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Original Research Article

Understanding amino acids and bioactive amines changes during on-farm cocoa fermentation

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ABSTRACT

Fermentation is of utmost relevance in cocoa processing. Limited information is available on the impact of fermentation on nitrogenous compounds which are associated with relevant health benefits and aroma quality of chocolate. The objective of this study was to investigate the changes on free amino acids and bioactive amines during cocoa on-farm fermentation. In unfermented cocoa 14 amino acids and five amines were detected, with predominance of glutamic acid, leucine, phenylalanine, serine and spermidine and serotonin, respectively. Fermentation followed its due course, confirmed by total titratable acidity, pH and temperature changes. Amino acids levels varied during fermentation and the final levels were higher compared to the unfermented cocoa. Hydrophobic amino acids were prevalent at the end of fermentation. Total levels of most amines decreased (serotonin disappeared) during fermentation; whereas putrescine remained constant and phenylethylamine increased. Multivariate analysis differentiated three distinct phases for these nitrogenous compounds during fermentation: (i) from 0 to 48 h – high serotonin, tryptamine, and pH; (ii) from 60 to 132 h – high phenylalanine, lysine, leucine, alanine, threonine, phenylethylamine, and acidity; and (iii) 144 h – high glycine, histidine, arginine and phenylethylamine. The possibility of using blends with cocoa from different fermentation times, can lead to fermented cocoa with unique functional and sensory properties.

1. Introduction

Cocoa, the main ingredient of chocolate, undergoes different processing stages, including fermentation, drying, and roasting, which are essential for chocolate quality. Fermentation is of utmost importance because it is responsible for the generation of several chemical compounds which are precursors of reactions that take place during roasting, leading to proper chemical and sensorial quality of chocolate (Afoakwa et al., 2012; Barišíc et al., 2019; Do Carmo Brito et al., 2017; Predan et al., 2019; Spizzirri et al., 2019; Żyżelewicz et al., 2018). Cocoa fermentation is mainly conducted as a traditional, indigenous process, which takes place at the cocoa farm, as a part of cocoa bean production. Different microorganisms, including yeast, lactic acid bacteria (LAB), acetic acid bacteria (AAB), spore forming bacteria and molds are involved in cocoa fermentation (Barišíc et al., 2019; Castro-Alayo et al., 2019; Nielsen et al., 2015). Several chemical and biochemical reactions take place resulting in the formation and liberation of compounds which are relevant in the generation of cocoa flavor and aroma during cocoa roasting (Ho et al., 2014; Kumari et al., 2018; Predan et al., 2019; Spizzirri et al., 2019). Some changes which occur during fermentation are well documented in the literature. Initially, yeast and LAB convert pulp sugar into ethanol and, as aeration starts, the oxygen tension increases and AAB produce acetic acid from ethanol; both reactions are highly exothermic, increasing the temperature of the cocoa mass (Afoakwa et al., 2012; Delgado-Ospina et al., 2020; Efraim et al., 2010). There is a decrease in pH and an increase in titratable acidity. Ethanol and acetic acid diffuse into the bean causing embryo death and cell wall breakdown with liberation of endogenous enzymes, leading to a cascade

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of enzymatic reactions. There is a decrease in phenolic contents by phenoloxidases activity, diminishing bitterness and astringency (Barišíc et al., 2019; Cruz et al., 2013; do Carmo Brito et al., 2017; De Taeye et al., 2017).

The fermentation-driven changes in cocoa also include protein hydrolysis, which is relevant in the generation of flavor and aroma characteristics during roasting (Adeyeye et al., 2010; Castro-Alayo et al., 2019; Ho et al., 2014; Rohsius et al., 2006). The low pH values achieved during fermentation activate endogenous proteolytic enzymes, aspartic endoprotease and carboxypeptidase which hydrolyze storage proteins (mainly vicilin) into free amino acids and peptides. These compounds, along with reducing sugars are necessary for Maillard reaction during drying and roasting (Adeyeye et al., 2010; Marseglia et al., 2014; Rohsius et al., 2006; Spizzirri et al., 2019). In addition, free amino acids liberated during cocoa fermentation are responsible for the characteristic taste (umami, sweet, bitter, among others) of the chocolate (Adeyeye et al., 2010; Rohsius et al., 2006).

The low pH achieved during fermentation also activates amino acid decarboxylases, which are responsible for the formation of bioactive amines. These reactions are additionally favored from the amino acids released during proteolysis (Do Carmo Brito et al., 2017; Restuccia et al., 2019; Spizzirri et al., 2019). The interest on bioactive amines during fermentation and in the final chocolate relies on the health promoting properties of some of them, but also on the adverse effects exerted mainly by histamine and tyramine. The polyamine spermidine is associated with antioxidant and anti-aging properties (do Carmo Brito et al., 2017; Kalač, 2014); and it can prevent cardiovascular diseases, regulate systemic inflammation, and induce cytoprotective autophagy, important to the maintenance of cellular homeostasis and lifespan; and it is involved in the maturation and recovery of the intestinal mucosa and production of immunoglobulin A (Kalač, 2014; Madeo et al., 2018). The other amines, e.g. phenylethylamine, tryptamine, tyramine and serotonin are neuroactive. Phenylethylamine is a catecholamine releasing agent, stimulator of the hypothalamus, inducing pleasurable sensations, affecting the levels of neurotransmitters in the brain, enhancing mood lifting and sexual drive (do Carmo Brito et al., 2017; Yılmaz and Gökmen, 2020). In addition, the lack of phenylethylamine has been associated with attention deficit and hyperactivity (Afoakwa et al., 2012; Irsfeld et al., 2014). Serotonin plays important roles in physiological and behavioral processes – cardiovascular regulation, reproductive behavior, pain sensitivity, regulation of appetite, body temperature, sleep, thirst, mood, sexual activity, aggression, anxiety, and of peripheral functions in the cardiovascular, gastrointestinal, endocrine and pulmonary systems (Spizzirri et al., 2019; Vadodaria et al., 2018; Yılmaz and Gökmen, 2020). Tyramine and tryptamine, at low concentrations, modulate vaso- and neural-activities. Tryptamine is considered a neuromodulator and neurotransmitter (Yılmaz and Gökmen, 2020). However, the presence of some amines, e.g., histamine and tyramine, at high concentrations in chocolate, can be detrimental to human health, especially in sensitive individuals or those under monoaminoxidase inhibitor drugs (EFSA, 2011).

There is limited information on bioactive amines formation during cocoa fermentation. In a preliminary study from our research group, during laboratory fermentation of Amazonian cocoa, four amines were detected and followed during fermentation – spermidine, spermine, tyramine and tryptamine. To the best of our knowledge, this is the first study addressing concomitant changes on free amino acids and bioactive amines during on-farm cocoa fermentation. In addition, the performance of the fermentation process was also monitored by temperature, pH, and total titratable acidity.

