) were not able to extract them from the matrix. The behavior observed for the other amines varied depending on the chocolate formulation, e.g., the proportion of under-fermented samples. For the diamines putrescine and cadaverine, lower levels, compared to the original chocolate, were detected for treatments C1 and C2 (higher proportions of fully fermented cocoa); whereas similar levels were found for the cholates with higher amounts of under-fermented cocoa. The levels of serotonin and agmatine found after oral digestion were lower compared to the original samples and they were not detected in the samples with higher proportion of under-fermented cocoa.

Based on these results, oral digestion conditions, make available in chocolate only some of the amines present in chocolate (5 % TCA extraction), with lower levels of putrescine, cadaverine, serotonin and agmatine. The last two amines were not even detected in the samples with higher proportion of under-fermented cocoa.

3.3.2. Gastric phase

After gastric digestion, there was a significant increase on the levels of the aromatic amines – tyramine and phenylethylamines for all samples, making more of them available compared to initial levels (Table 2). Histamine was not detected in any sample, suggesting that this amine is present in chocolate in a conjugated form and that even digestive conditions (pepsin and pH 2.0) were not able to extract them from the matrix. The availability of the other amines depended on the proportion of under-fermented cocoa in the chocolate. Spermidine levels were higher compared to original levels for chocolates with lower proportion of under-fermented cocoa. The diamines putrescine and cadaverine, were detected at lower levels compared to the original chocolate, but similar to oral results for treatments C1 and C2 (higher proportions of fully fermented cocoa); whereas similar levels were found for the chocolates with higher amounts of under-fermented cocoa. No change was observed for spermidine in the samples with higher proportions of under-fermented cocoa. The levels of serotonin found after gastric digestion were lower compared to the original samples C1 and C2 (lower under-fermented cocoa) but higher compared to oral results. The levels of agmatine found after gastric digestion were lower compared to the

Table 2
Contents of free bioactive amines in chocolate and solutions from the oral (O), gastric (G) and intestinal (I) *in vitro* digestion phases of chocolates made with different proportions of under-fermented (48 h) and fermented (144 h) cocoa beans.

