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Influence of cocoa clones on the quality and functional properties of chocolate – Nitrogenous compounds

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ABSTRACT

Cloning techniques have been used to improve agronomical traits and to answer to the new demand for fine chocolate. However, scarce information is available regarding their impact on chocolate quality. This study investigated amino acids and bioactive amines in nine cocoa clones and their impact on quality, safety and functional properties of chocolates. Most of the clones had 18 out of the 20 amino acids investigated (five lacked methionine, one phenylalanine and another alanine, proline and valine). Total levels varied from 28.57 to 84.5 g/kg and ~45% were essential amino acids. Bitter amino acids represented 55%, sweet 19% and umami 2% of total. Spermidine, putrescine, tryptamine, tyramine and phenylethylamine were present in all samples; agmatine was detected in five, cadaverine in eight, histamine in seven, and serotonin in six samples. Total levels varied from 9.10 to 44.09 mg/kg. The presence of spermidine, tryptamine, agmatine and phenylethylamines is relevant due to health promoting properties. Histamine and tyramine levels are not enough to cause adverse effects. Multivariate analyses separated the chocolates into five clusters. This information is relevant for further agronomical studies and in the formulation of cocoa blends for chocolate with unique and optimized nutritional, functional, safety and sensory qualities.

1. Introduction

Chocolate is an indulgent confection, much appreciated due to its sensory quality. Chocolate provides lipids, proteins and minerals (potassium, magnesium, copper and iron) (Arunkumar & Jegadeeswari, 2019; Barišić et al., 2019; Katz, Doughty, & Ali, 2011). It also provides functional and sensory properties which can be attributed mostly to phenolic and nitrogenous compounds including amino acids and bioactive amines (Do Carmo Brito, Chisté, Pena, Gloria, & Lopes, 2017; Barišić et al., 2019; Spizzirri et al., 2019). Phenolic compounds are widely investigated and known to affect antioxidant activity and sensory properties of chocolate (Katz et al., 2011); whereas scarce information is available regarding amino acids and bioactive amines. Some amino acids are building blocks for proteins in the body, thereby fundamental for recovery and survival. In addition, free amino acids are responsible for the characteristic taste (umami, sweet, bitter, acidic) of chocolate (Adeyeye, Akinyeye, Ogunlade, Olaofe, & Boluwade, 2010). Bioactive amines can impart beneficial effects to human health. Spermidine has antioxidant activity (Kalač, 2014; Do Carmo Brito et al., 2017); and phenylethylamine, tryptamine and serotonin are neuroactive (Vadodaria, Stern, Marchetto, & Gage, 2017; Yılmaz & Gökmen, 2020). However, histamine and tyramine at high concentrations can cause adverse effects to human health, especially in sensitive individuals (EFSA, 2011; Silva et al., 2020).

Some free amino acids and bioactive amines are inherent to unfermented cocoa, but they can change during processing. According to previous studies (Adeyeye et al., 2010; Barišić et al., 2019; Crafack et al., 2014; Gloria, Deus, & Bispo, 2019; Marseglia, Palla, & Caligiani, 2014), unfermented Forastero cocoa had 14 free amino acids. During on-farm fermentation, total amino acids increased 2-fold, with higher increases

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for histidine, alanine, arginine, asparagine, phenylethylamine and threonine. Overall, there was predominance of acidic amino acids in unfermented cocoa, and hydrophobic ones in the fermented product (phenylalanine, leucine and alanine). Free amino acids are relevant as they are precursors for the Maillard reaction which takes place during roasting, leading to the characteristic chocolate flavor; and free amino acids remaining in the chocolate contribute to taste characteristics (Adeyeye et al., 2010; Castro-Alayo, Idrogo-Vásquez, Siche, & Cardenas-Toro, 2019; John et al., 2016).

Amino acids are also precursors of free bioactive amines. Some amines are inherent to unfermented cocoa, but they change during fermentation and roasting. Unfermented Forastero cocoa from Bahia, Brazil, had spermidine, serotonin, tyramine, putrescine and tryptamine (total of 30.87 mg/kg). During on-farm fermentation, total amines, spermidine, tryptamine, tyramine and serotonin decreased; and phenylethylamine was produced and accumulated (Gloria, Deus, & Franca, 2019).

Chocolate is consumed worldwide, with values as high as 8.8 kg per capita/year (Statista, 2020). Cocoa, the key raw material for chocolate manufacturing, is a cash crop of great economic significance (Katz et al., 2011). During most of the twentieth century, Brazil occupied the second position in cocoa production. However, the witches' broom disease, caused by the fungus *Moniliophtora perniciosa*, drastically reduced production (Cruz, Fontes, Leite, Soares, & Bispo, 2013; De Souza, Monteiro, Ferreira, Gramacho, & Luz, 2018), with serious socio-economic and ecological consequences. The development of witches' broom resistant clones, with durable resistance and desirable agronomical traits, has been undertaken by CEPLAC (Comissão Executiva de Planejamento da Lavoura Cacaueira, Ilhéus, Bahia). To date, several clones have been released to farmers; however, additional research is needed for the chemical characterization of the beans to assure its suitability to produce good quality chocolate (Cruz et al., 2013). Furthermore, with the recent popularity of chocolate with higher cocoa percentage, seeds with specific characteristics are needed (ICCO, 2019).

The present study aimed at evaluating the functional, safety and sensory potential of nine cocoa beans clones regarding amino acids and bioactive amines. The cocoa clones were produced and submitted to the same post-harvest procedures, including fermentation and roasting; and used to make 70% cocoa chocolate at the same farm (Bahia, Brazil). The chocolates were analyzed for free amino acids and bioactive amines, and multivariate approaches - principal component analysis and hierarchical cluster analysis were used to separate the clones according to these characteristics.