2. Materials and methods

2.1. Materials

A commercial blend of Forastero cocoa (Theobroma cacao L.) seeds was used. They came from ripe fruits (total of 1500 kg) and were from the same farm were fermentation was performed (South of Bahia, Brazil). The reagents were of analytical grade, except HPLC solvents (LC grade). Ultrapure-water was from Milli-Q Plus (Millipore Corp., Milford, MA, USA). L-amino acids standards (aspartic acid, serine, asparagine, glycine, glutamic acid, glutamine, histidine hydrochloride, threonine, arginine hydrochloride, alanine, proline, cystine, tyrosine, tryptophan, valine, methionine, lysine hydrochloride, leucine, isoleucine, phenylalanine and norvaline), bioactive amines standards (putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, cadaverine dihydrochloride, serotonin hydrochloride, histamine dihydrochloride, tyramine hydrochloride, tryptamine, 2-phenylethylamine hydrochloride) and the derivatization agent for amines (*o*-phthalaldehyde) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). AccQ.FluorTM pre-column derivatization kit for amino acids was from Waters (Milford, MA, USA).

2.2. Cocoa fermentation

Spontaneous commercial cocoa fermentation was performed at the farm (Southern Bahia, Brazil, 14°41′96″ S and 39°12′109″ W), following standard protocols. Immediately after harvest, the fruits were cut open (stainless steel knives), and the beans and pulp, without the peel and placenta, were placed in 100 × 100 × 120 cm wooden boxes (500 kg capacity). The boxes were covered with banana leaves and burlap. After 48 h fermentation, the seeds were mixed every day to allow aeration of the mass. Fermentation was concluded at 144 h based on aspects, color and aroma typical of fermented cocoa beans. The experiment was performed in triplicate. The average local daily temperature during fermentation varied from 22.3 °C to 29.6 °C.

Throughout fermentation (0–144 h, 12 h intervals), the temperature was measured 24 cm deep in the wooden boxes (digital stick thermometer, MINIPA MT-450, Joinville, SC, Brazil) and samples (~300 g) were randomly taken from each fermentation box. The samples were aseptically packed and taken immediately to the laboratory for physico-chemical analysis. The samples were frozen and kept at −18 °C until analysis for free amino acids and bioactive amines.

2.3. Determination of physico-chemical characteristics

The efficiency of the fermentation process was determined by measuring pH and determining total titratable acidity (AOAC, 2016). The temperature was taken using a digital thermometer. The moisture content (AOAC, 2016) was also determined. The analyses were performed in triplicate.

2.4. Determination of amino acids by UPLC™

Free amino acids were extracted by three successive extractions of 5 g ground cocoa with 7 ml 5% trichloroacetic acid followed by centrifugation at 11,180 *g* at 4 °C for 10 min (Deus et al., 2020). The internal standard norvaline (25 pmol on column) was added to the extract, and the volume was brought up in a 25-mL volumetric flask. The extract was centrifuged at 16,000 *g* at 4 °C for 10 min (Jouan CR3i refrigerated centrifuge, Saint-Herblain, France), neutralized using an equal volume of 1 M NaOH and derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl – AQC (Moreira et al., 2017). The contents of free amino acids were determined after filtering the extract (PTFE 0.22 μm membrane, Minisart SRP 4®, Sartorius, Goettingen, Germany). A Waters Acquity™ Ultra Performance LC (UPLC™) system (Waters, Milford, MA, USA) was used (Marseglia et al., 2014; Moreira et al., 2017). The UPLC™ system was equipped with an Acquity™ tunable ultra-violet (TUV) detector at 249 nm (40 points sec^{-1}). A BEH C18 column (50 × 2.1 mm, 1.7 μm, Acquity UPLC™) and a gradient elution of A – 0.1 mol L^{-1} sodium acetate (pH 4.80 with acetic acid) and B – acetonitrile were used. The concentrations of the analytes were

calculated by interpolation in analytical curves ($R^2 \geq 0.997$). The method was fit for the purpose for the analysis of 20 amino acids and ammonia in cocoa (Table S1 and Fig. S1).

2.5. Determination of bioactive amines by HPLC

The determination of bioactive amines was carried out by ion-pair reverse phase liquid chromatography (do Carmo Brito et al., 2017). The samples (5 g) were extracted as described for amino acids analysis. A Shimadzu HPLC system (Shimadzu®, Kyoto, Japan) and a Novapak C18 column (3.9 × 300 mm, 4 μm, Waters, MA, USA) were used. A gradient of 0.2 M sodium acetate and 0.3 mM sodium octane sulfonate, pH 4.9 and acetonitrile was used. Amines were quantified fluorometrically (340 and 445 nm of excitation and emission, respectively) after post-column derivatization with *o*-phthalaldehyde. The concentrations of the amines were calculated by interpolation in analytical curves ($R^2 \geq 0.998$). The method was fit for the purpose for the analysis of nine bioactive amines in cocoa (Table S2 and Fig. S2).

2.6. Statistical analysis

The results were submitted to analysis of variance and the means were compared by the Tukey test (95 % significance). Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for the characterization of cocoa beans during fermentation. In PCA, contents of individual amines and amino acids, total amines, total amino acids, temperature, pH and titratable acidity were used as active variables and the supplementary variable (time of fermentation) was projected onto the factor space. The dendrogram for HCA analysis was obtained by clustering variables. The statistical software Minitab® (16.2.3, SP, Brazil) was used.

3. Results and discussion

3.1. Characterization of the unfermented cocoa

The unfermented Forastero cocoa was characterized by pH 6.64, total titratable acidity of 5.57 mEq NaOH 100 g^{-1}, moisture content of 5.42 g 100 g^{-1} and temperature of 27.4 °C (Table 1). Fourteen free amino acids were detected (Table 2) at total levels of 423 mg 100 g^{-1}. The sulfurous amino acids (cystine and methionine), isoleucine, tryptophan, glutamine, asparagine and ammonia were not detected. The predominant amino acid (Fig. 1) was glutamic acid (20.4 % of total), followed by leucine (12.2 %), phenylalanine (10.6 %), serine (9.9 %) and arginine (7.9 %); the others accounted for less than 6% of total levels. Six essential amino acids were detected (167 mg 100 g^{-1}, 39.4 % of total). The acidic and the hydrophobic amino acids were 104 and 176 mg 100 g^{-1} (24.5 and 41.6 %), respectively.

Among the nine amines investigated, five were present in the unfermented cocoa, with total mean levels of 30.9 mg kg^{-1} (Table 3). The predominant amine was spermidine (43.4 % of total), followed by serotonin (28.5 %), tyramine (18.2 %), putrescine and tryptamine, with ~5% contribution each (Fig. 2).

The pH and total titratable acidity of unfermented cocoa are like literature values for Forastero cocoa beans (Cruz et al., 2013; Efraim et al., 2010).

This is the first report on the simultaneous characterization of free amino acids and free bioactive amines in unfermented cocoa. Total levels of free amino acids were high, with a high percentage of acidic followed by hydrophobic amino acids. To the best of our knowledge, no information regarding the profile and levels of free amino acids in unfermented cocoa is available. Data in the literature for free amino acids refers to cocoa available in the market, which is already fermented (Marseglia et al., 2014; Rohsius et al., 2006; Spizzirri et al., 2019); or to total amino acids, after protein hydrolysis (Adeyeye et al., 2010).

