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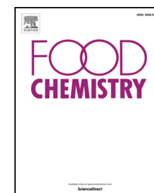
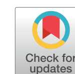


Analytical Methods

Chemical implications and time reduction of on-farm cocoa fermentation by *Saccharomyces cerevisiae* and *Pichia kudriavzevii*



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A R T I C L E I N F O

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A B S T R A C T

The use of starters during fermentation has been gaining momentum as it can warrant high-quality chocolate. The objective of this study was to investigate the influence of *Saccharomyces cerevisiae* (Sc) and *Pichia kudriavzevii* (Pk) during on-farm fermentation on physico-chemical and microbiological characteristics and levels of methylxanthines and bioactive amines of cocoa. Four treatments were used: ScPk (1:1), only Sc, only Pk, and no starter (control). The starters lead to changes throughout fermentation, but provided fermented cocoa with similar pH, titratable acidity, reducing sugars and phenolic compounds. ScPk shortened fermentation time by 24 h. The ScPk fermented and dried cocoa had higher levels of monomeric phenols, methylxanthines, phenylethylamine and lower levels of the putrefactive amines – putrescine and cadaverine ($p < 0.05$). The results were confirmed by multivariate analysis. Based on these results, the mixture of both yeasts species is a promising starter for cocoa fermentation decreasing duration time and modulating high-quality components.

1. Introduction

Cocoa bean is the main raw material for chocolate production. It undergoes several important processing stages from fruit opening to industrial processing. However, fermentation is outstanding, due to its role in the formation of precursors for high-quality chocolate aroma and flavor (Ho, Zhao, & Fleet, 2015).

Cocoa fermentation is a natural and spontaneous process, involving different microorganisms, including yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Each group develops stepwise according to the environmental conditions in which they are present. Yeasts and LAB hydrolyze fermentable sugars (glucose, fructose and sucrose) transforming them into ethanol and lactic acid, leading to increased temperature (Figueroa-Hernández, Mota-Gutierrez, & Ferrocino, 2019). Yeasts, e.g. *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kluyveromyces marxianus*, also produce pectinolytic enzymes. These enzymes break down pectin which is responsible for the viscosity and stickiness of cocoa pulp, causing the collapse of the pulp and allowing the formation of void space between the beans, favoring air percolation (Schwan & Wheals, 2004; Ouattara, Koffi, Karou, Sangaré, Niamke, & Diopoh, 2008; Ho, Zhao, & Fleet, 2014; Vuyst & Weckx, 2016). The aeration conditions are needed for the growth of AAB which convert ethanol into acetic acid, increasing the temperature and leading to the death of the seed embryo (Moreira, Vilela, Miguel, Santos, Lima, & Schwan, 2017). The high temperature and low pH are needed for protein breakdown and release of amino acids, which are precursors of

Abbreviations: AAB, Acetic acid bacteria; ANOVA, Analysis of variance; CF, Control fermentation; GYC, Glucose yeast medium with calcium carbonate; HCA, Hierarchical Cluster Analysis; LAB, Lactic acid bacteria; LOD, Limit of detection; LOQ, Limit of quantification; meq. NaOH 0.1N/100 g, milliequivalent sodium hydroxide solution 0.1N per 100 g sample; mg ECE/g, milligram equivalent epicatechin per gram sample; MRS, De Man, Rogosa & Sharpe; PCA, Principal Component Analysis; Pk, Fermentation with *Pichia kudriavzevii*; PPO, polyphenoloxidase; YPD, Yeast extract peptone dextrose; Sc, Fermentation with *Saccharomyces cerevisiae*; ScPk, Fermentation with both species of yeasts (Sc + Pk); TPC, Total phenolic compounds; TRS, Total reducing sugars; TTA, Total titratable acidity

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typical chocolate aromas and flavors and also of bioactive amines (Hernández-Hernández, López-Andrade, Ramírez-Guillermo, Guerra Ramírez, & Caballero Pérez, 2016).

The development of starter cultures for use in cocoa fermentation has been gaining momentum due to the numerous benefits they can exert on process standardization and harmonization of chocolate quality. Studies indicate that some yeast species are valued due to the high capacity to produce ethanol and to hydrolyze pectin and proteins, facilitating the release of sugars and nitrogenous compounds which are relevant precursors for high-quality chocolate, mainly regarding flavor compounds, including alcohols, esters, pyrazines (Visintin et al., 2017). Among them, *Saccharomyces cerevisiae* and *Pichia kudriavzevii* were highlighted for their ability to produce a wide variety of esters, alcohols and aldehydes that impart desirable aromas to chocolate, such as 'fruity', 'floral' and 'sweet' aromas (Koné et al., 2016). Furthermore, they have high capacity to adapt to diverse environmental conditions and are capable of inhibiting the growth of putrefactive microorganisms (Batista, Ramos, Ribeiro, Pinheiro, & Schwan, 2015). In addition, these species can facilitate drainage of the pulp, increasing aeration, allowing fast microbial succession of LAB to AAB, thereby reducing fermentation time (Sandhya et al., 2016). However, scarce information is available regarding the influence of added starter culture on the fermentation process and on the formation of compounds which are responsible for high-quality chocolate quality, including bioactive amines, phenolic compounds and methylxanthines.

The objective of this study was to investigate, for the first time, the influence of yeast starter cultures, comprised of *Saccharomyces cerevisiae* and *Pichia kudriavzevii*, on the evolution of on-farm cocoa fermentation and on the formation of compounds which are responsible for high-quality chocolate. Four fermentation treatments were undertaken (only *S. cerevisiae*; only *P. kudriavzevii*; both *S. cerevisiae* and *P. kudriavzevii*; and control – no yeast added). Fermentation was followed by determination of the changes on temperature, pH, total titratable acidity, total phenolic compounds and microbial counts. The fermented cocoa was dried and analyzed for methylxanthines (theobromine, theophylline and caffeine), biogenic amines, catechin and epicatechin in the fermented and dried cocoa beans.

2. Material and methods

2.1. Chemicals and reagents

Reagents and solvents were of analytical grade (Synth, Diadema, SP, Brazil; Dinâmica, SP, Brazil). Acetonitrile and methanol were HPLC grade (J.T. Baker, Radnor, PA, USA). Glucose, Folin-Ciocalteu reagent, 3,5-dinitrosalicyclic acid and the standards [spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride, agmatine sulfate, cadaverine dihydrochloride, 5-hydroxytryptamine (serotonin), histamine dihydrochloride, tyramine hydrochloride, 2-phenylethylamine hydrochloride, tryptamine, *o*-phthalaldehyde, (+)-catechin, (−)-epicatechin, theobromine, theophylline and caffeine] were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ultrapure water was from Milli-Q (Millipore Corp., Milford, MA, USA). For chromatographic analysis, HPLC solvents were filtered through 0.45 μm nylon membrane (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and the extracts through 0.22 μm (Analítica, São Paulo, SP, Brazil).