2. Material and methods

2.1. Reagents

The reagents were of analytical grade, except high performance liquid chromatography – HPLC solvents. Ultrapure-water was from Milli-Q Plus (Millipore Corp., Milford, MA, USA). Bioactive amines (putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, cadaverine dihydrochloride, serotonin hydrochloride, histamine dihydrochloride, tyramine hydrochloride, tryptamine, 2-phenylethylamine hydrochloride), ammonium chloride, and L-amino acids (aspartic acid, serine, asparagine, glycine, glutamic acid, glutamine, histidine hydrochloride, threonine, arginine hydrochloride, alanine, proline, cystine, tyrosine, valine, methionine, lysine hydrochloride, leucine, isoleucine, phenylalanine and norvaline) standards were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). AccQ.FluorTM pre-column derivatization kit was from Waters (Milford, MA, USA). Analytical grade reagents were purchased from Merck (Rio de Janeiro, RJ, Brazil) and Hexis Científica (Jundiaí, SP, Brazil).

2.2. Raw material and chocolate production

Cocoa samples were obtained from a farm located in the South of Bahia, Brazil (14°41′96 "S and 39°12′109″ W) and the chocolates were prepared in the chocolate production facility at the same farm. The cocoa seeds were from nine different clones (Table 1) from CEPLAC based on resistance to witches' broom disease, productivity, technological quality and potential for high quality fine cocoa: TSH 1188, BN 34, PH 16, CEPEC 2002 (CEPEC), CCN 51, Pará/Parazinho (Pará/P), Ipiranga, PS 1319 and SR 162 Catongo (Catongo). The different clones were submitted to similar post-harvest processing conditions, including fermentation, drying, and roasting; however, during fermentation, the times and temperatures varied depending on the clone (Table 1). Chocolate was prepared following standard procedures using the same formulation for all chocolates – 70% cocoa (67 g/100 g nibs and 3 g/100 g cocoa butter), 29.6 g/100 g sucrose and 0.4 g/100 g soy lecithin. After fermentation, cocoa was sun dried (3 days), roasted in a rotating electric roaster (cylindrical roaster, Jaf Inox, Tambaú, SP, Brazil) from 90 to 120 °C for 80 min, peeled for peel and germ removal. The resulting nibs were ground (FX grinder Jaf Inox, Tambaú, SP, Brazil) with sugar, and the mixture was refined in a five-roll mill (MX roll refiner, Jaf Inox, Tambaú, SP, Brazil), producing a cocoa paste with 20 μm granulation. The refined paste was transferred to a longitudinal conche (JAF Inox, Tambaú, SP, Brazil) at 70 °C for 24 h, for conching. Cocoa butter and lecithin were added. The chocolate was transported to a tempering wheel (JAF Inox, Tambaú, SP, Brazil) where it was kept until reaching 42 °C. The chocolate was molded in rectangular polyethylene molds, packaged and kept at −18 °C until analyses.

2.3. Analysis of amino acids and bioactive amines by ultra-high-performance liquid chromatography (UHPLC)

Free amino acids and amines were extracted from samples by three successive extractions of 5 g ground chocolate with 7 mL 5 g/100 mL trichloroacetic acid followed by centrifugation at 11,180 *g* at 4 °C/10 min (Do Carmo Brito et al., 2017). The internal standard norvaline (25 pmol in 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/10 min, neutralized with an equal volume of 1 mol/L NaOH and derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl – AQC (Marseglia et al., 2014).

The levels of free amino acids and amines were determined simultaneously using a Waters Acquity™ UPLC system (Waters, Milford, MA, USA) equipped with an AcquityTM tunable ultra-violet (TUV) detector (249 nm). A BEH C18 column (50 × 2.1 mm, 1.7 μm, Acquity UPLC™) and a gradient elution of A – 0.1 mol/L sodium acetate (pH 4.80) and B – acetonitrile was used: initial–2.5 min/0–0% B; 2.8–4.5 min/0–3% B; 4.5–10.0 min/3–30% B; 10.0–11.0 min/30–100% B; 11.0–11.75 min/100–100% B; 11.75–12.5 min/100–0% B, and further re-equilibration at initial conditions for another 2.5 min. The concentrations of amines and amino acids were calculated by interpolation in analytical curves (Table 2).

2.4. Statistical analysis

Analysis were undertaken in duplicate. The results were submitted to one-way ANOVA and the means were compared by the Tukey test at 5% significance (Minitab® 17.3.1). Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for the characterization of chocolate from the different clones. In PCA, the contents of individual free amino acids, amines and total levels were active variables in the derivation of the principal components. The supplementary variable chocolates from the different cocoa clones were projected onto the factor space. The dendrogram for HCA analyses was obtained by clustering the same variables used for PCA (Minitab® 16.2.3).

Table 1
Characteristics of the nine different Theobroma cacao clones resistant to witches' broom disease used for chocolate production (Bahia, Brazil).

Clone	Characteristics				
	Origin	Type	Fruit color/format	Seed color	Fermentation time (h)/temp (°C)
CEPEC 2002	Brazil	Forastero hybrid	yellow	Purple + white	131/49
BN 34	Brazil	Trinitarian	red	Purple	159/50
Ipiranga	Brazil	Trinitarian hybrid	red	Purple	156/50
Pará/Parazinho	Brazil	Forastero	yellow	Purple	156/49
CCN 51	Ecuador	Crossing ICSAS x IMC67	red	Purple	159/48
SR162 Catongo	Brazil	Catongo	yellow	White	159/48
TSH 1188	Trinidad &Tobago	Trinitarian hybrid	reddish	Purple	132/47
PS 1319	Brazil	Trinitarian hybrid	reddish	Purple	144/48
PH 16	Brazil	Forastero hybrid	reddish	Purple	156/48

* All of the 9 clones were cultivated, processed and used for chocolate production under the same conditions, in the same farm.