Among bioactive amines detected in unfermented cocoa, the polyamine spermidine is inherent to plants and living organisms. It is involved in cell division, organogenesis, and stress response (Kalač, 2014). Putrescine is an intermediate of spermidine synthesis. Spermidine and putrescine play important roles in plant development, growth and survival under drought, heat, salt and oxidative stress (Handa et al., 2018). Serotonin participates in various processes such as reproduction, germination, vegetative growth, melatonin production and seed longevity (Hwang and Back, 2020). Tryptamine is linked to plant protection against predators (Gloria and Engeseth, 2017). Data on free bioactive amines in unfermented cocoa is scarce in the literature, as cocoa available in the market is already fermented. Do Carmo Brito et al. (2017) detected only four amines in Amazonian Forastero cocoa after 24 h fermentation (spermidine, spermine, tryptamine and tyramine). The presence of additional free amines in Forastero cocoa from Bahia (serotonin and putrescine) suggests the influence of genotype, origin (soil characteristics, climatic conditions) and farming practices on the chemical components of cocoa as reported in the literature (Castro-Alayo et al., 2019; Deus et al., 2020; Marseglia et al., 2014; Spizzirri et al., 2019).

Table 1
Physico-chemical changes during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil.

Fermentation time (h)	Temperature* (°C)	pH	Titratable acidity (mEq NaOH 100 g^{-1})	Moisture (g 100 g^{-1})
0	27.4 ± 0.0^{f}	6.64 ± 0.03^{a}	5.57 ± 0.26^{d}	5.42 ± 0.14^{c}
12	30.0 ± 0.1^{ef}	6.61 ± 0.02^{ab}	5.65 ± 0.82^{d}	6.11 ± 0.08^{abc}
24	32.0 ± 0.1^{de}	6.56 ± 0.02^{b}	6.84 ± 0.52^{d}	6.04 ± 0.12^{abc}
36	31.4 ± 0.1^{e}	6.44 ± 0.01^{c}	6.65 ± 0.41^{d}	5.72 ± 0.29^{bc}
48	32.2 ± 0.4^{de}	6.16 ± 0.02^{d}	8.48 ± 1.11^{cd}	5.54 ± 0.20^{bc}
60	34.6 ± 1.7^{d}	5.60 ± 0.00^{e}	12.8 ± 0.91^{c}	5.64 ± 0.27^{bc}
72	46.6 ± 0.6^{a}	4.98 ± 0.01^{f}	23.2 ± 1.22^{b}	6.81 ± 0.37^{a}
84	42.5 ± 0.5^{c}	4.83 ± 0.01^{g}	26.6 ± 2.29^{ab}	6.31 ± 0.06^{ab}
96	45.1 ± 0.4^{ab}	4.75 ± 0.01^{h}	30.1 ± 1.32^{a}	6.71 ± 0.17^{a}
108	42.2 ± 0.3^{c}	4.70 ± 0.00^{hi}	30.7 ± 0.86^{a}	6.11 ± 0.05^{abc}
120	42.7 ± 0.7^{bc}	4.67 ± 0.01^{i}	30.7 ± 0.42^{a}	6.29 ± 0.06^{ab}
132	44.2 ± 0.9^{abc}	4.68 ± 0.01^{i}	26.5 ± 2.18^{ab}	5.56 ± 0.40^{bc}
144	44.7 ± 1.2^{abc}	4.72 ± 0.01^{hi}	26.5 ± 2.52^{ab}	5.82 ± 0.16^{bc}

n = 3 trials, and analyses in triplicate.
Means (± standard deviations) with different superscripts in the same columns are significantly different (Tukey test, $p<0.05$).
* 24 cm deep.

3.2. Physico-chemical changes during on-farm spontaneous fermentation

Throughout fermentation, there was an increase in temperature from 27 °C, reaching a maximum of 46.6 °C at 72 h, followed by a reduction to 44.7 °C at the end of fermentation (Table 1). The pH decreased significantly throughout fermentation, from 6.64 to 4.72 and the total titratable acidity increased up to 96 h (~30 mEq NaOH 100 g^{-1}) keeping similar values until the end of fermentation. The moisture content changed ($p < 0.05$) throughout fermentation, with values ranging from 5.42 to 6.81 g 100 g^{-1}. All these changes are typical of cocoa fermentation and reassure that cocoa was successfully fermented (Castro-Alayo et al., 2019; Do Carmo Brito et al., 2017; Ho et al., 2014; Rohsius et al., 2006).

Table 2
Changes on the levels of free, total, acidic, hydrophobic, and essential amino acids during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil.