Yeast extract, De Man, Rogosa & Sharpe (MRS) agar, peptone water, bacteriological peptone and bacteriological agar were from Kasvi (São José dos Pinhais, PR, Brazil); calcium carbonate and chloramphenicol were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); and nystatin was from Prati-Donaduzzi (Toledo, PR, Brazil). These components were used to make the culture media: Yeast Extract Peptone Dextrose Agar – YPD [20 g glucose, 20 g bacteriological peptone, 10 g yeast extract (Batista, Ramos, Dias, Pinheiro, & Schwan, 2016), adapted with 20 g bacteriological agar in 1 L distilled water, pH 5.6]; YPD broth [20 g glucose, 20 g bacteriological peptone, 10 g yeast extract in 1 L distilled water (Batista et al., 2016), pH 5.6]; and Glucose Yeast Medium with calcium carbonate – GYC [50 g glucose, 10 g yeast extract, 30 g calcium carbonate, 20 g bacteriological agar in 1 L distilled water, pH 5.6 (Moreira, Miguel, Duarte, Dias, & Schwan, 2013)].

2.2. Yeasts strains and starter cultures preparation

Cultures of *Saccharomyces cerevisiae* (GenBank Access KY794742) and *Pichia kudriavzevii* (GenBank Access KY794725) were used. These strains were isolated and identified during fermentation of cocoa beans at the same farm in 2016 (Chagas Junior, 2016). They were adequately stored in the Bank of Microorganisms of the Laboratory of Biotechnology Processes, Federal University of Pará (LABIOTEC, UFPA, Brazil).

The preserved strains were activated in Petri dishes containing sterile YPD agar incubated in inverted position at 28 °C for 72 h (Baffi et al., 2011). After incubation, colonies were transferred to Erlenmeyers containing 100 mL of sterile YPD broth and subsequently incubated in an orbital shaker at 150 rpm, 30 °C for 12 h. The contents of each Erlenmeyer were aseptically transferred to 900 mL of sterile YPD broth in a bench bioreactor (FerMac 320, Electrolab Biotech, Tewkesbury, UK) at 30 °C and 150 rpm (Ramos, Dias, Miguel, & Schwan, 2014) for cell growth ($\approx 10^8$ cells/mL). Subsequently, the contents were centrifuged in sterile falcon-type conical tubes at 10.000 × *g* for 14 min at 4 °C. The supernatant was discarded, and the biomass pellet stored under refrigeration (4 ± 2 °C) for up to 48 h prior to use.

2.3. Cocoa beans fermentation and dehydration

Forastero cocoa fruits were produced, fermented and dried at a farm in Tomé-Açu, PA, Brazil (02°28′41.3 "S and 48°16′50.7″ W), in September of 2017. On-farm cocoa fermentation was conducted according to commercial practices. Immediately after harvest, the fruits were cut open with stainless steel knives and the beans and pulp, without peel and placenta, were placed in wooden boxes with a capacity of 50 kg of cocoa beans/each.

Four fermentation treatments were carried out simultaneously: (i) a control without inoculum (CF), (ii) fermentation with 10^6 cells of *S. cerevisiae* (Sc), (iii) fermentation with 10^6 cells of *P. kudriavzevii* (Pk) and (iv) fermentation with the two species all together (ScPk), 1:1, 10^6 cells each. All experiments were performed in triplicate with a total of 12 fermentation boxes. The starter cultures (10^6 cells/g cocoa beans) (Visintin et al., 2017) were suspended in 500 mL sterile peptone water and they were sprayed uniformly onto the seeds, layer by layer, as they were placed into the wooden boxes. Banana leaves were placed on the surface of the fermentation mass.

Throughout fermentation (0, 24, 48, 72, 96, 120, 144 and 168 h), the temperature was measured with a digital thermometer (model HT-600, Instrutherm, São Paulo, SP, Brazil) at the middle of the wooden boxes. Samples from each fermentation box were taken from five random points, totaling $\approx$200 g of samples per fermentation time per box. The samples were aseptically packed in sterile bags and taken immediately to the laboratory for microbiological analysis and, afterwards, they were stored at −18 °C for chemical analyses. Fermentation was terminated based on aspects, color and aroma typical of fermented cocoa beans.

At the end of fermentation (168 h), except for ScPk, which ended at 144 h, the cocoa beans were transferred to trays, and exposed to sunlight for natural dehydration. The drying period lasted approximately 72 h. The dried samples were packed in sterile plastic bags and transported to the laboratory for analysis.

2.4. Methods of analysis

2.4.1. Microbiological analysis during the fermentation

Ten grams of cocoa beans were aseptically ground and homogenized in 90 mL peptone water (0.1% w/v), providing 10^{-1} dilutions. Subsequently, serial decimal dilutions were obtained for the quantification of yeasts in YPD agar with chloramphenicol (100 mg/L) for inhibition of bacterial growth. The spread plate technique was used and the plates were incubated at 28 °C for 72 h (Baffi et al., 2011).

For acetic acid bacteria (AAB), serial decimal dilutions were prepared in GYC agar with 0.2% nystatin for the inhibition of fungal growth. The spread plate technique was used, and the plates were incubated at 30 °C for 96 h (Ho et al., 2014). For lactic acid bacteria (LAB), the dilutions were made on MRS agar (pH 6.2) with 0.2% nystatin for inhibition of fungal growth. The pour plate technique was used followed by incubation at 30 °C for 72 h (de Melo Pereira, Magalhães, de Almeida, da Silva Coelho, & Schwan, 2013).

2.4.2. Physico-chemical analysis of cocoa beans during fermentation

The peel and germs were removed from the cocoa seeds, which were ground in an analytical mill (A11, IKA Staufen, Germany). The analysis of moisture content (method 931.04), pH (method 970.21) and total titratable acidity (TTA, method 31.06.06) were carried out according to the Association of Official Analytical Chemists (Horwitz, & Latimer, 2006) and total reducing sugars (TRS) by the 3,5-dinitrosalicylic acid method (Miller, 1959). All analyzes were performed in triplicate.

2.4.3. Total phenolic compounds in cocoa beans during fermentation and in the dried cocoa beans

The fermented and dried cocoa seeds were freeze-dried (L101 Liotop, São Paulo, SP, Brazil) and defatted with *n*-hexane (do Carmo Brito, Campos Chisté, da Silva Pena, Abreu Gloria, & Santos Lopes, 2017). Total phenolic compounds (TPC) were determined by the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) using an UV–visible spectrophotometer (EVO 60, Thermo Fisher Scientific, Waltham, MA, USA) at 760 nm. The results were obtained by interpolation in epicatechin calibration curves (20–100 mg/L, $R^2 \geq 0.99$) and expressed in milligram equivalent epicatechin per gram (mg ECE/g). The analyses were performed in triplicate.

2.4.4. Methylxanthines and monomeric phenols in the fermented and dried cocoa beans by HPLC

Dried and defatted samples (250 mg) were placed in polypropylene tubes containing 2.5 mL aqueous ethanol solution (1:1, v/v) and dissolved using an ultrasonic bath for 10 min at 25 °C (He, Lv, Zhou, & Shi, 2010; Sandhya et al., 2016). The tubes were centrifuged at 1.500 × *g* for 10 min and the extraction was repeated. The supernatants were combined and filtered through 0.22 µm membrane prior to HPLC analysis.

Aliquots of 20 µL were automatically injected into a HPLC coupled with a diode array detector at 280 nm (He et al., 2010) (1260 Infinity, Agilent Technologies, La Jolla, CA, USA) and a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies, La Jolla, CA, USA) at 25 °C. The mobile phases were (A) water: acetonitrile (99.8:0.2, v/v) and (B) methanol, at a linear gradient of 0 to 50% for 0–12 min, 50 to 100% for 13–20 min at a flow rate of 1.2 mL/min. Identification was based on retention times of the compounds compared to those of standards and addition of the suspected standards to the sample. Quantification was undertaken by interpolation in analytical curves of each standard (Table 1).