Table 2
Limits of detection (LOD) and quantification (LOQ), range of analytes' concentrations for calibration curves, equations and determination coefficients (R^2) of analytical curves for each amino acid, bioactive amines and NH_3 by UHPLC-UV.

Analyte	Limits (mg/100 g)		Range	Linear equations [a]
	LOD	LOQ	(mg/100 g)	(R^2)
Amino acids				
Alanine	0.21	0.71	0.71–21.38	y = 0.0328x – 0.0108 (0.999)
Arginine	0.42	1.39	1.39–41.81	y = 0.0357x – 0.0069 (0.997)
Aspartic acid	0.32	1.06	1.06–31.94	y = 0.0313x – 0.0165 (0.998)
Glutamic acid	0.32	1.06	1.06–31.71	y = 0.0025x – 0.0192 (0.9901)
[b] Gly + gln	0.18	0.60	0.60–18.02	y = 0.0416x – 0.0979 (0.986)
Histidine	0.28	0.92	0.92–27.64	y = 0.0356x – 0.0055 (0.999)
Isoleucine	0.31	1.05	1.05–31.48	y = 0.0393x – 0.0012 (0.999)
Leucine	0.31	1.05	1.05–31.48	y = 0.0395x – 0.005 (0.999)
Lysine	0.35	1.17	1.17–35.09	y = 0.0671x – 0.0053 (0.999)
Methionine	0.36	1.19	1.19–35.81	y = 0.0386x + 0.0078 (0.999)
Phenylalanine	0.40	1.32	1.32–39.65	y = 0.041x – 0.013 (0.999)
Proline	0.28	0.92	0.92–27.63	y = 0.0325x – 0.0078 (0.998)
Serine	0.25	0.84	0.84–25.22	y = 0.0149x – 0.317 (0.992)
Threonine	0.29	0.95	0.95–28.59	y = 0.0333x – 0.0032 (0.999)
Tyrosine	0.43	1.45	1.45–43.49	y = 0.0359x – 0.0076 (0.998)
Valine	0.28	0.94	0.94–28.12	y = 0.0357x + 0.0013 (0.999)
Bioactive amines				
Agmatine	0.31	1.04	1.04–31.25	y = 0.0345x – 0.0129 (0.999)
Cadaverine	0.25	0.82	0.82–24.52	y = 0.07535x – 0.0002 (0.999)
Histamine	0.27	0.89	0.89–26.68	y = 0.0326x – 0.088 (0.999)
Phenylethylamine	0.29	0.97	0.97–29.08	y = 0.0437x + 0.0011 (0.999)
Putrescine	0.21	0.71	0.71–21.16	y = 0.074x – 0.0245 (0.998)
Serotonin	0.42	1.41	1.41–42.29	y = 0.0258x – 0.0845 (0.994)
Spermidine	0.35	1.16	1.16–34.86	y = 0.1009x – 0.0528 (0.998)
Tryptamine	0.38	1.28	1.28–38.45	y = 0.0444x + 0.025 (0.998)
Tyramine	0.33	1.10	1.10–32.92	y = 0.0411x + 0.0022 (0.999)
NH_3	0.04	0.14	0.14–4.08	y = 0.1016x – 0.0453 (0.996)

[a] y = peak area/internal standard peak area (25 pmol), x = analyte concentration in pmol.
[b] Gly + Gln – glycine + glutamine.

3. Results and discussion

3.1. Free amino acids

Among the 20 amino acids investigated, 18 were detected in the chocolates (Table 3). Ammonia was not detected in the samples. Cystine and asparagine were not detected in any sample, and glycine and glutamine were reported together. Most of the clones had all 18 amino acids, except for alanine, proline and valine which were not detected in PS 1319; phenylalanine was not detected in CEPEC 2002; whereas methionine was only detected in BN 34, Ipiranga, CCN 51 and PS 1319. Based on these results, PS 1319 had the least diversity of amino acids (15), followed by CEPEC 2002 (16).

The total levels of free amino acids varied from 28.57 to 84.49 g/kg for Catongo and Ipiranga, respectively (mean – 62.28 g/kg). Leucine was the prevalent amino acid, contributing the most to total levels (13.1–19.7%), except for Catongo and PS 1319, which had prevalence of alanine (14.2%) and arginine (27%), respectively. The second prevalent amino acid was phenylalanine (10.6–15.0%), except for CEPEC 2002 and PS 1319, in which this amino acid was not detected and represented only 4.9% of total levels, respectively. Schumacher et al. (2010) also reported leucine as a prevalent free amino acid in 40% cocoa chocolate.

Only three clones contained all nine essential amino acids – BN 34, Ipiranga and CCN 51; four lacked methionine – Pará/Parazinho, Catongo, TSH 1188 and PH 16; one lacked valine – PS 1319; and another one lacked both methionine and phenylalanine – CEPEC 2002. The total levels of essential amino acids varied from 10.72 g/kg (Catongo) to 37.39 g/kg (Ipiranga) (mean – 27.61 g/kg). Even though phenylalanine is an essential amino acid, its absence can be interesting for chocolate designed for phenylketonurics.

The sum of hydrophobic amino acids varied from 15.42 to 47.58 g/kg for Catongo and Ipiranga, respectively (mean – 34.50 g/kg); and the sum of acidic amino acids varied from 4.42 to 14.28 g/kg for Catongo and Ipiranga and Pará/Parazinho, respectively (mean – 10.76 g/kg). The highest levels of hydrophobic amino acids were observed for Ipiranga, Pará/Parazinho and PH 16 (~45 g/kg), which were fermented for 156 h at temperatures of 48–50 °C (Table 1). This result suggests that these clones were more susceptible to aspartic endopeptidase and carboxypeptidase activities (Crafack et al., 2014; Marseglia et al., 2014; Spizzirri et al., 2019); which could have resulted from the acidity and temperature reached during fermentation. The prevalence of hydrophobic over acidic amino acids was observed for all chocolates, with mean ratio of 3.2 (hydrophobic/acidic). Ratios were higher than the mean for CCN 51 and Catongo (~3.6); and lower for CEPEC and PS1319.