Amino	Levels (mg 100 g^{-1}) during cocoa fermentation (h)												
acids	0	12	24	36	48	60	72	84	96	108	120	132	144
Ala‡	27.4 ± 0.22^{f}	23.4 ± 0.27^{g}	26.9 ± 0.92^{f}	24.6 ± 0.17^{fg}	27.3 ± 0.61^{f}	71.3 ± 0.44^{b}	73.8 ± 0.14^{b}	67.4 ± 0.17^{c}	72.3 ± 1.78^{b}	58.2 ± 0.20^{e}	68.1 ± 0.15^{c}	64.2 ± 1.33^{d}	112 ± 0.49^{a}
Arg	34.0 ± 1.05^{g}	9.11 ± 0.28^{i}	10.1 ± 0.14^{i}	16.0 ± 0.81^{h}	17.5 ± 0.26^{h}	38.5 ± 0.84^{f}	39.1 ± 0.15^{f}	52.4 ± 0.17^{d}	57.1 ± 0.33^{c}	46.4 ± 0.21^{e}	55.1 ± 0.17^{cd}	101 ± 1.86^{b}	133 ± 0.58^{a}
Asp†	17.4 ± 0.28^{j}	37.6 ± 0.29^{i}	46.8 ± 0.12^{g}	43.5 ± 0.37^{h}	54.9 ± 0.33^{f}	75.1 ± 0.30^{b}	0.00 ± 0.00^{l}	0.00 ± 0.00^{l}	116 ± 0.62^{a}	69.2 ± 0.22^{d}	58.5 ± 0.13^{e}	6.70 ± 0.40^{k}	71.1 ± 0.31^{c}
Glu†	86.3 ± 0.76^{h}	114 ± 0.28^{g}	131 ± 1.45^{f}	147 ± 4.70^{e}	152 ± 0.39^{e}	190 ± 1.93^{c}	190 ± 1.93^{c}	192 ± 0.19^{c}	211 ± 1.04^{a}	173 ± 0.21^{d}	203 ± 0.12^{b}	178 ± 3.41^{d}	35.2 ± 0.15^{i}
Gly	5.20 ± 0.31^{f}	7.10 ± 0.18^{e}	7.10 ± 0.18^{de}	7.05 ± 0.10^{de}	7.24 ± 0.38$^{c.d}$	6.99 ± 0.02^{de}	6.93 ± 0.22^{e}	7.10 ± 0.17^{c}	7.09 ± 0.17^{c}	7.13 ± 0.20^{de}	7.07 ± 0.13$^{d.e}$	7.24 ± 0.38^{b}	16.2 ± 0.07^{a}
His*	5.10 ± 0.39^{f}	7.94 ± 0.28^{e}	8.50 ± 0.03^{de}	8.46 ± 0.14^{de}	9.69 ± 0.23$^{c.d}$	9.29 ± 0.01^{de}	10.9 ± 0.14^{c}	10.7 ± 0.16^{c}	10.8 ± 0.69^{c}	8.35 ± 0.21^{de}	9.13 ± 0.13$^{d.e}$	20.6 ± 0.83^{b}	26.5 ± 0.12^{a}
Leu*‡	51.7 ± 0.09^{f}	15.6 ± 0.28^{i}	17.5 ± 0.10^{i}	25.8 ± 0.01^{h}	31.0 ± 0.80^{g}	113 ± 0.71^{d}	106 ± 0.14^{e}	121 ± 0.18^{c}	141 ± 1.24^{a}	107 ± 0.21^{e}	127 ± 0.13^{b}	107 ± 1.40^{e}	120 ± 0.09^{c}
Lys*	28.5 ± 0.08^{e}	4.02 ± 0.27^{g}	6.28 ± 0.43^{g}	10.9 ± 0.57^{f}	13.2 ± 0.80^{f}	41.6 ± 0.08^{c}	38.6 ± 0.14^{d}	47.9 ± 0.17^{b}	54.2 ± 2.22^{a}	45.4 ± 0.21^{b}	51.9 ± 0.13^{a}	52.6 ± 0.49^{a}	41.6 ± 0.05^{c}
Phe*‡	44.8 ± 3.75^{f}	9.92 ± 0.28^{h}	11.2 ± 0.24^{h}	17.4 ± 0.02^{g}	21.0 ± 0.79^{g}	101 ± 0.37^{d}	89.6 ± 0.14^{e}	99.7 ± 0.17^{d}	130 ± 0.04^{b}	90.3 ± 0.22^{e}	107 ± 0.15^{c}	90.0 ± 2.20^{e}	179 ± 0.78^{a}
Pro	17.3 ± 0.16^{g}	18.7 ± 0.28^{g}	20.5 ± 0.41^{f}	23.2 ± 0.47^{d}	27.3 ± 0.45^{c}	33.2 ± 0.67^{a}	32.6 ± 0.16^{ab}	28.0 ± 0.17^{c}	31.3 ± 0.58^{b}	22.6 ± 0.21^{de}	27.9 ± 0.13^{c}	21.1 ± 0.70^{ef}	28.2 ± 0.17^{c}
Ser	42.0 ± 1.75^{h}	59.2 ± 0.26^{ef}	56.8 ± 0.64^{fg}	45.0 ± 0.52^{g}	61.0 ± 0.84^{e}	79.0 ± 0.72^{a}	31.0 ± 0.14^{i}	54.1 ± 0.17^{g}	75.9 ± 1.43^{b}	65.7 ± 0.20^{d}	69.9 ± 0.13^{c}	56.2 ± 0.00^{g}	17.5 ± 0.08^{j}
Thr*	11.9 ± 0.27^{g}	8.69 ± 0.28^{i}	9.59 ± 0.06^{hi}	13.2 ± 0.70^{g}	14.2 ± 0.41^{g}	27.3 ± 0.90^{de}	28.5 ± 0.14^{cde}	29.2 ± 0.19^{cd}	31.2 ± 1.98^{c}	22.9 ± 0.21^{f}	26.1 ± 0.15^{e}	37.8 ± 0.79^{b}	47.3 ± 0.21^{a}
Tyr‡	26.9 ± 0.94^{d}	22.6 ± 0.28^{de}	27.0 ± 0.61^{d}	27.5 ± 1.21^{d}	31.0 ± 0.09^{d}	69.3 ± 12.57^{a}	56.9 ± 0.14^{bc}	59.2 ± 0.17^{abc}	71.4 ± 1.97^{a}	52.4 ± 0.21^{c}	62.0 ± 0.13^{abc}	29.1 ± 1.56^{d}	10.6 ± 0.05^{e}
Val‡	25.2 ± 0.11^{gh}	18.5 ± 0.19^{i}	22.0 ± 1.27^{hi}	25.6 ± 1.36^{g}	30.9 ± 1.14^{f}	66.1 ± 0.55^{b}	60.1 ± 0.17^{c}	56.9 ± 0.20^{cd}	79.4 ± 2.11^{a}	55.8 ± 0.19^{d}	64.7 ± 0.13^{b}	48.1 ± 0.61^{e}	10.2 ± 0.04^{j}
Total	423 ± 10.2^{fg}	356 ± 3.09^{h}	401 ± 6.60^{g}	435 ± 11.0^{f}	499 ± 7.50^{e}	922 ± 20.1^{b}	764 ± 3.70^{d}	826 ± 2.20^{c}	1090 ± 16.2^{a}	824 ± 3.00^{c}	937 ± 1.90^{b}	820 ± 15.9^{c}	849 ± 3.20^{c}
Acidic†	104 ± 1.00^{i}	152 ± 0.56^{h}	178 ± 1.57^{g}	191 ± 5.07^{ef}	207 ± 0.72^{d}	265 ± 2.23^{b}	190 ± 1.93^{ef}	192 ± 1.66^{e}	327 ± 0.95^{a}	242 ± 0.42^{c}	261 ± 0.26^{b}	185 ± 2.26^{f}	106 ± 0.46^{i}
Hyd.‡	176 ± 5.10^{g}	90.1 ± 1.40^{j}	105 ± 3.10^{j}	121 ± 2.80^{i}	141 ± 3.40^{h}	421 ± 14.6^{b}	387 ± 0.70^{d}	404 ± 0.80^{c}	494 ± 7.10^{a}	363 ± 1.50^{e}	428 ± 0.60^{b}	338 ± 7.10^{f}	433 ± 1.50^{b}
Ess.*	167 ± 4.69^{d}	64.7 ± 1.58^{e}	75.1 ± 2.13^{e}	101 ± 2.80^{e}	120 ± 4.10^{de}	359 ± 2.62^{c}	334 ± 0.87^{c}	365 ± 1.07^{c}	447 ± 8.28^{a}	329 ± 1.25^{c}	385 ± 0.82^{bc}	356 ± 6.32^{c}	425 ± 1.29^{ab}

n = 3 fermentation trials.
Mean values ± standard deviation (dry weight basis) with different superscripts in the same line are significantly different (Tukey test, $p<0.05$).
Ala – alanine, Arg – arginine, Asp – Aspartic acid, Glu – glutamic acid, Gly – glycine, His – histidine, Leu – leucine, Lys – lysine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine.
† Acidic.
‡ Hyd. – hydrophobic.
* Ess. – essential amino acids.