Sample/ Phase	Free bioactive amines (mg/kg) SPD	PUT	CAD	HIM	SRT	AGM	TYM	PHM
Chocolate								
C1	4.52 ± 0.48^{ghi}	5.57 ± 0.47^{a}	7.21 ± 0.60^{a}	1.20 ± 0.12^{abc}	8.02 ± 0.65^{ab}	2.27 ± 0.21^{a}	2.59 ± 0.13^{fg}	2.15 ± 0.14^{efg}
C2	5.88 ± 0.02^{cdef}	5.42 ± 0.39^{a}	6.99 ± 0.28^{a}	1.16 ± 0.10^{abc}	8.43 ± 0.82^{a}	2.16 ± 0.23^{a}	2.77 ± 0.32^{efg}	1.87 ± 0.10^{fg}
C3	3.16 ± 0.05^{j}	2.49 ± 0.03^{de}	2.95 ± 0.09^{fghij}	0.59 ± 0.01^{e}	4.33 ± 0.18^{e}	0.96 ± 0.03^{b}	1.41 ± 0.05^{g}	1.09 ± 0.02^{hi}
C4	3.70 ± 0.07^{hij}	2.43 ± 0.24^{de}	2.63 ± 0.07^{ghij}	1.20 ± 0.01^{abc}	4.09 ± 0.08^{e}	0.87 ± 0.01^{b}	1.47 ± 0.08^{g}	1.03 ± 0.01^{hi}
C5	5.30 ± 0.10^{defg}	2.33 ± 0.01^{e}	2.24 ± 0.02^{ij}	0.97 ± 0.02^{cd}	0.97 ± 0.02^{fg}	0.78 ± 0.01^{bc}	1.49 ± 0.03^{g}	0.81 ± 0.01^{i}
Oral								
C-O1	4.50 ± 0.21^{ghi}	2.52 ± 0.61^{cde}	3.85 ± 0.29^{def}	nd	1.54 ± 0.08^{f}	0.37 ± 0.04^{e}	1.68 ± 0.13^{g}	1.61 ± 0.04^{gh}
C-O2	6.91 ± 0.80^{bc}	2.33 ± 0.17^{e}	2.08 ± 0.01^{j}	nd	3.50 ± 0.61^{e}	0.44 ± 0.02^{de}	1.32 ± 0.14^{g}	0.98 ± 0.04^{hi}
C-O3	4.98 ± 0.41^{defgh}	2.64 ± 0.09^{cde}	3.68 ± 0.01^{defg}	nd	nd	nd	1.38 ± 0.09^{g}	1.94 ± 0.11^{fg}
C-O4	3.44 ± 0.65^{ij}	2.06 ± 0.01^{e}	2.56 ± 0.03^{hij}	nd	nd	nd	1.37 ± 0.01^{g}	1.80 ± 0.05^{fg}
C-O5	4.57 ± 0.60^{fghi}	2.70 ± 0.16^{cde}	2.57 ± 0.03^{hij}	nd	nd	nd	1.19 ± 0.05^{g}	1.03 ± 0.01^{hi}
Gastric								
C-G1	8.68 ± 0.39^{a}	3.18 ± 0.02^{cde}	3.99 ± 0.30^{cdef}	nd	6.01 ± 0.53^{cd}	0.47 ± 0.17^{cde}	5.69 ± 0.39^{abcd}	3.38 ± 0.20^{ab}
C-G2	6.81 ± 0.17^{bc}	2.66 ± 0.15^{cde}	3.22 ± 0.12^{fghi}	nd	4.92 ± 0.07^{de}	0.45 ± 0.01^{de}	4.38 ± 0.06^{cdef}	2.95 ± 0.01^{bcd}
C-G3	4.05 ± 0.01^{ghij}	3.20 ± 0.11^{cde}	4.65 ± 0.69^{bcd}	nd	nd	nd	3.85 ± 0.06^{def}	2.45 ± 0.01^{cdef}
C-G4	6.30 ± 0.01^{cd}	2.47 ± 0.51^{de}	3.14 ± 0.23^{fghij}	nd	nd	nd	4.81 ± 0.01^{bcde}	2.83 ± 0.01^{bcde}
C-G5	4.57 ± 0.00^{fghi}	3.64 ± 0.26^{bcd}	5.05 ± 0.01^{bc}	nd	nd	nd	3.06 ± 0.69^{efg}	1.96 ± 0.01^{fg}
Intestinal								
C-I1	4.95 ± 0.22^{efgh}	4.65 ± 0.48^{ab}	5.49 ± 0.28^{b}	1.29 ± 0.17^{ab}	6.82 ± 1.02^{bc}	0.70 ± 0.06^{bcd}	7.63 ± 1.28^{a}	4.01 ± 0.14^{a}
C-I2	6.72 ± 0.02^{bc}	3.16 ± 0.04^{cde}	3.56 ± 0.02^{defgh}	1.21 ± 0.01^{abc}	4.11 ± 0.04^{e}	0.66 ± 0.01^{bcde}	6.61 ± 0.83^{ab}	2.97 ± 0.05^{bcd}
C-I3	7.81 ± 0.10^{ab}	3.76 ± 0.11^{bc}	3.48 ± 0.25^{efgh}	1.31 ± 0.04^{a}	nd	nd	5.96 ± 1.41^{abc}	3.08 ± 0.07^{bc}
C-*I4*	6.75 ± 0.18^{bc}	4.59 ± 0.03^{ab}	4.59 ± 0.41^{bcd}	0.87 ± 0.03^{d}	nd	nd	7.32 ± 0.05^{a}	3.23 ± 0.30^{b}
C-I5	5.94 ± 0.07^{cde}	3.68 ± 0.68^{bcd}	4.42 ± 0.03^{bcde}	1.05 ± 0.13^{bcd}	nd	nd	4.06 ± 0.38^{cdef}	2.32 ± 0.62^{def}

SPD-spermidine; AGM-agmatine; PUT-putrescine; CAD-cadaverine; HIM-histamine; TYM-tyramine; PHM-phenylethylamine; SRT-serotonin.
Proportion of under-fermented (48 h) and fermented (144 h) cocoa, respectively: C1-0 % and 100 %; C2-20 % and 80 %; C3-35 % and 65 %; C4-50 % and 50 %; C5-65 % and 35 %.
Mean values ± standard deviations with different letters in the same columns are significantly different (Tukey test, $p \leq 0.05$).
nd - < limit of quantification = 0.3 mg/kg.

original samples and similar to oral results for samples with lower proportion of under-fermented cocoa. But serotonin and agmatine were not detected after gastric digestion of chocolate samples with higher proportion of under-fermented cocoa, corroborating with the results described by Dala-Paula et al. (2021a) in 70 % chocolate. The release of the amines in this digestion phase is probably due to pepsin activity on chocolate protein, as well as to the pH change, which could enhance breakdown of conjugated forms of amines (Casal et al., 2004).

Based on these results, gastric digestion (pepsin and pH 2.0) was able to release tyramine and phenylethylamine from chocolate samples, irrespective of the amount of under-fermented cocoa used during processing. Furthermore, it released serotonin and spermidine, in special for samples with lower proportion of under-fermented cocoa. However, it did not affect the release of the other amines in a significant way.