2.4.5. Bioactive amines in the fermented and dried cocoa beans by HPLC

The determination of bioactive amines was carried out with 5 g samples, after trichloroacetic acid extraction (do Carmo Brito et al., 2017). A Shimadzu HPLC system (LC-10AD, Shimadzu, Kyoto, Japan) with a fluorometric detector (340 and 445 nm of excitation and emission, respectively) and a Novapak C18 column (3.9 × 300 mm, 4 µm, Waters Co., Milford, MA, USA). A gradient of 0.2 M sodium acetate and 0.3 mM sodium octanesulfonate, pH 4.9 and acetonitrile was used (do Carmo Brito et al., 2017; do Carmo Brito, Campos Chisté, Santos Lopes, Abreu Gloria, & da Silva Pena, 2019). Amines were identified by comparison of retention times and co-elution with standards. The quantification was performed after post column derivatization with *o*-phthalaldehyde, using analytical curves for each amine (Table 1).

2.5. Statistical analysis

The results were submitted to analysis of variance (ANOVA) and the means were compared by the Duncan test at 5% significance. Multivariate analysis was used to further analyze data. For Principal Component Analysis (PCA), temperature, microbial counts, pH, total acidity, TPC and TRS values, were used as active variables during fermentation with each of the four different treatments (starter cultures). The Hierarchical Cluster Analysis (HCA) was obtained considering the same PCA active variables and the groups were formed based on Euclidean distances (Ward's method). PCA and HCA were also carried out for the fermented and dried cocoa beans considering as active variables: pH, TPC, monomeric phenols, methylxanthines and bioactive amines. The statistical analyses were undertaken using the Statistica 7.0 software (StatSoft Inc., Tulsa, USA).

3. Results and discussion

3.1. Influence of starter culture on the physico-chemical characteristics during fermentation

During fermentation using the different starter culture, all treatments showed evolution typical of cocoa fermentation (Table 2): the temperature increased reaching a maximum followed by a decrease; there was significant increase on TTA and decreases on pH, TRS and TPC.

The end of fermentation, determined by the processor, was 168 h for most treatments (CF, Sc, and Pk) whereas for treatment ScPk fermentation ended earlier, at 144 h. This result indicates that by using the combination of the starter cultures *S. cerevisiae* and *P. kudriavzevii*, the cocoa beans were ready for drying 24 h earlier. This represents advancement in cocoa fermentation, but the desired quality must be kept.

The increase in temperature during cocoa fermentation is important as it causes the seed to lose its germinative power, which is essential for the formation of aroma precursors and characteristic flavors of chocolate (Ramos et al., 2014; Visintin et al., 2017). The increase in the temperature of the cocoa mass during fermentation is associated with the metabolism of ethanol (produced from yeasts) by AAB. These reactions are highly exothermic and raise the temperature of the fermenting mass (do Carmo Brito et al., 2017). Maximum temperatures (≈45 °C) were reached at 72 h fermentation for control (CF), decreasing afterwards. However, the treatments with added starter cultures reached higher temperatures earlier in the fermentation process (at 48 h) and it remained high for 96, 120 and 144 h for ScPk, Pk and Sc, respectively. Similar trends were also observed when using the combination of *S. cerevisiae* and *Torulaspora delbrueckii* during cocoa fermentation (Visintin et al., 2017). Afterwards, the temperatures decreased reaching similar values (≈34 °C) at 168 h fermentation for most treatments (CF, SC and Pk) and at 144 h fermentation for ScPk.

Based on these results, by adding the yeast starter cultures, typical high temperatures were reached earlier, compared to CF. Moreover, the temperature remained higher for a longer period of time, probably due to the more intense yeast activity during initial fermentation times (Fig. 1) favoring the numerous biochemical reactions that are inherent to cocoa fermentation (Ho et al., 2014). The behavior observed for the ScPk treatment indicates that a faster microbial succession took place.

Table 1
Concentrations range of the analytes used for construction of the calibration curves and respective coefficients of correlation and limits of quantification for HPLC analysis.

Compound	Standard curve		Limit of
	Concentration range (μg/mL)	Linear equation (R^2)	quantification
Methylxanthines			
Theobromine	3.125–50	$y = 0.0000001x - 3.4713$ (> 0.999)	0.64 mg/g
Theophylline	3.125–100	$y = 0.0000002x - 0.055$ (> 0.999)	0.02 mg/g
Caffeine	3.125–100	$y = 0.0000002x - 0.1899$ (> 0.999)	0.04 mg/g
Monomeric phenols			
Catechin	3.125–50	$y = 0.000001x + 0.5831$ (> 0.999)	0.31 mg/g
Epicatechin	3.125–100	$y = 0.0000004x + 0.9615$ (> 0.999)	0.11 mg/g
Bioactive amines			
Putrescine	0.71–21.16	$y = 0.074x - 0.0245$ (≥ 0.998)	0.07 mg/kg
Cadaverine	0.82–24.52	$y = 0.0535x - 0.0002$ (≥ 0.999)	0.08 mg/kg
Phenylethylamine	0.97–29.08	$y = 0.0437x + 0.0011$ (≥ 0.999)	0.10 mg/kg

R^2 - Correlation coefficient of linear regression.

The pH values did not differ statistically at the beginning of fermentation for all treatments ($p > 0.05$). However, the pH decreased reaching lower levels at 72 h fermentation (3.30–3.89), with lower levels found for Pk (3.30–3.35). The pH increased afterwards, and higher values were found for the Pk treatment (5.15) compared to the others (4.90–4.96). The pH values are within the range found in other cocoa fermentation studies (Nazaruddin, Seng, Hassan, & Said, 2006).

In the opposite way, as expected, TTA increased up to 72 h for all treatments (Table 2), and higher levels were observed for treatments without *S. cerevisiae* (CF and Pk treatments). After 72 h fermentation, TTA decreased and, at the end of fermentation, higher values were found for the treatments with starter culture compared to CF ($p \leq 0.05$). The increase in TTA is mainly due to the production of lactic and acetic acids from lactic and acetic acid bacteria, respectively during metabolism of the microorganisms. The acetic acid produced, along with ethanol, at high temperature, diffuse through the seed tissues and cotyledon, killing the germ and activating proteolytic enzymes (Efraim et al., 2010; Ramos et al., 2014; do Carmo Brito et al., 2017; Visintin et al., 2017). The increase in pH and concomitant decrease in TTA at the end of fermentation can result from the evaporation of volatile acids, mainly acetic acid, which takes place by stirring the cocoa mass (Lima, Almeida, Nout, & Zwietering, 2011).

Table 2
Changes on temperature, pH, total titratable acidity (TTA), reducing sugars and total phenolic compounds throughout on-farm cocoa fermentation with different starters CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.