The increase in temperature is typical of spontaneous fermentation and results from the release of heat during anaerobic fermentation, and from the metabolism of ethanol by AAB bacteria. The increase in titratable acidity and concomitant decrease in pH are due to the acids produced during fermentation – lactic and acetic acids resulting, respectively, from LAB and AAB activities. The latter takes place after revolving the cocoa mass, which started at 48 h fermentation, as it allowed aeration favoring growth and activity of AAB (do Carmo Brito et al., 2017). Ethanol, produced from sugars by yeast, and acetic acid diffuse into the beans causing death of the embryo and cell wall breakdown with liberation of endogenous enzymes (Barišíc et al., 2019; Castro-Alayo et al., 2019; Roshius et al., 2006). There is activation of endogenous proteolytic enzymes (carboxypeptidase and aspartic endo-protease), releasing free amino acids and oligopeptides (Barišíc et al., 2019; Castro-Alayo et al., 2019). There is also activation of amino acid decarboxylases resulting in the formation of amines (Barišíc et al., 2019; Do Carmo Brito et al., 2017; Spizzirri et al., 2016).

3.3. Amino acids changes during on-farm spontaneous fermentation

The same free amino acids detected in the unfermented cocoa (Table 2), were found throughout fermentation; however, the contents varied significantly. At the beginning of the anaerobic phase, up to 12 h fermentation, there was a significant decrease (16 % loss) on total levels of free amino acids. During this period of time, there was a decrease in some individual amino acids including arginine, leucine, lysine, phenylalanine, and threonine, all of them essential amino acids (Spizzirri et al., 2019). Some amino acids did not change at this period (alanine, proline, tyrosine and valine), whereas all of the others increased. These changes in amino acids during anaerobic fermentation are probably associated with nitrogen and carbon metabolism by the growing LAB and yeast (Castro-Alayo et al., 2019; Liu et al., 2020).

Afterwards, there was an increase on total levels of amino acids, reaching higher values ($p < 0.05$) at 60 h (2.2-fold higher than initial levels) and again at 96 h fermentation (2.6-fold higher). At the end of fermentation, total levels were higher compared to initial levels; but lower compared to the high levels observed at 60 and 96 h fermentation. At 60 h fermentation, there was a significant increase on the levels of every amino acid with higher increases observed for aspartic acid (4.3-fold), followed by alanine, glutamic acid, leucine, phenylalanine, threonine, tyrosine and valine (2.2–2.6-fold). At this point, there was prevalence of glutamic acid (21 %), followed by leucine (12.3 %), phenylalanine (11 %), serine (9%) and aspartic acid (8%), the other amino acids contributing with less than 8% of total levels (Fig. 1).

At 96 h fermentation, the highest increases in individual amino acids were for aspartic acid (6.7-fold compared to initial levels), followed by

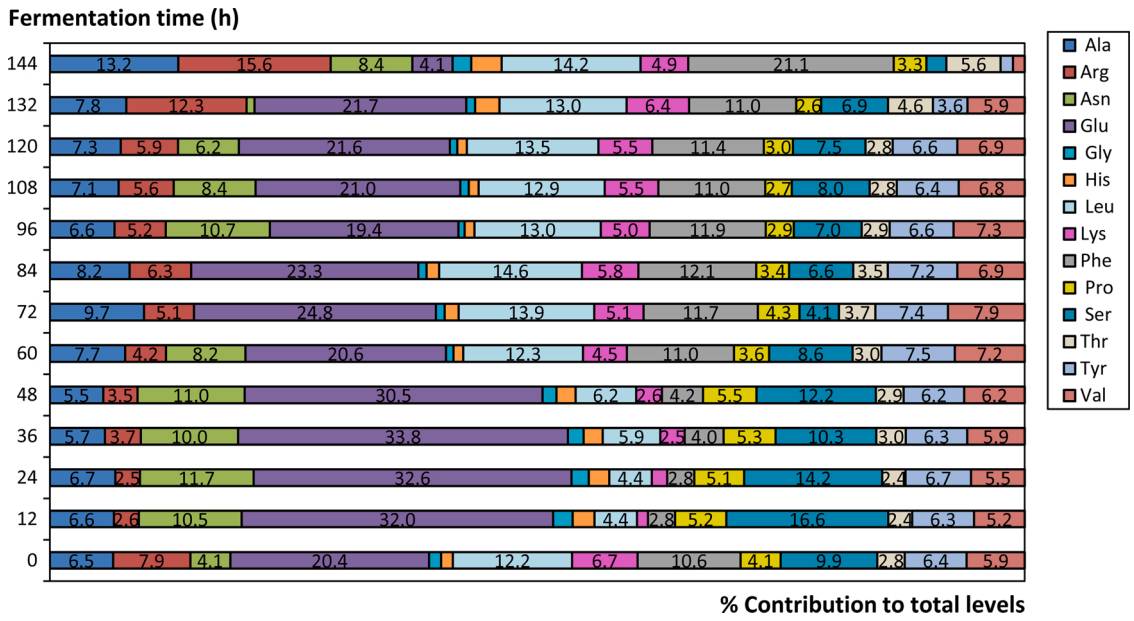

Fig. 1. Percent contribution of each amino acid to the total contents during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil. Ala – alanine, Arg – arginine, Asn – Asparagine, Glu – glutamic acid, Gly – glycine, His – histidine, Leu – leucine, Lys – lysine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine.

Table 3
Changes on the profile and levels of free bioactive amines during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil.