3.3.3. Intestinal phase

During intestinal digestion, total amine levels increased for all chocolate formulations compared to the previous phases (oral and gastric) (Table 2). The levels of phenylethylamine were higher compared to initial chocolate levels, but similar to levels after gastric digestion, suggesting that intestinal digestion did not affect phenylethylamine levels. Putrescine and cadaverine levels were higher compared to those from gastric digestion. However, when comparing results with initial levels in the chocolates, higher levels were observed for samples containing higher proportions of under-fermented cocoa; whereas in samples with a lower proportion of under-fermented, similar levels were found for putrescine, but lower for cadaverine. Spermidine levels, after intestinal digestion were similar to initial chocolate levels for C1 and C2 (lower under-fermented) but higher for C3, C4 and C5 (higher under-fermented). In this way, when a higher proportion of under-fermented cocoa was used, spermidine which was present in conjugated form became available after intestinal digestion. Serotonin and agmatine were only detected in the samples with a lower proportion of under-fermented cocoa (C1 and C2). The levels of these amines were not affected by the intestinal phase. Tyramine levels were higher after intestinal digestion (4.06 to 7.63 mg/kg) compared to initial chocolate levels (1.41 to 2.77 mg/kg), and also compared to samples after gastric digestion (3.06 to 5.69 mg/kg). This result suggests tyramine is released from conjugates under both gastric and intestinal digestions. Histamine, which was not detected after the other digestions (oral and gastric), was detected (0.87 to 1.31 mg/kg) at levels similar to those found in the original chocolate (0.59 to 1.20 mg/kg). Based on this result, histamine was only released from conjugates after intestinal digestion (pancreatin and bile salts, pH 6.9). It is known that histamine and tyramine, at high levels can cause adverse effects on human health (EFSA, 2011). However, despite the increase in total levels during the digestion process, in the intestinal phase, these values do not compromise the health of the consumer, since they do not reach the maximum limits observed (NOAEL) for healthy individuals.

Studies regarding the bioaccessibility of amines are scarce and considering the specificities of a complex matrix like chocolate, even harder to find; however, the release of free bioactive amines from dark chocolate after gastric and intestinal digestions, corroborates with findings by Dala-Paula et al. (2021a). The increase in total amines after these phases can be explained by the breakdown of conjugated forms of the amines by the conditions available (e.g., pepsin solution, pH 2.0; and bile salts and pancreatin, respectively for gastric and intestinal). In this way, the release of tyramine, serotonin, spermidine (lower % under-fermented) agmatine and phenylethylamine from conjugated forms were most significant during gastric digestion, whereas putrescine, cadaverine, histamine and spermidine (higher % under-fermented) were most significant during intestinal digestion. Therefore, the specificity of the digestive enzymes associated with each phase may affect amine availability. During digestion, there is breakdown of proteins which is initiated in the stomach by pepsin and HCl, and still occurs in the intestinal phase due to the action of pancreatic proteases (Kulp, Fortson, Knize, & Felton, 2003), both being important for the bioaccessibility of the amines. It is likely that the conditions prevalent during digestion, also allow liberation of amines conjugated with proteins and also with phenolic compounds. Furthermore, the increased pH (6.9) in this phase and the high Na^+ content in the simulated duodenal juice, can affect electrostatic interaction between amines and their conjugates, contributing to their release (Dala-Paula et al., 2021a).

It would be interesting to carry out research on interaction mechanisms and structure-affinity relationships between bioactive amines and the main protein fractions and phenolic compounds of chocolate. Computer simulation methods and m ultispectral techniques have been used to investigate possible interactions between bioactive compounds and proteins (Wu et al., 2021). Considering the possible interactions between bioactive amines and protein and phenolic compounds in chocolate, future research could contribute to a better understanding of its bioaccessibility, bioavailability and biotoxicity.

3.3.4. Multivariate analyses

Multivariate analysis of auto-scaled data of individual and total bioactive amines during digestion of chocolates indicated that a two principal component (PC) model explained 95.5 % of the variance, total amines as the main variable (0.91) of PC1 (86.9 %) and serotonin content as the main variable (0.70) of PC2 (8.6 %) as shown in Fig. 4a. The formation of three groups in multivariate statistics, with 74.92 % of similarity, allowed samples separation through total amine contents. Three main groups were differentiated based on total amines levels: the first with the highest contents, formed by the chocolates with the highest proportion of fully fermented cocoa and some C1 digestion stages (C-I1, C2, C1, and C-G1); followed by the second with intermediate levels (C-I2, C-*I*4, C-I3 and C-G2), and the third one with the lowest levels (CI-5, C-G4, C-G5, C-G3, C-O4, C-O5, C-O3, C5, C-O1, C3, C4, and C-O2).