Parameters/ treatments	Mean values*/Fermentation time (h)							
	0	24	48	72	96	120	144	168
Temperature (°C)								
CF	29.7 ± 0.52^{eA}	33.2 ± 0.48 ^{dB}	40.0 ± 0.62^{bB}	44.2 ± 1.43^{aA}	41.4 ± 2.76^{bB}	41.9 ± 2.28^{bA}	37.2 ± 2.48^{cB}	32.4 ± 2.39^{dA}
Sc	30.9 ± 0.59^{cA}	34.9 ± 0.51^{bA}	43.7 ± 2.61^{aA}	43.9 ± 1.97^{aA}	44.1 ± 2.10^{aAB}	42.6 ± 1.15^{aA}	43.0 ± 0.82^{aA}	34.3 ± 1.58^{bA}
Pk	30.4 ± 1.42^{cA}	35.2 ± 1.38^{bA}	45.2 ± 1.79^{aA}	44.3 ± 1.93^{aA}	44.6 ± 0.87^{aA}	45.3 ± 0.91^{aA}	36.7 ± 1.88^{bB}	35.5 ± 2.11^{bA}
ScPk	31.2 ± 1.03^{bA}	34.0 ± 0.87^{bA}	44.0 ± 1.59^{aA}	43.1 ± 1.24^{aA}	44.1 ± 1.46^{aAB}	34.4 ± 2.84^{bB}	31.4 ± 2.95^{bC}	n.a.
pH								
CF	6.57 ± 0.36^{aA}	4.70 ± 0.03^{bC}	4.20 ± 0.01^{cdB}	3.89 ± 0.03^{eA}	4.03 ± 0.04^{deA}	4.34 ± 0.02^{cA}	4.73 ± 0.03^{bA}	4.90 ± 0.01^{bB}
Sc	6.46 ± 0.04^{aA}	5.42 ± 0.04^{bA}	4.42 ± 0.06^{eA}	3.84 ± 0.04^{gA}	4.09 ± 0.01^{fA}	4.10 ± 0.03^{fB}	4.71 ± 0.01^{dA}	4.96 ± 0.03^{cB}
Pk	6.27 ± 0.04^{aA}	5.30 ± 0.09^{bB}	3.52 ± 0.02^{fC}	3.30 ± 0.04^{gB}	3.34 ± 0.05^{gC}	3.88 ± 0.02^{eC}	4.05 ± 0.06^{dC}	5.15 ± 0.02^{cA}
ScPk	6.44 ± 0.08^{aA}	5.37 ± 0.02^{bAB}	3.57 ± 0.04^{eC}	3.35 ± 0.03^{fB}	3.57 ± 0.04^{eB}	4.11 ± 0.04 ^{dB}	4.28 ± 0.04^{cB}	n.a.
TTA (meq. NaOH 0.1 N/100 g)[1]								
CF	4.85 ± 0.49^{fA}	16.06 ± 1.01^{dA}	29.00 ± 0.59^{bA}	39.68 ± 0.55^{aA}	27.68 ± 1.47^{bB}	23.18 ± 1.07^{cA}	16.60 ± 1.03^{dA}	12.53 ± 0.09^{eB}
Sc	4.76 ± 0.29^{gA}	11.01 ± 0.46^{fB}	18.82 ± 1.97^{dC}	32.77 ± 1.24^{aB}	23.93 ± 0.84^{bC}	21.86 ± 1.36^{cA}	12.63 ± 0.96^{fB}	14.75 ± 0.90^{eA}
Pk	4.00 ± 0.11^{gB}	8.55 ± 0.52^{fC}	28.15 ± 0.32^{cA}	40.41 ± 0.75^{aA}	33.32 ± 0.55^{bA}	20.98 ± 0.36^{dA}	17.20 ± 2.95^{eA}	16.85 ± 0.16^{eA}
ScPk	5.28 ± 0.47^{dA}	6.50 ± 0.50^{dD}	25.68 ± 1.35^{bB}	30.83 ± 1.62^{aB}	24.93 ± 1.44^{bC}	17.42 ± 1.70^{cB}	14.90 ± 1.95^{cA}	n.a.
Reducing sugars (mg/g)								
CF	11.72 ± 0.02^{aB}	9.51 ± 0.75^{bB}	2.62 ± 0.39^{eC}	4.87 ± 0.10^{dD}	6.30 ± 0.06^{cB}	4.93 ± 0.08^{dA}	1.59 ± 0.02^{fC}	1.85 ± 0.04^{fA}
Sc	11.70 ± 0.26^{bB}	14.41 ± 0.36^{aA}	5.15 ± 0.40^{dA}	5.27 ± 0.10^{dC}	6.68 ± 0.19^{cA}	4.21 ± 0.02^{eB}	1.93 ± 0.08^{fB}	1.92 ± 0.04^{fA}
Pk	10.61 ± 0.08^{aC}	9.41 ± 0.42^{bB}	3.93 ± 0.00 ^{dB}	6.37 ± 0.26^{cB}	2.90 ± 0.03^{eD}	2.94 ± 0.29^{eC}	2.08 ± 0.03^{fA}	1.30 ± 0.05^{gB}
ScPk	13.42 ± 0.60^{aA}	9.78 ± 0.44^{bB}	4.20 ± 0.16^{eB}	6.72 ± 0.06^{cA}	5.77 ± 0.16^{dC}	1.43 ± 0.00^{fD}	1.60 ± 0.00^{fC}	n.a.
Total phenolic compounds (mg ECE/g)[2]								
CF	63.99 ± 0.97^{aA}	65.26 ± 1.22^{aB}	50.10 ± 1.44^{bA}	50.13 ± 0.21^{bA}	35.76 ± 2.90^{cB}	38.00 ± 1.55^{cB}	38.80 ± 0.48^{cB}	36.95 ± 1.88^{cB}
Sc	62.20 ± 0.14^{bB}	70.00 ± 0.83^{aA}	52.72 ± 2.99^{cA}	49.53 ± 1.03^{cdA}	47.12 ± 0.68^{deA}	45.78 ± 0.69^{eA}	41.33 ± 0.06^{fA}	41.55 ± 1.22^{fA}
Pk	62.10 ± 0.14^{aB}	45.78 ± 1.78^{cD}	50.91 ± 3.60^{bA}	51.29 ± 0.84^{bA}	30.29 ± 0.94^{eC}	35.37 ± 0.95 ^{dB}	35.95 ± 0.33^{dC}	30.25 ± 0.47^{eC}
ScPk	63.05 ± 0.06^{aA}	53.40 ± 0.89^{bC}	39.22 ± 1.46 ^{dB}	45.86 ± 0.02^{cB}	36.98 ± 0.03^{eB}	26.21 ± 0.04^{fC}	25.10 ± 0.70^{fD}	n.a.

* Means (± standard deviation) with different capital letters in the same columns (treatments) and with different lower case letters in the same line (fermentation time) are statistically different (Duncan test, $p \leq 0.05$).
n.a.: not analyzed as fermentation for ScPk was completed in 144 h.
[1] meq. NaOH 0.1 N/100 g: milliequivalent sodium hydroxide solution 0.1 N per 100 g sample.
[2] mg ECE/g: milligram equivalent epicatechin per gram sample.