Fermentation time (h)	Mean levels ± standard deviation (mg kg^{-1})						
	Spd	Put	Srt	Trm	Tym	Phm	Total
0	13.5 ± 0.31^{ab}	1.63 ± 0.10^{b}	8.85 ± 0.31^{a}	1.44 ± 0.10^{cde}	5.65 ± 0.03^{a}	nd	30.9 ± 0.16^{a}
12	11.5 ± 0.45^{cd}	0.68 ± 0.04^{de}	8.01 ± 0.10^{b}	1.24 ± 0.06^{ef}	1.50 ± 0.06^{e}	nd	22.9 ± 0.38^{cde}
24	10.4 ± 0.38^{de}	0.83 ± 0.09^{cde}	4.50 ± 0.11^{d}	2.17 ± 0.22^{ab}	0.75 ± 0.04^{g}	nd	19.0 ± 1.18^{fg}
36	7.51 ± 0.08^{f}	0.55 ± 0.03^{e}	4.47 ± 0.11^{d}	1.78 ± 0.02^{bc}	0.92 ± 0.06^{fgk}	nd	14.9 ± 0.73^{i}
48	13.1 ± 0.69^{ab}	1.28 ± 0.21^{bcd}	5.25 ± 0.22^{c}	2.45 ± 0.03^{a}	1.35 ± 0.14^{df}	nd	23.4 ± 0.37^{bcd}
60	10.2 ± 0.41^{de}	1.17 ± 0.22^{bcd}	3.25 ± 0.33^{e}	1.12 ± 0.11^{efg}	3.44 ± 0.05^{bc}	1.66 ± 0.17^{d}	20.8 ± 0.81^{defg}
72	10.0 ± 0.36^{e}	1.30 ± 0.09^{bcd}	nd	1.26 ± 0.05^{def}	3.75 ± 0.11^{b}	4.06 ± 0.10^{ab}	20.4 ± 0.69^{efg}
84	10.8 ± 0.70^{de}	1.35 ± 0.24^{bc}	nd	1.31 ± 0.48^{cde}	3.45 ± 0.22^{bc}	4.20 ± 0.25^{ab}	21.1 ± 1.62^{def}
96	14.4 ± 0.14^{a}	1.80 ± 0.45^{b}	nd	1.08 ± 0.13^{efg}	3.53 ± 0.21^{b}	4.51 ± 0.07^{a}	25.7 ± 1.27^{b}
108	11.3 ± 0.69^{cde}	1.52 ± 0.13^{b}	nd	1.03 ± 0.02^{efg}	3.72 ± 0.27^{b}	3.86 ± 0.18^{b}	21.4 ± 0.70^{def}
120	12.4 ± 0.24^{bc}	3.33 ± 0.30^{a}	nd	1.76 ± 0.14^{bcd}	3.32 ± 0.30^{bc}	4.39 ± 0.20^{ab}	25.2 ± 0.35^{bc}
132	8.26 ± 0.44^{f}	1.39 ± 0.22^{bc}	nd	0.73 ± 0.12^{g}	3.01 ± 0.02^{cd}	2.67 ± 0.33^{c}	16.1 ± 0.96^{hi}
144	9.92 ± 0.67^{e}	1.62 ± 0.25^{b}	nd	0.78 ± 0.09^{fg}	2.68 ± 0.25^{d}	3.16 ± 0.25^{c}	18.2 ± 1.30^{gh}

n = 3 fermentation trials.
nd (not detected, < limit of quantification – 0.141 and 0.097 mg kg^{-1} for serotonin and phenylethylamine, respectively).
Mean values (dry weight basis) with different superscripts in the same column are significantly different (Tukey test, $p<0.05$).
Spd – spermidine, Put – putrescine, Srt – serotonin, Trm – tryptamine, Tym – tyramine, Phm – phenylethylamine.

valine (3.2-fold). This fermentation time was characterized by prevalence of glutamic acid (19 %), followed by leucine (13 %), phenylalanine (12 %), aspartic acid (11 %), valine (7.3 %) and serine (7%), and others with less than 6.6 % of total (Fig. 1), similar to that at 60 h, but with increased valine levels.

At the end of fermentation (144 h), total amino acids levels were lower compared to 60 h and 96 h fermentation, but significantly higher compared to the unfermented cocoa. Most of the individual amino acids were present at higher levels compared to initial levels, including histidine (5.2-fold), alanine, arginine, aspartic acid, phenylalanine, and threonine (4-fold), followed by glycine (3.1-fold), leucine (2.3-fold), lysine and proline (1.5-fold). However, the levels of glutamic acid, serine, tyramine and valine, were only 60 % of initial levels. Fermented cocoa (144 h) was characterized by predominance of phenylalanine (21 %), followed by arginine (16 %), leucine (14 %), alanine (13.2 %), aspartic acid (8.4 %); the others with less than 5% of total levels (Fig. 1). Based on these results, there was a significant increase on total (2.0-fold), essential and hydrophobic amino acids (2.5-fold each), whereas acidic amino acids remained the same (Table 2). At the end of fermentation hydrophobic and acidic amino acids represented 51.0 and 12.5 % of total levels, respectively.

The increase on total free amino acids during aerobic fermentation (after 48 h) can be due to microbial metabolism – AAB and yeast (Castro-Alayo et al., 2019; Liu et al., 2020). In addition, as the pH decreases and the temperature increases, there is activation of endogenous and microbial proteolytic enzymes. Aspartic endoprotease splits cocoa proteins into hydrophobic peptides, which are hydrolyzed by carboxypeptidase releasing predominantly hydrophobic amino acids, e.g., phenylalanine, alanine and leucine (Marseglia et al., 2014; Spizzirri et al., 2019; Yusep et al., 2002).

Based on the results, fermentation improved the levels of free essential and hydrophobic amino acids. Even though different studies showed a different profile of some amino acids after fermentation, they are unanimous in reporting the prevalence of phenylalanine, leucine and alanine (Adeyeye et al., 2010; Ho et al., 2014; Kumari et al., 2018; Marseglia et al., 2014; Rohsius et al., 2006). The liberation of amino

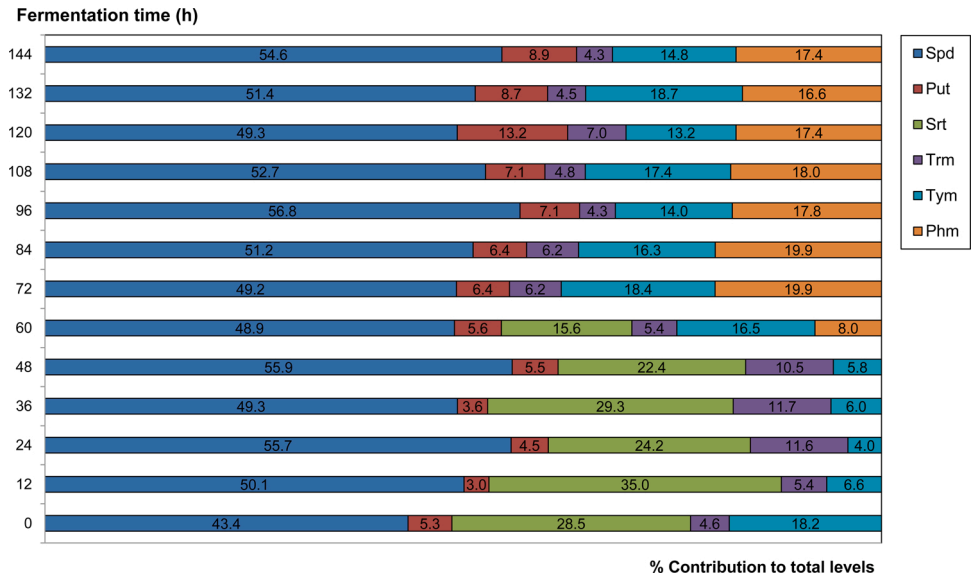

Fig. 2. Percent contribution of each bioactive amine to the total contents during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil. Spd – spermidine, Put – putrescine, Srt – serotonin, Trm – tryptamine, Tym – tyramine, Phm – phenylethylamine.

acids during fermentation is important because these compounds are essential for the formation of cocoa flavors through Maillard reaction and Strecker degradation during drying and roasting (Barisi et al., 2019; Castro-Alayo et al., 2019; Rohsius et al., 2006; Spizzirri et al., 2019). The free hydrophobic amino acids, especially leucine, alanine and phenylalanine, are desirable as they are precursors of 3-methylbutanal and phenylacetaldehyde with honey, green and floral notes (Barišíc et al., 2019; Castro-Alayo et al., 2019; Spizzirri et al., 2019). Leucine and glucose produce aromatic notes described as sweet chocolate. In addition, linalool, which is responsible for fine chocolate aroma, can be produced by *S. cerevisiae* from leucine catabolism (Castro-Alayo et al., 2019).