The profile and levels of free amino acids in the chocolate can affect its organoleptic characteristics (John et al., 2016; Kumari et al., 2018). When grouping amino acids according to their contribution to sensory characteristics (Table 3), the umami glutamic acid was present at low levels, from 0.06 (BN 34) to 4.60 g/kg (TSH 1188), mean of 1.18 g/kg. The total levels of sweet amino acids varied from 3.58 (PS 1319) to 19.0 g/kg (PH 16 and Ipiranga), average of 11.73 g/kg; and of the bitter amino acids varied from 13.72 (Catongo) to ~40 g/kg (Ipiranga, Pará/Parazinho, CN 51 and PH 16), mean of 34.58 g/kg. Overall, the bitter amino acids contributed the most to total levels (55.5%), followed by the sweet ones (18.8%), and umami (only 1.9%). In this context, the chocolates with a tendency for bitter taste due to amino acids are Ipiranga, Pará/Parazinho, CCN 51 and PH 16; whereas those with a tendency for sweet would be PH 16 and Ipiranga. TSH 1188 and Catongo were the chocolates with higher levels of glutamic acid (4.60 and 2.32 g/kg, respectively), which can provide an umami (savory) taste, enhancing the sensory impression.

This is the first report on the profile and levels of amino acids in chocolate made with different commercial cocoa clones. The results emphasize the role of different cocoa clones cultivated under the same conditions, at the same farm, on free amino acids in chocolate.

Table 3
Levels of free individual amino acids and total (classified according to the nutritional and sensory characteristics: essential, sweet, bitter, umami – glutamic acid, tasteless, acidic, hydrophobic, overall) in chocolates prepared with nine different cocoa clones resistant to witches' broom disease cultivated and processed under the same conditions (Bahia, Brazil).