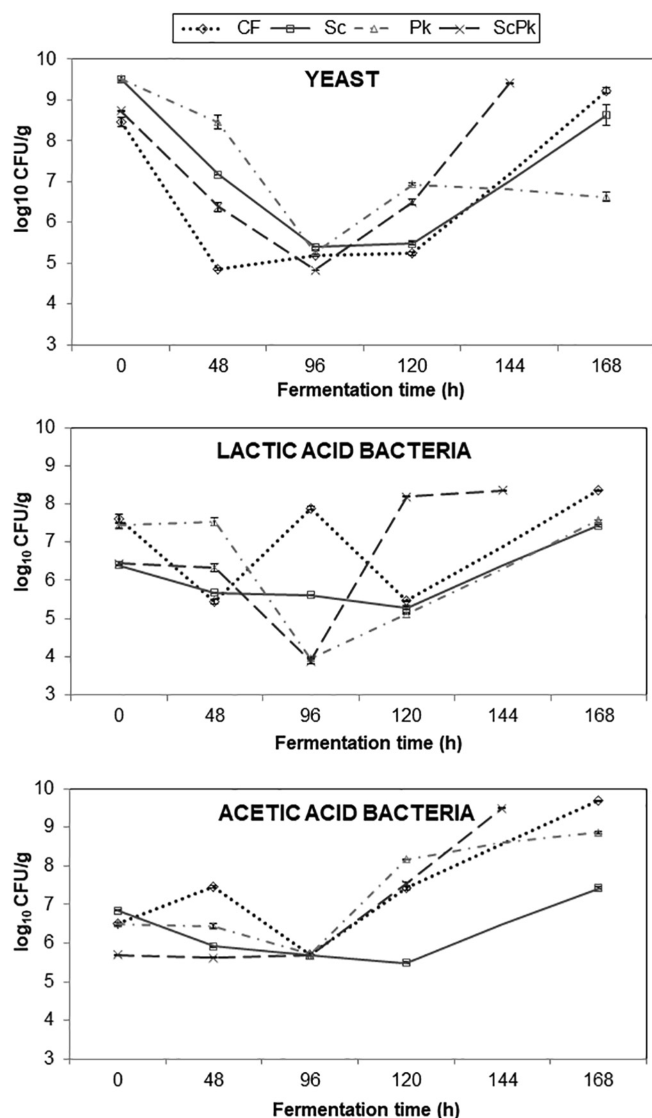

Fig. 1. Counts of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) during on-farm cocoa fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.

The maintenance of the cocoa pH around 5.0 during fermentation is relevant as it allows activity of proteases which are important in the formation of chocolate aroma (Ho et al., 2014). Protein hydrolysis liberates small peptides and amino acids which are substrates for the Maillard reaction during drying and roasting (Figueroa-Hernández et al., 2019). In addition, the acid environment induces the decarboxylation of free amino acids by microbial enzymes producing amines. Under favorable conditions (low pH environment, for example), some microbial species can produce decarboxylase enzymes and produce biogenic amines as a result of the decarboxylation of free amino acids. Some bacterial species of the genus *Lactobacillus*, *Salmonella* and *Staphylococcus* can produce enzymes such lysine, ornithine and phenylalanine decarboxylases, thus warranting survival under this stressful situation, by buffering the pH due to the production of cadaverine, putrescine and phenylethylamine, respectively (Gloria, 2006; do Carmo Brito et al., 2017).

With respect to the reducing sugars, during fermentation there was a significant decrease on TRS levels ($p \leq 0.05$), due to the use of sugar by microorganisms (Moreira et al., 2017). However, at 72 and 96 h fermentation, there were increases on reducing sugars which could be related to the hydrolysis of sucrose by lactic and acetic acids, produced by lactic and acetic acids bacteria, respectively (Schwan & Wheals, 2004; Ho et al., 2014; Vuyst & Weckx, 2016). Glucose, fructose and sucrose are the carbohydrates that are most readily available in the cocoa pulp during the first fermentation times (Schwan & Wheals, 2004). However, sucrose probably is not assimilated by some species of yeast and bacteria. When there is production of lactic and acetic acids there is hydrolysis of the sucrose molecule into glucose and fructose, and these monosaccharides become available for fermentation. Invertase secreted by yeasts also breakdown sucrose in glucose and fructose (Schwan & Wheals, 2004; Ho, Zhao, & Fleet, 2014; Vuyst & Weckx, 2016). In this study, this behavior can be suggested by increasing the amount of TRS between 72 h and 96 h (Table 1).

There was reduction on the contents of TPC during fermentation, with an overall loss of 33 to 60%, which is within the ranges reported in the literature (Efraim et al., 2010; do Carmo Brito et al., 2017). The loss of phenolic compounds is associated with polyphenoloxidase (PPO) activity, which is responsible for the enzymatic browning reactions and the typical brown color of cocoa beans. According to the literature (Hernández-Hernández et al., 2016), the ideal temperature for PPO activity is between 42 and 45 °C. Longer times at this temperature range were observed for treatment Sc (96 h), followed by Pk and CF (72 h), and then ScPk (48 h). Therefore, lower TPC levels would be expected in the Sc treatment, which was not the case. In this context, there might be other factors affecting PPO activity besides temperature, for example, polymerization and exudation of the liquid formed during fermentation (Nazaruddin et al., 2006), which can affect phenolic compounds. The understanding of the reactions behind these changes is important as they affect color, bitterness and astringency of chocolate. At the end of fermentation (144 h), higher TPC levels were found for the Sc treatment, followed by CF and Pk, and by fermentation with ScPk ($p \leq 0.05$).

3.2. Changes on microbial counts during fermentation

The microbial population dynamics among the three major groups of cocoa fermentation microorganisms, yeast, LAB and AAB (Fig. 1), show the change in the predominance of these microorganisms throughout fermentation. This is relevant to warrant the desirable cocoa and chocolate quality. The counts of yeast during on-farm indigenous fermentation (CF) decreased in the first 48 h, remained constant for up to 120 h, and increased afterwards (Fig. 1). The yeast count decrease is probably due to the consumption of readily fermentable carbohydrates by the yeasts in the first fermentation times (Schwan & Wheals, 2004), suggesting that there are no more readily substrates available after 48 h fermentation. However, the microbial count increases again after 96 h can be associated with invertase activity and conversion of sucrose into glucose and fructose (Vuyst & Weckx, 2016), once again providing substrate for microbial growth. However, when yeasts were used as starter cultures (treatments Sc, Pk and ScPk), higher yeasts counts were observed at the beginning of fermentation compared to control and it took longer (96 h) for the yeasts counts to decrease, reaching lower counts at 96 h. This behavior can be explained by the yeast species present in the control fermentation (CF) and in the Sc (*Saccharomyces cerevisiae*) fermentation. It is known that *S. cerevisiae* is one of the most common yeast species present in cocoa fermentations in Brazil (Figueroa-Hernández, Mota-Gutierrez, & Ferrocino, 2019). The inoculum of *S. cerevisiae* may have found a synergy with *S. cerevisiae* naturally present in the CF treatment at 96 h. Future studies on molecular biology are needed to elucidate the microbial dynamics with these inoculants. There was a significant increase on yeasts counts afterwards, and the rates were higher for the treatments with *P. kudriavzevii* (Pk and ScPk) compared to control (CF) and *S. cerevisiae* (Sc) treatments. The final yeast counts (168 h) were lower for Pk compared to the others. In the treatment with both yeasts (ScPk), the higher counts were reached at 144 h, 24 h earlier compared to the other treatments.

For the lactic acid bacteria – LAB, during control fermentation (CF), the counts varied throughout fermentation (Fig. 1), decreasing up to 48 h, increasing up to 96 h, decreasing again up to 120 h, and increasing until the end of fermentation. However, when yeasts were inoculated, no increase on LAB counts was observed at 96 h; moreover, there was a decrease on LAB counts at 96 h when *P. kudriavzevii* was inoculated (both Pk and ScPk treatments). This result suggests that the inoculation of yeast inhibited the initial growth of LAB (up to 96 h), and that the inhibition was more pronounced when *P. kudriavzevii* was in the starter culture. However, the combination of yeasts (ScPk) provided a faster recovery of LAB counts, reaching higher counts earlier, at 120 h fermentation.