On the other hand, some amino acids, mainly essential amino acids are metabolized by microorganisms during fermentation, which explains the amino acids loss in the anaerobic fermentation (first 48 h) and after 96 h fermentation (Barišíc et al., 2019; Liu et al., 2020). Amino acids can also condensate with phenolic compounds (Barišíc et al., 2019; De Taeye et al., 2017). Furthermore, amino acids can be decarboxylated by microbial enzymes (amino acids decarboxylases) liberating biogenic amines (do Carmo Brito et al., 2017; Spizzirri et al., 2019).

The decreased glutamic acid levels in fermented cocoa might be disadvantageous as this amino acid is considered umami, desirable in food products. In addition, when considering sweet (alanine, glycine, proline, serine and threonine) and bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine) amino acids, from 60 h up to 144 h fermentation, total sweet amino acids doubled, whereas total bitter amino acids doubled at 60 h and tripled at 144 h fermentation, which can affect cocoa and chocolate aroma. However, the impact of roasting on the levels of these amino acids, as well as the impact of these amino acids in the final product must be ascertained (Zyzelewicz et al., 2018; Delgado-Ospina et al., 2020).

Therefore, the levels of free amino acids varied significantly throughout fermentation, and the understanding of these changes can be used in the modulation of cocoa processing to obtain cocoa and chocolate with unique flavor and health promoting properties. In addition, it will allow introduction of the low-processed food concepts, a recent trend in food science to answer to the new consumers demand (Zyzelewicz et al., 2018).

3.4. Bioactive amines changes during on-farm fermentation

During fermentation, six amines were detected (Table 3), four of them were found throughout fermentation (spermidine, tyramine, tryptamine and putrescine), whereas serotonin was only detected up to 72 h, and phenylethylamine was detected from 60 h fermentation on. In our previous study (do Carmo Brito et al., 2017), spermidine, tryptamine and tyramine were detected, but spermine was also present and putrescine, serotonin and phenylethylamine were not during laboratory fermentation of Forastero Amazonian cocoa. Based on these results, the occurrence of amines in fermented cocoa can vary within genotype, origin, production practices and fermentation conditions.

During fermentation, there were significant changes on total levels, as well as on individual amines. Total levels were higher at 96–120 h, but, at the end of fermentation, they were only 59 % of initial levels. Significantly higher spermidine levels were observed at 48 and 96 h; however, the fermented cocoa had lower levels compared to unfermented (26 % loss). The levels of putrescine decreased during the first 36 h, increased up to 120 h, and, at the end of fermentation, it reached values like initial levels. The levels of serotonin decreased continuously and were not detected at 72 h fermentation. Tryptamine levels were higher at 24–48 h fermentation, decreasing afterwards. The levels at the end of fermentation represented 45.8 % loss from initial levels. Tyramine levels decreased significantly (24–36 h fermentation), increased (60–120 h), and, at the end of fermentation, the levels were 52.6 % lower from initial levels. However, phenylethylamine, which was not detected in the unfermented cocoa, was first detected at 60 h, increased (96–120 h), decreasing at the end of fermentation to 3.16 mg/kg. The fermented cocoa (Fig. 2) had predominance of spermidine (54.6 %), followed by phenylethylamine (17.4 %), tyramine (14.8 %), putrescine (8.9 %) and tryptamine (4.3 %). When comparing these changes with those from our previous laboratory studies (Do Carmo Brito et al., 2017), they were unanimous in showing a decrease in tyramine levels and an initial increase followed by a decrease in tryptamine.

Tyrosine and phenylalanine are the amino acid precursors for tyramine and phenylethylamine, respectively. At the beginning of fermentation, there was no change on these amino acids, but a decrease on pre-existing amines. From 48 h fermentation on, there were increases on both amino acids and amines levels. The highest levels of tyrosine and phenylalanine were observed 48 h before the highest levels of the amines were reached. At the end of fermentation, both phenylalanine

and phenylethylamine were 3-fold higher than initial levels, whereas tyrosine and tyramine were 40 % of initial levels.

When evaluating the influence of fermentation on bioactive amines, it is important to consider the health promoting and adverse effects associated with them. Even though tyramine levels increased significantly during fermentation, the levels in the fermented cocoa are below the no observed adverse effect levels (NOAEL) for healthy individuals; however, for those taking classical MAOI drugs the levels found could be of concern (EFSA (European Food Safety Authority), 2011).

During fermentation, amines are formed from free amino acids, through microbial decarboxylase activity. The increase on free amino acids was demonstrated in this study and several microorganisms associated with cocoa fermentation (yeasts, LAB and AAB) are known to possess amino acid decarboxylase activity. The pH decrease during fermentation induces amine formation as a protection mechanism against the acid medium (Do Carmo Brito et al., 2017; Nielsen et al., 2015; Restuccia et al., 2019; Spizzirri et al., 2019). Moreover, contaminating bacteria, which can be introduced during processing, can also decarboxylate amino acids providing an additional source of amines (Do Carmo Brito et al., 2017; Restuccia et al., 2019; Spizzirri et al., 2019). Therefore, fermentation of cocoa beans favors amines formation. However, there can also be degradation of amines present in fermented cocoa because some microorganisms metabolize amines (Castro-Alayo et al., 2019; Do Carmo Brito et al., 2017).

Based on these results, fermentation affected amine profile and the possibility of modulating the formation of amines during fermentation is a promising tool to achieve high quality chocolate with respect to functional properties and safety.

3.5. *Multivariate analysis of changes occurring during cocoa fermentation*

Multivariate analysis of auto scaled data (temperature, pH, total titratable acidity, moisture, free amino acids and free bioactive amines) indicated that a two-principal component (PC) model explained 75 % of the variance. The first – PC1 explained 55 % of the variance (Fig. 3a and b) and it differentiated unfermented cocoa up to 48 h fermentation (negative values) from fermented cocoa beans from 60 to 144 h (positive values). The first group, corresponding to the anaerobic fermentation, was characterized by higher pH and higher levels of the free amines tryptamine and serotonin. The second group included longer fermentation times, after aeration of the cocoa mass. It was characterized by higher temperature and higher total titratable acidity and higher levels of the free amino acids - leucine, phenylalanine, lysine, alanine, threonine, followed by arginine, proline and valine; and by the higher levels of the free bioactive amines - phenylethylamine, putrescine, and tyramine. This behavior confirms that from 60 h fermentation on, the increases on acidity and temperature favored proteolytic activity and liberation of amino acids from protein hydrolysis. The amino acids liberated are mainly the hydrophobic ones, which result from aspartic endopeptidase and carboxypeptidase activities (Crafack et al., 2014; Yusep et al., 2002).