Amino acid [a]	Levels of free amino acids [b] (g/kg)								
	CEPEC	BN 34	Ipiranga	Pará/P	CCN 51	Catongo	TSH 1188	PS 1319	PH 16
Alanine‡£	3.37 ± 0.15^{cd}	3.04 ± 0.17^{de}	9.58 ± 0.19^{a}	4.16 ± 0.21^{b}	3.44 ± 0.16^{d}	4.00 ± 0.23^{bc}	2.71 ± 0.13^{e}	0.00^{f}	9.68 ± 0.21^{a}
Arginine§	5.21 ± 0.22^{de}	4.55 ± 0.15^{e}	8.77 ± 0.13^{b}	6.40 ± 0.21^{c}	5.28 ± 0.14^{de}	2.98 ± 0.12^{f}	5.63 ± 0.24^{cd}	13.74 ± 0.46^{a}	8.05 ± 0.23^{b}
Aspartic acid+	3.71 ± 0.03	3.16 ± 0.05^{d}	4.96 ± 0.07^{a}	3.54 ± 0.11^{bc}	3.33 ± 0.04^{cd}	1.06 ± 0.01^{g}	1.66 ± 0.10^{f}	2.61 ± 0.01^{e}	2.75 ± 0.11^{e}
Glutamic acid#	1.03 ± 0.09^{c}	0.06 ± 0.01^{e}	0.62 ± 0.10^{d}	0.44 ± 0.03^{d}	0.46 ± 0.02^{d}	2.32 ± 0.11^{b}	4.60 ± 0.17^{a}	0.53 ± 0.04^{d}	0.55 ± 0.08^{d}
[c]Gly + Gln†£	1.20 ± 0.09^{cd}	0.96 ± 0.03^{d}	1.60 ± 0.08^{b}	2.28 ± 0.13^{a}	1.45 ± 0.10^{bc}	0.54 ± 0.04^{e}	1.34 ± 0.02^{bc}	0.20 ± 0.02^{f}	1.31 ± 0.06^{bc}
Histidine*§	1.28 ± 0.07^{cd}	1.20 ± 0.07^{d}	1.73 ± 0.04^{b}	2.02 ± 0.10^{a}	1.46 ± 0.04^{bc}	0.46 ± 0.08^{e}	1.19 ± 0.09^{cd}	1.39 ± 0.07^{bcd}	1.64 ± 0.08^{b}
Isoleucine*‡§	4.09 ± 0.02^{b}	3.29 ± 0.05^{d}	3.91 ± 0.14^{b}	4.29 ± 0.14^{b}	3.76 ± 0.13^{bc}	1.28 ± 0.06^{e}	3.40 ± 0.08^{cd}	7.62 ± 0.23^{a}	3.94 ± 0.12^{bc}
Leucine*‡§	11.40 ± 0.31^{c}	9.55 ± 0.17^{d}	12.04 ± 0.37^{bc}	13.16 ± 0.30^{a}	12.00 ± 0.25^{ab}	3.59 ± 0.11^{f}	9.35 ± 0.21^{d}	5.26 ± 0.13^{e}	10.04 ± 0.19^{d}
Lysine*+	4.54 ± 0.04^{c}	5.53 ± 0.28^{b}	6.56 ± 0.15^{a}	6.80 ± 0.02^{a}	5.86 ± 0.14^{b}	1.90 ± 0.21^{e}	3.89 ± 0.04^{d}	2.00 ± 0.06^{e}	5.79 ± 0.03^{b}
Methionine*‡§	0.00^{e}	1.70 ± 0.15^{c}	1.04 ± 0.01^{d}	0.00^{e}	2.01 ± 0.07^{b}	0.00^{e}	0.00^{e}	2.76 ± 0.02^{a}	0.00^{e}
Phenylalanine*‡§	0.00^{e}	8.79 ± 0.15^{c}	10.84 ± 0.25^{ab}	11.08 ± 0.37^{a}	10.58 ± 0.26^{b}	3.02 ± 0.14^{d}	8.18 ± 0.12^{c}	2.34 ± 0.15^{d}	8.54 ± 0.24^{c}
Proline‡£	2.99 ± 0.13^{b}	2.74 ± 0.15^{bc}	2.96 ± 0.15^{b}	3.76 ± 0.24^{a}	3.23 ± 0.14^{b}	1.05 ± 0.16^{d}	2.14 ± 0.10^{c}	0.00^{e}	3.32 ± 0.16^{b}
Serine†£	2.36 ± 0.01^{a}	1.82 ± 0.04^{cd}	2.09 ± 0.04^{abc}	2.39 ± 0.08^{ab}	2.02 ± 0.13^{abc}	0.97 ± 0.04^{e}	1.81 ± 0.13^{d}	1.09 ± 0.09^{e}	1.99 ± 0.08^{bc}
Threonine*†£	1.34 ± 0.04^{de}	1.09 ± 0.03^{e}	3.41 ± 0.13^{b}	1.26 ± 0.03^{c}	0.95 ± 0.10^{de}	0.98 ± 0.01^{f}	1.02 ± 0.05^{cd}	2.29 ± 0.11^{a}	3.66 ± 0.11^{b}
Tryptophan*‡+	0.79 ± 0.08^{d}	0.89 ± 0.01^{cd}	1.12 ± 0.06^{b}	1.27 ± 0.08^{b}	1.17 ± 0.03^{bc}	0.09 ± 0.00^{e}	1.09 ± 0.06^{bc}	2.63 ± 0.14^{a}	1.07 ± 0.07^{bc}
Tyrosine†+	6.47 ± 0.13^{cd}	5.46 ± 0.23^{e}	7.17 ± 0.12^{ab}	7.88 ± 0.20^{a}	6.97 ± 0.16^{bc}	1.93 ± 0.16^{f}	5.19 ± 0.17^{e}	5.65 ± 0.14^{de}	6.68 ± 0.20^{bc}
Valine*‡§	6.83 ± 0.22^{a}	5.57 ± 0.15^{c}	6.09 ± 0.21^{ab}	6.59 ± 0.15^{a}	5.85 ± 0.18^{bc}	2.39 ± 0.15^{c}	5.63 ± 0.20^{c}	0.00^{d}	6.45 ± 0.19^{ab}
Total									
Essential*	17.25 ± 0.47^{f}	27.05 ± 0.89^{d}	37.39 ± 0.91^{a}	33.13 ± 0.95^{ab}	31.06 ± 0.91^{c}	10.72 ± 0.62^{g}	24.40 ± 0.68^{e}	34.77 ± 1.24^{b}	32.68 ± 0.88^{bc}
Sweet£	11.26 ± 0.42^{bc}	9.65 ± 0.42^{cd}	19.65 ± 0.59^{a}	13.85 ± 0.69^{b}	11.08 ± 0.63^{bc}	7.55 ± 0.48^{d}	9.03 ± 0.43^{cd}	3.58 ± 0.22^{e}	19.96 ± 0.62^{a}
Bitter§	28.81 ± 0.84^{e}	34.66 ± 0.89^{d}	44.41 ± 1.15^{a}	43.55 ± 1.27^{ab}	40.93 ± 1.07^{bc}	13.72 ± 0.66^{f}	33.39 ± 0.94^{d}	33.10 ± 1.06^{d}	38.65 ± 1.05^{c}
Tasteless+	15.52 ± 0.28^{cd}	15.03 ± 0.57^{d}	19.81 ± 0.40^{a}	19.48 ± 0.41^{a}	17.33 ± 0.37^{b}	4.99 ± 0.38^{f}	11.82 ± 0.37^{e}	12.88 ± 0.35^{e}	16.29 ± 0.41^{bc}
Hydrophobic‡	29.47 ± 0.91^{d}	35.57 ± 1.00^{c}	47.58 ± 1.38^{a}	44.32 ± 1.49^{b}	42.03 ± 1.22^{b}	15.42 ± 0.85^{f}	32.51 ± 0.90^{cd}	20.61 ± 0.67^{e}	43.03 ± 1.18^{b}
Acidic†	11.37 ± 0.27^{b}	9.33 ± 0.33^{c}	14.28 ± 0.37^{a}	13.81 ± 0.44^{a}	11.38 ± 0.49^{b}	4.42 ± 0.25^{d}	9.36 ± 0.37^{c}	9.23 ± 0.36^{c}	13.64 ± 0.45^{a}
Overall	56.62 ± 1.64^{d}	59.40 ± 1.89^{d}	84.49 ± 2.24^{a}	77.32 ± 2.39^{b}	69.80 ± 2.08^{c}	28.57 ± 1.64^{f}	58.83 ± 1.91^{d}	50.09 ± 1.66^{e}	75.44 ± 2.14^{b}

[a] Amino acids were discriminated according to the following characteristics, individually and as a total *Essential (Histidine, Isoleucine, Leucine, Lysine, Methionine, Threonine, Tryptophan, Valine);# Umami (Glutamic acid); £Sweet (Alanine, Glycine + Glutamine, Proline, Serine, Threonine);§Bitter (Arginine, Histidine, Isoleucine, Leucine, Methionine, Phenylalanine, Valine); +Tasteless (Aspartic acid, Lysine, Tryptophan, Tyrosine); †Acidic (Glycine + Glutamine, Serine, Threonine, Tyrosine); and ‡Hydrophobic amino acids (Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Tryptophan, Valine).

[b] Means ± standard deviation with different superscripts in the same lines are significantly different (Tukey test. $p \leq 0.05$).

[c] Gly + Gln – glycine + glutamine.

Table 4
Levels of individual and total bioactive amines (mg/kg) chocolates prepared with nine different cocoa clones resistant to witches' broom disease cultivated and processed under the same conditions (Bahia, Brazil).