The changes on acetic acid bacteria – AAB also varied throughout fermentation. There was an initial increase on AAB counts in the control fermentation (CF) in the first 48 h, followed by a decrease at 96 h and increased afterwards (Fig. 1). However, when yeasts starter cultures were inoculated (Sc, Pk and ScPk), the counts remained similar up to 96 h, increasing afterwards with a sharper increase for the ScPk treatment, reaching higher counts (≈10 log10 CFU/g) at 144 h. Similar counts were obtained for CF and Pk at 168 h; whereas in the Sc treatment, final AAB counts only reached 7 log10 CFU/g at the end of fermentation.

Briefly, during natural cocoa fermentation (CF), the succession of microorganisms was as follows: initially, AAB, LAB and yeasts were present with higher counts for yeasts, followed by LAB and AAB (10^9, 10^7 and 10^6, respectively); at 48 h there was predominance of AAB; followed by LAB at 96 h; and, at the end of fermentation (168 h), AAB, LAB and yeast were present with higher counts for yeasts and AAB (10^9) followed by LAB (10^8) (Fig. 1). These results are similar to literature reports (Visintin, Alessandria, Valente, Dolci, & Cocolin, 2016). The use of yeasts as starter cultures modulated the counts of microorganisms throughout fermentation. It attenuated the increases on LAB and AAB counts, reduced the decrease in yeasts, resulting in similar counts at the end of fermentation. In addition, by adding both *S. cerevisiae* and *P. kudriavzevii* simultaneously, the time required for fermentation was shortened.

3.3. Influence of starter culture on the physico-chemical characteristics of the fermented and dried beans

The reduction on the moisture content immediately after fermentation is required to terminate microbial activities; some chemical reactions start or continue during the drying process, including Maillard reaction, which plays an important role in cocoa and chocolate quality (Efraim et al., 2010). The characteristics of the on-farm fermented and dried cocoa are indicated in Table 3. Fermented cocoa beans had moisture content values from 5.56% to 5.72%, which were not significantly different ($p > 0.05$, Duncan's test) among treatments. However, significant difference was observed for the other parameters analyzed ($p < 0.05$, Duncan's test).

The pH values varied from 5.26 to 5.56, with higher values for control (CF) and the treatment containing both yeasts (ScPk) (5.56 and 5.54, respectively), whereas the treatments with individual yeasts provided lower values, with the lowest pH for the Sc treatment. Total titratable acidity varied from 17.55 to 22.86 meq NaOH/100 g. In accordance with pH values, lower acidity was observed for CF and for the treatment containing both yeasts (ScPk), and higher values were observed for the Sc treatment. Based on these results, the treatment with both yeasts (ScPk) was not different ($p > 0.05$) from the typical natural on-farm fermentation (CF), and had the lowest acidity, which is desirable for high quality chocolate. The product with the highest acidity was obtained with *S. cerevisiae*, suggesting that the predominance of this yeast can lead to lower pH compared to control fermentation.

The levels of total phenolic compounds varied from 26.35 to 32.25 mg ECE/g, with lower levels for CF and Sc treatments (≈26 mg ECE/g) and higher levels for the Pk treatment (32.25 mg ECE/g). Higher levels of total phenolic are desirable due to the antioxidant properties of these compounds, but they are also associated with the bitterness of the product (Lima et al., 2011). Therefore, the possibility of modulating total phenolic levels in cocoa during fermentation would be relevant in the optimization of chocolate quality.

Catechin and epicatechin are also phenolic compounds which contribute to the antioxidant activity, bitterness and astringency (Efraim et al., 2010). The CF treatment provided the lowest levels (0.05 and 0.32 mg/g, respectively), whereas the treatment with both yeasts (ScPk) the highest levels (2.19 and 3.14 mg/g, respectively). These compounds also affect product quality, therefore, the possibility of modulating their concentration in the final product is desirable. During cocoa fermentation, the microorganisms can hydrolyze phenolic complexes (which are linked to sugars reducing availability) into free and simple-soluble phenols increasing the levels of these compounds. The modification of the cellular structure of the cocoa cotyledon during the drying process, can release these compounds thus increasing their quantity (Haile & Kang, 2019; Ooi, Ting, & Siow, 2020). There is evidence that yeasts species like *Pichia kudriavzevii* provide the release of these compounds as well, as the drying process in recent laboratory scale studies (Ooi et al., 2020).

Among the three methylxanthines investigated (Table 3), only theobromine and caffeine were detected, whereas theophylline was not (LOQ = 0.02 mg/g). Higher levels of methylxanthines were present in the ScPk treatment (10.83 mg/g) compared to the others. These methylxanthines are exuded from the cocoa bean testa (cocoa bean shell) (Nazaruddin et al., 2006) and the use of both yeasts (ScPk) could have facilitated its release. The possibility of modulating theobromine and caffeine levels in cocoa is also interesting as these compounds are associated with several functional and pharmacological properties, including reduction of stress and beneficial neurophysiological effects (Sansone et al., 2017; Todorovic, Redovnikovic, Todorovic, Jankovic, Dodevska, & Sobajic, 2015). However, in large amounts, caffeine can trigger agitation, hypertension and insomnia (Martínez-López et al., 2014).

Among the nine bioactive amines investigated, only three were detected in the fermented and dried cocoa beans: putrescine, cadaverine and phenylethylamine (Table 3). Higher total amines levels were found in the Sc treatment (7.10 mg/kg) followed by CF and Pk (4.6–4.8 mg/kg), which were higher compared to the treatment with mixed starter culture – ScPk (2.42 mg/kg).

The highest levels of cadaverine and putrescine were observed in the Sc treatment, suggesting that added *S. cerevisiae* could induce production of these amines during fermentation. Putrescine can be formed directly from ornithine by decarboxylase activity and it is the precursor in the formation of the polyamines which are relevant in several vital functions for cell growth and maintenance (Tachihara, Uemura, Kashiwagi, & Igarashi, 2005; Gloria, 2006). The formation of cadaverine was reported by *S. cerevisiae* exposed to 12% ethanol for 24 h (Walters & Cowley, 1998). Since both putrescine and cadaverine can contribute with putrefactive sensory characteristics to the product (Tufariello et al., 2019), the minimization of the formation and accumulation of these amines is desirable. When using the treatment with both yeast – ScPk, putrescine and cadaverine were not detected, therefore, it would be the treatment of choice to prevent the formation and accumulation of these putrefactive amines.

Phenylethylamine was also detected in every dried fermented cocoa. Higher levels (2.30–2.42 mg/kg) were detected in *P. kudriavzevii* treatments (Pk and ScPk), suggesting that this yeast can be associated with the buildup of this amine. Furthermore, phenylethylamine can be formed during cocoa dehydration, due to release from conjugated amines or to the oxidative decarboxylation of the precursor amino acid (phenylalanine) at high temperatures (Oracz & Nebesny, 2014). Phenylethylamine is an important hypothalamic stimulating amine and, thus, can induce feelings of well-being and affect the levels of serotonin

Table 3
Mean values of moisture content, pH, total titratable acidity, methylxanthines and bioactive amines in fermented and dried cocoa beans submitted to on-farm fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017.