According to PC1 loadings, total amines, serotonin and tyramine were the amines most affected by *in vitro* digestion, especially in the intestinal phase. PC2 explained 8.6 % of the variance, mainly by serotonin and negatively by tyramine, spermidine and phenylethylamine (Fig. 4b and 4c). The dendrogram-HCA (Fig. 4d) based on the variances and Euclidean distances between vectors confirmed PCA results, reinforced the data in Table 2, demonstrating that there was no clear separation of the chocolates and the phases.

4. Conclusion

Fermentation of cocoa results in loss of phenolics compounds and antioxidant activity and in the change in the profile of bioactive amines. In addition, fermentation is a lengthy process which can be a burden to cocoa processing. In this study, different proportions (0, 20, 35, 50 and 65 %) of under-fermented (48 h) were used along with fully fermented (1 4 4) cocoa in the production of dark chocolate. Total levels of amines were higher in samples with lower proportion of under-fermented cocoa – 0 and 20 % (~34 mg/kg) compared to those with 30 to 65 % under-fermented cocoa (~17 mg/kg). The same amines were detected in the chocolates, and serotonin was the prevalent amine in all of them, except for the one with 65 % under-fermented cocoa, in which spermidine was the predominant amine. The chocolates made with lower amounts of under-fermented cocoa (0 and 20 %) had significant higher levels of agmatine, phenylethylamines, and tyramine compared to the ones with higher amounts of under-fermented cocoa. As expected, the levels of total phenolic compounds in the chocolate increased significantly as the proportion of under-fermented cocoa increased.

During *in vitro* digestion of the different chocolates, the changes on total amines could also be grouped into samples with higher (35 to 65 %) and lower (0 and 20 %) proportions of under-fermented cocoa. In the first group, total levels were significantly higher after intestinal digestion compared to the original chocolate and after oral and gastric digestions. In the latter, after oral digestion, low levels of total amines were found, but levels increased significantly after gastric and them