PC2 explained 20 % of the variance and it differentiated the fermented beans (60–144 h) into two groups, separating 144 h fermentation from the remaining. From 60–132 h fermentation, cocoa was characterized by high levels of the amino acids serine, glutamine, tyrosine and valine and the amines spermidine, whereas at 144 h there was higher levels of glycine, histidine and arginine.

The HCA dendrogram (Fig. 3c) confirmed the existence of three clusters: (i) unfermented and fermented beans up to 48 h; (ii) fermented beans from 60 to 132 h, and (iii) 144 h fermented beans. The first – unfermented and anaerobic fermentation – with higher pH and prevalence of the bioactive amines serotonin and tryptamine; the second – actual fermentation – with higher temperatures and total titratable acidity and prevalence of hydrophobic amino acids; and, the third cluster – fermented cocoa – with predominance of the amino acids glycine and histidine, followed by arginine, threonine and alanine and

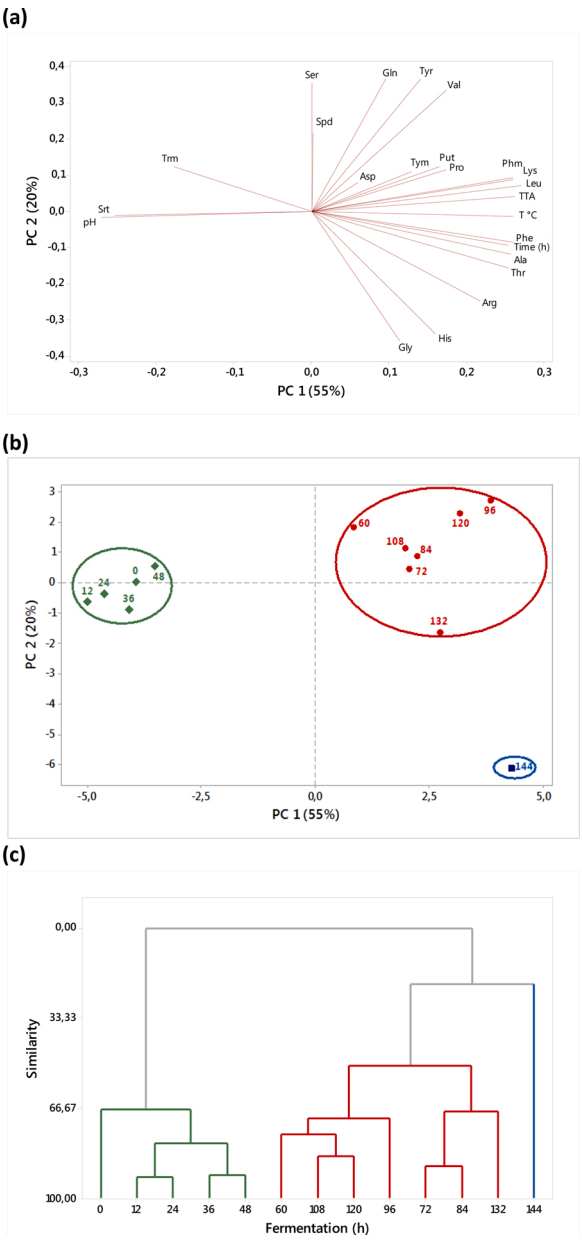

Fig. 3. Separation of cocoa beans by changes on amino acids, bioactive amines, pH and total titratable acidity during on-farm spontaneous fermentation of cocoa beans from Bahia, Brazil: (a) Variable projection by Principal Component Analysis (PCA), (b) Scatterplot for cocoa beans during each fermentation day by PCA with suggested grouping, in accordance with Hierarchical Cluster Analysis (HCA), and (c) dendrogram by HCA analysis. TTA – total titratable acidity, pH, C – temperature °C, Ala – alanine, Arg – arginine, Asp – Aspartic acid, Glu – glutamic acid, Gly – glycine, His – histidine, Leu – leucine, Lys – lysine, Phe – phenylalanine, Pro – proline, Ser – serine, Thr – threonine, Tyr – tyrosine, Val – valine, Spd – spermidine, Put – putrescine, Srt – serotonin, Trm – tryptamine, Tym – tyramine, Phm – phenylethylamine. Fermentation time: (♦) 0, 12, 24, 36, 48 h; (●) 60, 72, 84, 96, 108, 120, 132 h; and (■) 144 h.

the amine phenylethylamine. In this way, each one of the clusters would have specific functional properties, e.g., the first with high levels of amines with neuroactive and antioxidant properties (Spizzirri et al., 2019; Yılmaz and Gökmen, 2020); the second, with hydrophobic amino acids, relevant for cocoa flavor formation (Adeyeye et al., 2010; Rohsius et al., 2006) and with high levels of spermidine, which has antioxidant and intestinal mucosa maturation (Kalač, 2014; Madeo et al., 2018); and the last with higher levels of the amine with mood modulation activity –

phenylethylamine (Irsfeld et al., 2014; Yılmaz and Gökmen, 2020).

Therefore, multivariate analysis using the contents of free bioactive amines and amino acids can be a tool in the differentiation of fermentation time. The knowledge of these changes throughout fermentation is needed to follow fermentation and to assure its quality. It can predict the potential quality, safety and functional properties of cocoa and products.

4. Conclusion

The changes on amino acids and bioactive amines, simultaneously, during cocoa on-farm fermentation were investigated for the first time. Fermentation followed its due course: there were a significant decrease in pH and increases on temperature and titratable acidity. During fermentation, the levels of total amino acids increased, and total amines decreased. The levels of amino acids varied along fermentation, decreasing in the first 12 h, increasing up to 96 h fermentation, decreasing until the end. At 96 h fermentation, the highest levels amino acids were observed. At the end of fermentation, there were higher levels of total, hydrophobic and several individual amino acids (except glutamic acid, serine, tyrosine and valine). Umami amino acids decreased whereas bitter and sweet ones increased. Among the six amines detected during cocoa fermentation, the levels of spermidine, serotonin, tryptamine and tyramine decreased; putrescine did not change, whereas phenylethylamine was formed and the levels increased, which is relevant due to the role of this amine in mood modulation and prevention of neurological diseases. Multivariate analysis (PCA and HCA) differentiated three clusters: (i) unfermented and fermented cocoa up to 48 h - high levels of serotonin, tryptamine and pH; (ii) 60–132 h - high levels of lysine, leucine, phenylalanine, alanine, threonine, phenylethylamine and titratable acidity; and (iii) 144 h fermented cocoa - high levels of glycine, histidine and arginine. Based on these results, by blending samples from different fermentation times, unique functional properties and sensory qualities can be warranted to chocolate.

CRediT authorship contribution statement

Valterney L. Deus: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **Eliete S. Bispo:** Conceptualization, Funding acquisition, Writing - review & editing. **Adriana S. Franca:** Data curation, Writing - review & editing. **Maria Beatriz A. Gloria:** Conceptualization, Methodology, Investigation, Writing - review & editing, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2020.103776.

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