Parameters	Mean values*/Fermentation treatment			
	CF	Sc	Pk	ScPk
Moisture content (%)	5.65 ± 0.08[a]	5.72 ± 0.07[a]	5.56 ± 0.12[a]	5.71 ± 0.06[a]
pH	5.56 ± 0.02[a]	5.26 ± 0.04[c]	5.39 ± 0.01[b]	5.54 ± 0.03[a]
Total titratable acidity (meq. NaOH 0.1 N/100 g)[1]	18.02 ± 0.60[c]	22.86 ± 0.57[a]	19.63 ± 0.06[b]	17.55 ± 0.99[c]
Total phenolic compounds (mg ECE/g)[2]	26.35 ± 0.93[c]	26.59 ± 1.07[c]	32.25 ± 0.35[a]	29.05 ± 0.54[b]
Monomeric compounds (mg/g)				
Catechin	0.05 ± 0.00[d]	0.61 ± 0.03[c]	1.88 ± 0.11[b]	2.19 ± 0.07[a]
Epicatechin	0.32 ± 0.05[c]	2.39 ± 0.01[b]	2.44 ± 0.09[b]	3.14 ± 0.13[a]
Methylxanthines (mg/g)*				
Theobromine	7.40 ± 0.02[a]	5.53 ± 0.74[b]	7.19 ± 0.01[a]	8.05 ± 0.64[a]
Caffeine	0.93 ± 0.18[c]	1.27 ± 0.04[b]	1.22 ± 0.05[b]	2.78 ± 0.09[a]
Total	8.33 ± 0.19[b]	6.8 ± 0.70[c]	8.41 ± 0.04[b]	10.83 ± 0.73[a]
Bioactive amines (mg/kg)**				
Putrescine	1.13 ± 0.08[b]	2.22 ± 0.07[a]	1.05 ± 0.14[b]	0[c]
Cadaverine	1.79 ± 0.22[b]	3.32 ± 0.12[a]	1.49 ± 0.36[b]	0[c]
Phenylethylamine	1.64 ± 0.20[b]	1.56 ± 0.02[b]	2.30 ± 0.00[a]	2.42 ± 0.02[a]
Total	4.56 ± 0.51[b]	7.10 ± 0.21[a]	4.84 ± 0.21[b]	2.42 ± 0.02[c]

*Means (± standard deviation) with different letters in the same line (treatment) are statistically different (Duncan test, $p \leq 0.05$).
*Theophylline was not detected in any treatment. LOQ = 0.02 mg/g.
**Only 3 out of 9 amines were detected. Tyramine, histamine, serotonin, agmatine, spermidine and tryptamine were not detected in any sample. LOQ = 0.04 mg/kg.
[1] meq. NaOH 0.1 N/100 g: milliequivalent sodium hydroxide solution 0.1 N per 100 g sample.
[2] mg ECE/g: milligram equivalent epicatechin per gram sample.

and brain endorphins, improving mood (Afoakwa, 2008). But at high levels (≥30 mg/kg), this amine can be detrimental to human health, inducing migraines (Oracz & Nebesny, 2014; Jeon, Lee, & Mah, 2018). The levels of phenylethylamine obtained with the different treatments are far below the values capable of causing adverse effect to human health.

Based on these results, by using the ScPk treatment, a product with better characteristics was obtained compared to control and the use of the yeasts individually: higher pH, levels of total phenolic compounds, monomeric compounds, methylxanthines and phenylethylamine, and lower titratable acidity and levels of the putrefactive amines (putrescine and cadaverine). Furthermore, this product would have better functional properties associated with methylxanthines, monomeric compounds and phenylethylamine.

3.4. Differentiation of fermented cocoa from different treatments by multivariate analysis

In order to better understand which factors were affected by the different starter cultures used during on-farm cocoa fermentation, PCA and HCA were used. In the four fermentation treatments, the first two components (PC1 + PC2) accounted for approximately 83, 88, 75 and 92% of the data variance for CF, Sc, Pk and ScPk, respectively (Fig. 2).

For all the treatments, fermentation was separated into three clusters (HCA), which, based on the parameters included in this study, represented three fermentation momentums: (i) group 1 – 0 and 24 h, which was characterized by highest pH, TRS and TPC, i.e., the beginning of the fermentation process; (ii) group 2 – 48, 72, 96 and 120 h for CF, Sc and Pk treatments; and 48, 72 and 96 h for ScPk treatment, intermediate fermentation times, which were characterized by lower TPC and pH, and higher TTA and temperature; and (iii) group 3 – 144 and 168 h for CF, Sc and Pk treatments; and 120 and 144 h for ScPk treatment, which were characterized by the high counts of yeasts, LAB and AAB. Based on these results, ScPk differed from the other treatments as the fermentation process was sped up and the end of fermentation was achieved faster (144 h compared to 168 h). Moreover, the three groups were better segregated compared to the others and PC1 + PC2 accounted for the highest percentage of the data variance (92%).

A second PCA and HCA study (Fig. 3) compared dried cocoa submitted to the four different fermentation treatments and involved several components but also included monomeric phenols, methylxanthines and bioactive amines. According to Fig. 3, PC1 + PC2 accounted for approximately 91% of the data variance. There was a negative relationship between pH and cadaverine ($r = -0.76$) and putrescine ($r = -0.79$), indicating that low pH is a favorable environment for the formation of these amines (Gloria, 2006). The same behavior was found in the fermentation of cocoa and manipueira for tucupi (do Carmo Brito et al., 2017, 2019). CF and Sc treatments were characterized by higher TTA, putrescine and cadaverine, whereas Pk and ScPk were characterized by higher concentrations of TPC, monomeric phenolic compounds (catechin and epicatechin), methylxanthines (theobromine and caffeine) and phenylethylamine. HCA separated well these two groups. Based on these results, the treatment with *S. cerevisiae* (Sc) was similar to control and the presence of *P. kudriavzevii* affected the quality of the dried cocoa, setting them apart from CF. In fact, the treatments containing *P. kudriavzevii* provided products with better functional properties, associated with methylxanthines (neurophysiological and cardiovascular properties), phenolic compounds (antioxidant activity) and phenylethylamine (mood modulation). These characteristics were restricted to fermented cocoa beans with the addition of *P. kudriavzevii* – Pk and ScPk, therefore, enhancing the role of this yeast in cocoa fermentation.

When considering the time taken for fermentation to end, the ScPk treatment achieved final desirable characteristics 24 h shorter compared to the other treatments. Based on these results, the use of the two yeast species, together and in equal proportions, provided conditions that favored the active microbial performance generating acceptable and unprecedented indices to the process, reported for the first time in the literature on yeast starter cultures for cocoa fermentation in the Amazon region.