Clone	Bioactive amines (mg/kg)									
	AGM	CAD	HIM	PHM	PUT	SPD	SRT	TRM	TYM	Total
CEPEC 2002	0.00^{d}	3.17 ± 0.13^{b}	2.97 ± 0.14^{b}	4.53 ± 0.27^{b}	2.60 ± 0.01^{c}	2.71 ± 0.05^{f}	3.54 ± 0.21^{a}	1.62 ± 0.33^{b}	5.45 ± 0.21^{b}	26.59 ± 1.35^{c}
BN 34	0.00^{d}	1.19 ± 0.05^{d}	2.06 ± 0.15^{c}	5.01 ± 0.50^{b}	1.74 ± 0.10^{de}	5.65 ± 0.43^{bc}	0.00^{b}	1.16 ± 0.13^{c}	3.98 ± 0.23^{c}	20.79 ± 2.94^{d}
Ipiranga	0.00^{d}	0.96 ± 0.00^{de}	1.41 ± 0.04^{d}	2.72 ± 0.02^{c}	1.95 ± 0.04^{cde}	8.93 ± 0.05^{a}	3.03 ± 0.19^{a}	1.17 ± 0.11^{c}	1.26 ± 0.02^{e}	21.43 ± 0.47^{d}
Pará/ Parazinho	1.41 ± 0.15^{c}	0.53 ± 0.03^{e}	1.45 ± 0.07^{d}	3.11 ± 0.08^{c}	0.90 ± 0.06^{fg}	4.92 ± 0.35^{cd}	3.45 ± 0.06^{a}	1.75 ± 0.03^{b}	2.50 ± 0.01^{d}	20.02 ± 0.84^{d}
CCN 51	0.00^{d}	0.00^{f}	0.00^{e}	3.33 ± 0.14^{c}	0.57 ± 0.00^{g}	3.77 ± 0.11^{ef}	0.00^{b}	0.86 ± 0.05^{cd}	0.57 ± 0.07^{f}	9.10 ± 0.37^{e}
Catongo	3.48 ± 0.07^{b}	2.42 ± 0.04^{c}	3.42 ± 0.04^{a}	7.00 ± 0.13^{a}	2.52 ± 0.05^{cd}	4.87 ± 0.07^{cd}	3.06 ± 1.06^{a}	0.63 ± 0.02^{d}	5.07 ± 0.07^{b}	32.47 ± 1.55^{b}
TSH 1188	4.33 ± 0.07^{a}	8.19 ± 0.48^{a}	2.61 ± 0.29^{b}	4.49 ± 0.41^{b}	6.39 ± 0.81^{a}	4.43 ± 0.09^{de}	2.88 ± 0.27^{a}	1.03 ± 0.14^{cd}	9.74 ± 0.39^{a}	44.09 ± 2.95^{a}
PS 1319	1.42 ± 0.10^{c}	0.74 ± 0.03^{de}	1.39 ± 0.12^{d}	2.91 ± 0.24^{c}	1.48 ± 0.14^{ef}	6.37 ± 0.53^{b}	2.75 ± 0.18^{a}	1.17 ± 0.11^{c}	1.54 ± 0.18^{e}	19.77 ± 0.90^{d}
PH 16	4.64 ± 0.35^{a}	3.58 ± 0.30^{b}	0.00^{e}	7.26 ± 0.69^{a}	4.48 ± 0.12^{b}	8.25 ± 0.79^{a}	0.00^{b}	4.20 ± 0.15^{a}	10.39 ± 0.42^{a}	42.80 ± 1.35^{a}

Means ± standard deviations with different superscripts in the same columns are significantly different (Tukey test, $p \leq 0.05$).
AGM – agmatine, CAD – cadaverine, HIM – histamine, PHM – phenylethylamine, PUT – putrescine, SPD – spermidine, SRT – serotonin, TRM – tryptamine, TYM – tyramine.

3.2. Bioactive amines

All nine amines investigated were detected and five of them were present in all chocolates: spermidine, putrescine, tryptamine, tyramine and phenylethylamine (Table 4). Sucrose, which was used during chocolate formulation (29.6 g/100 g), did not contain any amines. Lecithin was not analyzed for amines, but at the low amount used, it is unlikely that it would affect amine levels. Therefore, the amines found in the chocolates come from the cocoa, which represent 67% of the formulation. Total amine levels varied widely (9.1–44.1 mg/kg), with higher levels (>40 mg/kg) for PH 16 and TSH 1188 and lower levels (<10 mg/kg) for CC N51.

There were differences on the levels of individual amines among chocolates. When considering amines which were present in every sample, spermidine varied from 2.71 to 8.93 mg/kg (mean – 5.54 mg/kg), with lower levels in CEPEC 2002 and higher in Ipiranga and PH 16. High spermidine levels are desirable due to its antioxidant, anti-aging and cardio-protector effects (Madeo, Eisenberg, Pietrocola, & Kroemer, 2018). Putrescine, which is a precursor of spermidine, was present at levels from 0.57 to 6.39 mg/kg (mean – 2.51 mg/kg) for CCN 51 and TSH 1188, respectively. High putrescine levels can be associated with plant response to drought, heat, salt and oxidative stresses (Handa, Fatima, & Mattoo, 2018; Menéndez et al., 2019). Tryptamine varied from 0.63 to 4.20 mg/kg (mean – 1.51 mg/kg) with higher levels for PH 16 and lower for CCN 51 and Catongo; it is a neuromodulator and neurotransmitter (Yılmaz & Gökmen, 2020) and it also shows antioxidant activity (Estevão, Carvalho, Ferreira, Fernandes, & Marques, 2011). Phenylethylamine levels ranged from 2.72 to 7.26 mg/kg (mean – 4.48 mg/kg); Catongo and PH 16 had the highest levels, whereas Ipiranga, Pará/Parazinho and CCN 51 had the lowest. It is a catecholamine releasing agent, enhancing mood lifting and sexual drive (Yılmaz & Gökmen, 2020). Tyramine was present at levels from 0.57 to 10.39 mg/kg (mean – 4.50 mg/kg), with higher levels in PH 16 and TSH 1188. Studies are needed to ascertain the ideal levels of the neuroactive amines for mood modulation, which is important to optimize levels of these compounds in chocolates.