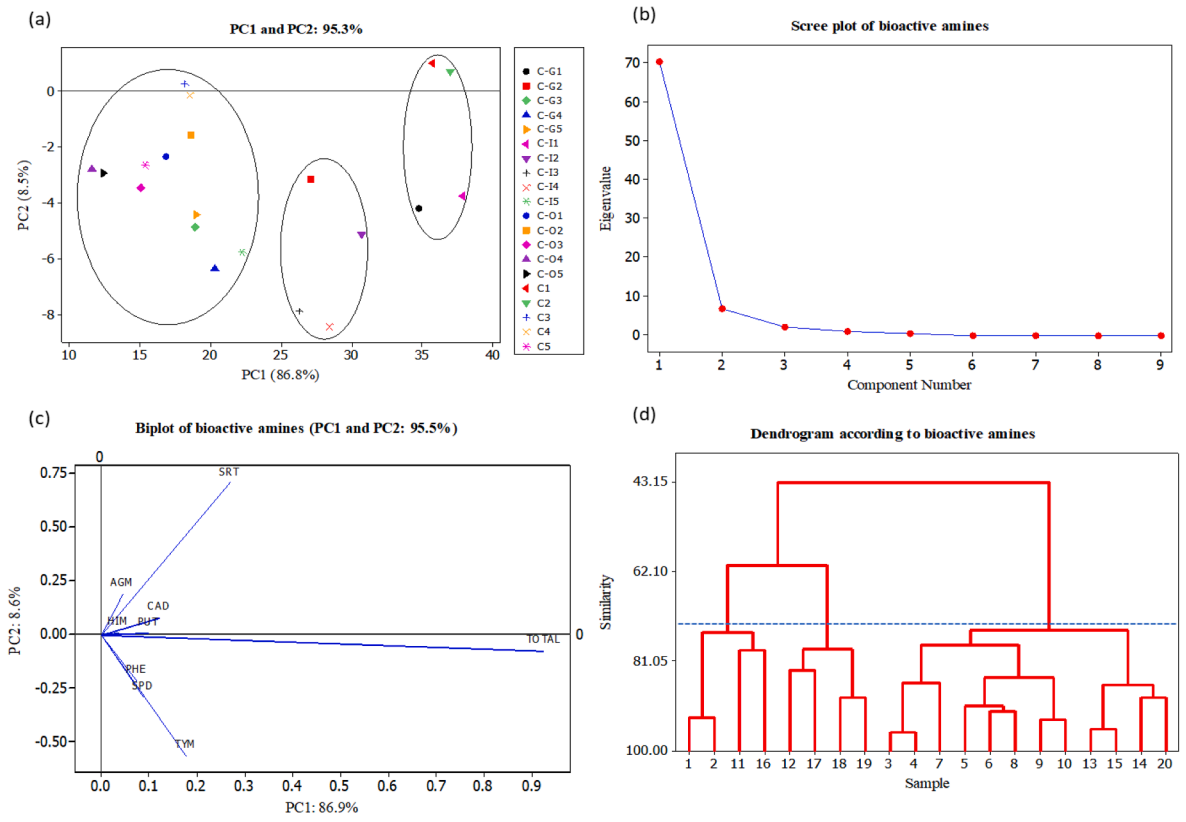

Fig. 4. Principal component analyses (PCA) and hierarchical cluster analyses (HCA) of free bioactive amines in chocolates (C) made with different proportions of under-fermented (48 h) and fermented (144 h) cocoa beans and respective digests after Oral (C—O), Oral + Gastric (C-G) and Oral + Gastric + Intestinal (C-I) *in vitro* digestions (a) PC1 and PC2 plots; (b) Biplot of bioactive amines (PC1 and PC2: 95.3 %); (c) Scree plot of bioactive amines; and (d) Dendrogram. Legend: AGM-agmatine, CAD-cadaverine, HIM-histamine, PHM-2-phenylethylamine, PUT-putrescine, SPD-spermidine, SRT-serotonin, TYM-tyramine, TOTAL-total amines. Dendrogram observations: 1, 2, 3, 4 and 5 (C1, C2, C3 C4 and C5 chocolates); 6, 7, 8, 9 and 10 (oral phase digests: C-O1, C-O2, C-O3, C-O4 and C-O5); 11, 12, 13, 14 and 15 (gastric phase digests: C-G1, C-G2, C-G3, C-G4 and C-G5); 16, 17, 18, 19 and 20 (after intestinal phase digests: C-I1, C-I2, C-I3, C-*I*4 and C-I5).

after intestinal digestion.

After oral digestion all amines were detected at levels similar or lower than levels in the original chocolate, except that serotonin and agmatine were not detected in the samples with higher proportion of under fermented cocoa (30–65 %) and histamine was not detected in any sample. Gastric digestion was important in the release of additional levels of the aromatic amines tyramine and phenylethylamine. It also allowed the release of serotonin and agmatine from samples with lower proportions of under-fermented cocoa; and it allowed the release of spermidine from samples with lower proportions of under-fermented cocoa. Intestinal digestion was the only phase capable of releasing histamine from all chocolates. It also allowed the additional release of putrescine and cadaverine; and spermidine for chocolates with higher percentage of under-fermented cocoa.

These results indicate, for the first time, that during *in vitro* digestion higher amounts of some aromatic amines (tyramine and phenylethylamine) are available for intestinal absorption compared with levels of free amines in the chocolate; the same observed for spermidine for chocolates with higher proportion of under-fermented cocoa. On the other hand, lower levels of serotonin and agmatine would be available for intestinal absorption for chocolates with lower percentage of under-fermented cocoa; and none for those with higher proportion of under-fermented cocoa.

These results are relevant in that some amines in chocolate, even though detected by analytical methods as free, would not be available for intestinal absorption (serotonin and agmatine for higher proportion of under-fermented cocoa; cadaverine for low percent under-fermented cocoa); whereas others will be available for absorption at higher amounts than analytical methods (phenylethylamine; tyramine; cadaverine and spermidine for chocolate with higher levels of under-fermented cocoa). Gastric digestion was relevant in the release of tyramine and phenylethylamine; and also serotonin and agmatine from chocolates with lower proportion of under-fermented cocoa. And intestinal digestion was relevant for the release of putrescine; cadaverine; histamine; and spermidine from chocolates with higher proportion of under-fermented cocoa.

Overall, the addition of under-fermented cocoa during chocolate production, could benefit the cocoa processing industry, optimizing processing time. By adding under-fermented cocoa to chocolate production one can modulate the profile and levels of amines in the final product. Similar results were observed for chocolates with 0 % (control) and 20 % under-fermented cocoa. However, when adding 30 to 65 % of under-fermented cocoa, the availability of some amines for intestinal absorption could be limited.

CRediT authorship contribution statement

Geisiane Santos Silva: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft. **Bruno M. Dala-Paula:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing. **Eliete S. Bispo:** Conceptualization, Funding acquisition, Project administration. **Maria Beatriz A. Gloria:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision.

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.

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