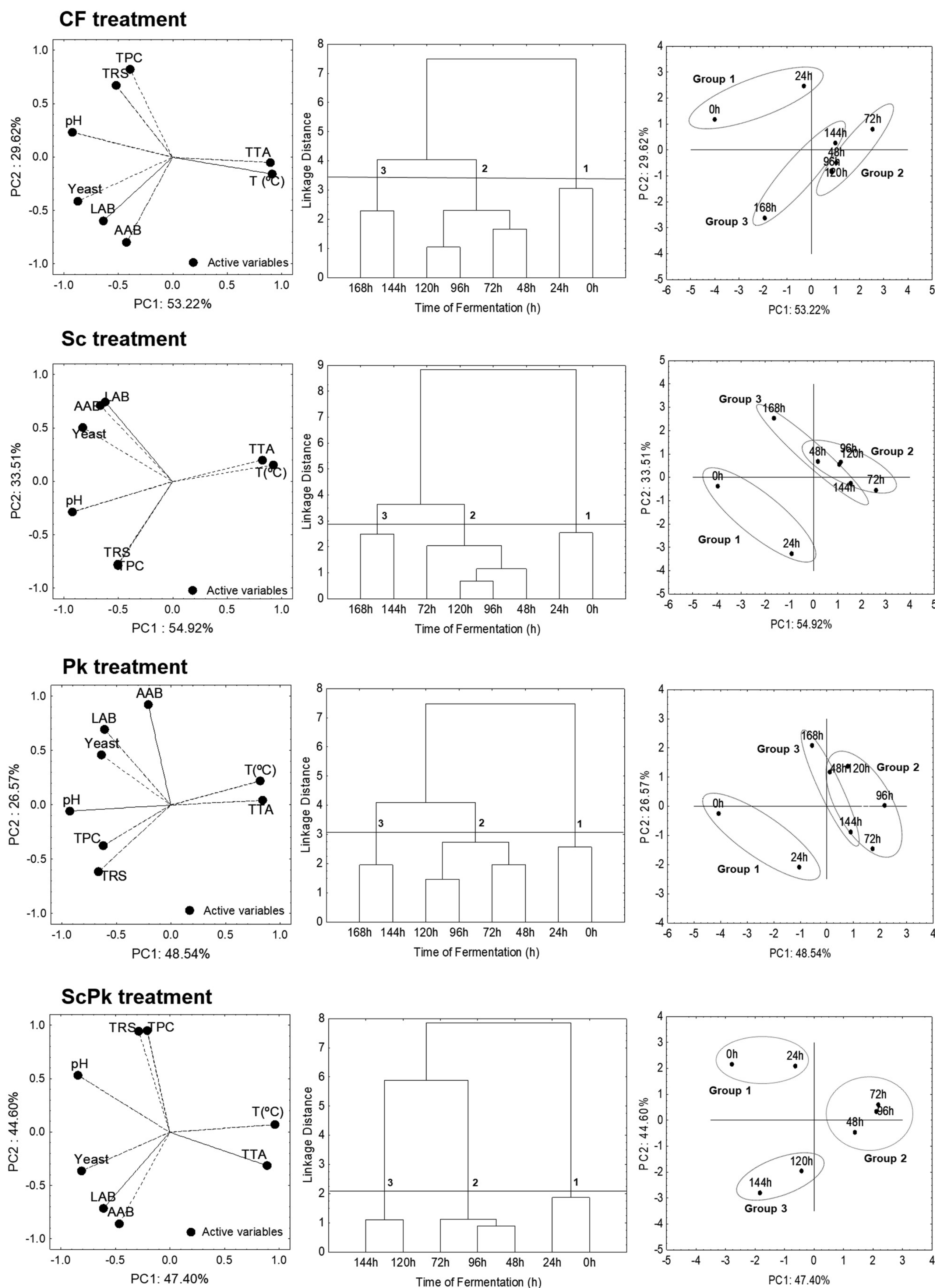

Fig. 2. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) during on-farm cocoa fermentation with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; and ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017. T – temperature, TTA – total titratable acidity, TPC – total phenolic compounds, TRS – total reducing sugars, LAB– lactic acid bacteria, AAB – acetic acid bacteria.

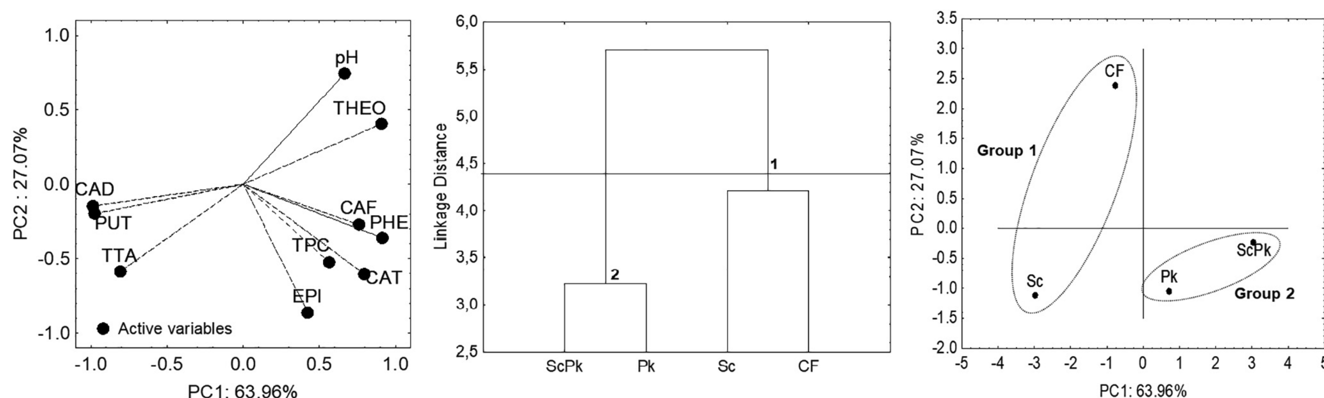

Fig. 3. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) of on-farm dried and fermented cocoa beans with different starters: CF – control, Sc – *Saccharomyces cerevisiae*, Pk – *Pichia kudriavzevii*; and ScPk – both *S. cerevisiae* and *P. kudriavzevii* in Tomé-Açu, PA, Brazil, 2017. TTA – total titratable acidity, TPC – total phenolic compounds, *EPI* – epicatechin, CAT – catechin, THEO – theobromine, CAF – caffeine, CAD – cadaverine, PUT –putrescine, PHE – phenylethylamine.

4. Conclusions

Significant differences were observed between the four treatments (CF, Sc, Pk, ScPk) with respect to pH, total titratable acidity, total phenolic compounds, total reducing sugars and temperature during fermentation. In addition, fermentation with ScPk was completed 24 h earlier. Fermented and dried cocoa beans from treatments with *P. kudriavzevii* (Pk and ScPk) showed higher amounts of phenolic compounds and methylxanthines, showing the influence of this species in increasing antioxidant capacity. The synergy of ScPk inhibited putrefactive amines and enhanced phenylethylamine, a mood modulating amine. PCA and HCA analyses confirmed previous results and provided clusters which clearly differentiated the characteristics of the treatments. Therefore, the addition of starter culture to cocoa prior to fermentation can modulate chocolate quality and functional properties. The promising results observed show the need of investment in studies aimed at modulating the quality of fermented and dried cocoa beans to warrant high-quality chocolate with desirable chemical and flavor characteristics associated with the new consumer demands.

CRediT authorship contribution statement

Gilson Celso Albuquerque Chagas Junior: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **Nelson Rosa Ferreira:** Conceptualization, Data curation, Methodology, Writing - review & editing. **Maria Beatriz A. Gloria:** Data curation, Formal analysis, Investigation, Funding acquisition, Methodology, Writing - review & editing. **Luiza Helena da Silva Martins:** Investigation, Writing - review & editing. **Alessandra Santos Lopes:** Conceptualization, Project administration, Methodology, Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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