When considering amines which occurred sporadically, agmatine was only present in five samples (1.41–4.64, mean– 1.70 mg/kg), with higher levels in PH 16 and TSH 1188. This amine, which is a precursor of spermidine from L-arginine by arginine decarboxylase (Akasaka & Fujiwara, 2019), has positive effects on numerous central nervous system-associated complications, such as depression, anxiety and convulsion (Olescowicz et al., 2020). Serotonin was present in six samples, all of them with levels around 3.0 mg/kg. This amine plays several important roles in physiological and behavioral processes such as regulation of anger, appetite, body temperature, blood pressure, mood, sexuality and sleep (Vadodaria et al., 2017; Yılmaz & Gökmen, 2020). Cadaverine was present in every sample, except for CCN 51, at levels up to 8.19 mg/kg in TSH 1188 (mean– 2.31 mg/kg). Histamine was present in seven of the chocolates at levels that varied from 1.39 to 3.42 (mean – 1.70) mg/kg, with higher levels found in Catongo.

Several studies investigated amines in milk chocolate, which, because of the several different ingredients added and low cocoa percentages, showed a wide range of amine profiles, depending on the respective formulation (Kosman, Stankevich; Makarov, & Tikhonov, 2007; Restuccia, Spizzirri, Puoci, & Picci, 2015). Information regarding bioactive amines in dark chocolate is scarce and limited due to the lack of history about the samples. In a study with three dark chocolates (60–70% cocoa), lower levels of spermidine and higher levels of tyramine and histamine were found and phenylethylamine was only present in one sample at low levels (Restuccia et al., 2015).

Some of the amines detected in this study were found in fermented Forastero cocoa (Bahia, Brazil) – spermidine, putrescine, tryptamine, tryptamine and phenylethylamine (Gloria, Deus, & Franca, 2019); whereas tryptamine, tyramine and spermidine were present in cocoa from Pará, Brazil (Do Carmo Brito et al., 2017). Agmatine was reported in dark chocolate for the first time. It can result from thermal decarboxylation of arginine during roasting. In a similar way, cadaverine and histamine can be formed by thermal decarboxylation of lysine and histidine, which were present in the chocolate.

High levels of putrescine and cadaverine in chocolate are undesirable as they can impart off flavor to the chocolate (EFSA, 2011); however, their threshold is not known, and, therefore, studies are needed to elucidate its impact on chocolate quality.

Regarding the safety aspects of amines, histamine and tyramine, at high levels, can cause adverse effects to human health. Histamine intolerance has symptoms including urticaria, headache, accelerated heart rate, blood pressure drop, nausea, and diarrhea (EFSA, 2011). No observed adverse effect level (NOAEL) levels of 25–50 mg per meal were set for histamine for healthy individuals, but any histamine can cause adverse effects in histamine sensitive individuals (EFSA, 2011). Tyramine can cause hypertension, vasoconstriction, pupil dilatation, migraines and cerebral hemorrhages (EFSA, 2011). NOAEL were established as 600, 50 and 6 mg of tyramine (per person per meal) for healthy individuals not taking monoamino oxidase inhibitors (MAOI), taking third generation MAOI and taking classical MAOI drugs, respectively (EFSA, 2011). Based on these results, even considering the highest levels of tyramine, an extremely large amount of chocolate would be necessary to elicit adverse effects. However, even though histamine levels were low, histamine sensitive individuals should be careful. Since histamine was not found in CCN 51 and PH 16, these clones can be used for histamine free chocolate.

3.3. Multivariate analysis

Multivariate analysis of auto-scaled data indicated that a model based on two principal components (PC) explained 68% of the variance (Fig. 1a). PC1 explained 45% of the variance and it was important in differentiating PH 16, Pará/Parazinho, Ipiranga (positive values) from Catongo and PS 1319 (negative values), mainly due to amino acids. The first group (positive values) was characterized mainly by high levels of total amino acids, several individual amino acids (leucine, lysine, serine, valine, proline, tyrosine, aspartic acid, glycine + glutamine, histidine, histamine, agmatine and phenylethylamine) and high levels of sweet, bitter, tasteless, hydrophobic and acidic amino acids (Fig. 1b). The second group (negative values) was highlighted by higher levels of glutamic acid, spermidine, methionine and tryptamine.

PC2 explained 23% of the variance and differentiated TSH 1188 and CEPEC 2002 (positive values) from PS 1319 and from BN 34 and CCN 51 (negative values), mostly related to bioactive amines. The differences were mainly due to putrescine, cadaverine, tyramine, total amines, agmatine and glutamic acid and valine (positive values) and to methionine, tryptophan, serotonin, essential amino acids, histidine and bitter amino acids (negative values) (Fig. 1c).

The dendrogram - HCA (Fig. 1d) based on the variances and Euclidian distances among vectors, confirmed PCA results and five clusters were obtained: (i) Ipiranga, PH 16, and Pará/Parazinho; (ii) BN 34 and CCN 51; (iii) PS 1319; (iv) CEPEC and TSH 1188; and (v) Catongo. Catongo had the lowest levels of amino acids, prevalence of alanine and leucine, and high levels of amines (total, phenylethylamine, histamine and serotonin). PS 1319 had the least diversity of amino acids (15), the lowest levels of sweet amino acids, phenylethylamine and hydrophobic/acidic ratio (2.2). TSH 1188 and CEPEC had higher leucine and lower methionine levels. BN 34 and CCN 51 had lower agmatine, serotonin and glutamic acid and higher leucine and phenylalanine levels. PH 16, Ipiranga and Pará/Parazinho had higher overall, essential, bitter, acidic and hydrophobic amino acids, higher levels of leucine, phenylalanine, valine and serotonin. These results confirm that different cocoa clones cultivated and processed under similar conditions give rise to chocolates with different nutritional, functional, safety and sensory characteristics.

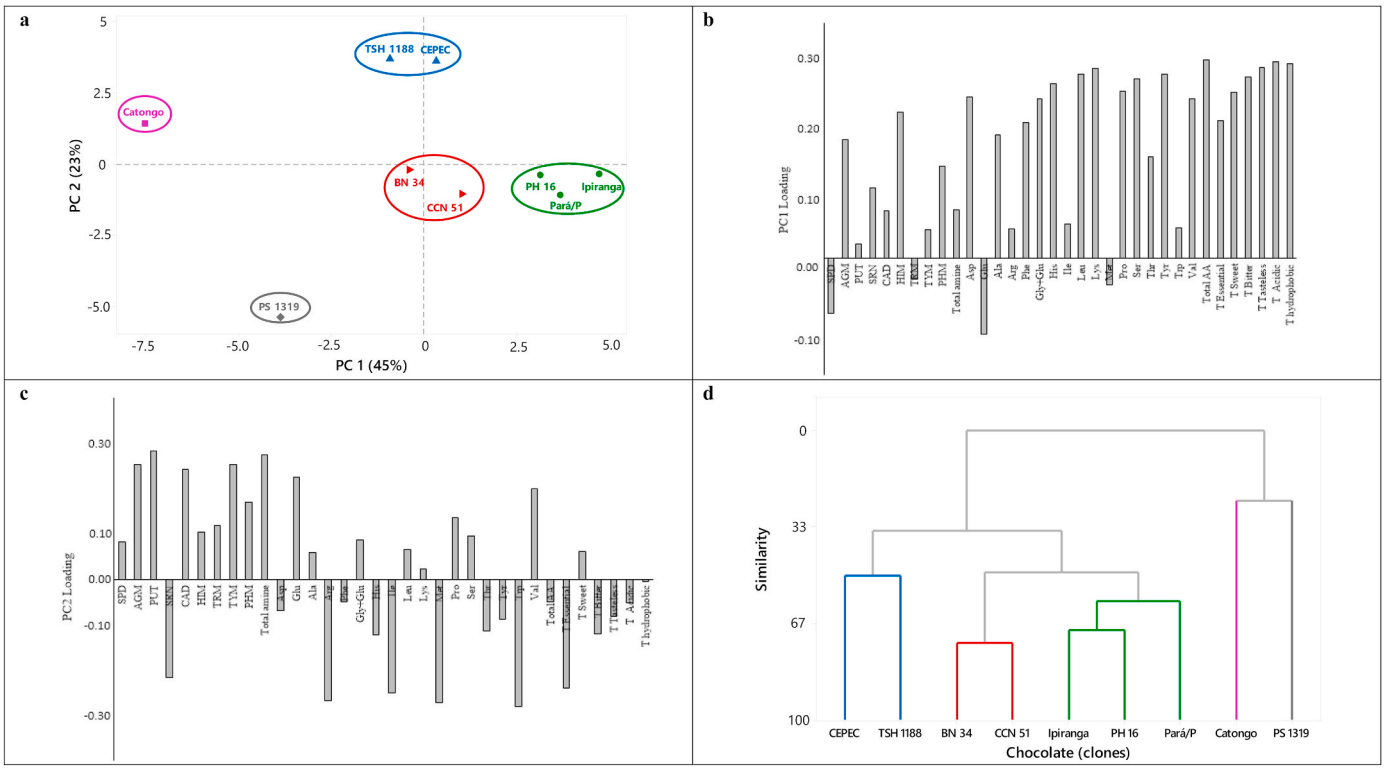

Fig. 1. Classification of chocolates prepared with nine different cocoa clones resistant to witches' broom disease cultivated and processed under the same conditions (Bahia, Brazil), according to according to the levels of amino acids, total amino acids (essential, sweet, bitter, umami, tasteless, hydrophobic and acidic), bioactive amines and total bioactive amines: (a) Variable projection (scatterplot), (b) loading plot of PC1, (c) loading plot of PC2, and (d) dendrogram obtained by Principal Component Analysis and Hierarchical Cluster A analysis. SPD – spermidine, AGM – agmatine, PUT – putrescine, CAD – cadaverine, HIM – histamine, SRT – serotonin, TRM – tryptamine, TYM – tyramine, PHM – phenylethylamine, Ala – Alanine, Gly + Gln – Glycine + Glutamine, Pro – Proline, Ser – Serine, Thr – Threonine, Arg – Arginine, His – Histidine, Ile – Isoleucine, Leu – Leucine, Met – Methionine, Phe – Phenylalanine, Val – Valine, Glu - Glutamic acid, Asp - Aspartic acid, Lys – Lysine, Trp – Tryptophan, Tyr – Tyrosine, Total AA – Total amino acids.

4. Conclusions

The chocolates made with the different cocoa clones cultivated in the same farm under the same edaphoclimatic conditions and post-harvest processing showed different profile and levels of amino acids and bioactive amines.

The results indicated that the levels and profile of amino acids and bioactive amines were significantly affected by the clone resulting in products with different nutritional, functional, safety and sensory attributes. These chocolates were well separated by principal components and hierarquical cluster analyses. The knowledge of the levels of these compounds in cocoa clones can be used for further agronomical purposes and also by industries in the definition of blends in the production of chocolate with specific nutritional, functional (health promoting properties), safety and sensorial quality according to customer demand.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript."

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Appendix A. Supplementary data

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