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Saccharomyces cerevisiae and *Pichia manshurica* from Amazonian biome affect the parameters of quality and aromatic profile of fermented and dried cocoa beans

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Abstract: The use of yeasts as starter cultures is a promising alternative to produce fermented cacao with particular characteristics regarding the quality of aromas and physical and chemical characteristics that are accepted by the chocolate market. This study aimed to evaluate the physical and chemical transformations of cocoa beans during fermentation after inoculation with starter cultures of yeast species *Pichia manshurica* (PF) and *Saccharomyces cerevisiae* (SF), both previously isolated in cocoa bean fermentations in the Brazilian Amazon, in comparison with a fermentation without the inoculum addition (CF). During the fermentation time, which was carried out on a cocoa farm in Igarapé-Miri (Amazon biome, Pará, Brazil), the contents of phenolic compounds (catechin and epicatechin), sugars (glucose, fructose, and sucrose), acetic acid, and ethanol were monitored by HPLC, and the volatile compounds profiles were assessed by GC-MS. The starter culture of *P. manshurica* was able to produce fermented cocoa beans with highly desirable characteristics for the production of good quality chocolate: low acidity, a broad variety of aromatic compounds with floral, fruity, and sweet characteristics, in addition to showing high contents of catechin and epicatechin, which are known by their antioxidant properties. Therefore, the use of starter cultures with species of yeasts isolated in the Amazon region, during cocoa fermentation, is an alternative to obtain fermented seeds with high quality favoring the commercial agreements in the chocolate market by cocoa producers.

KEYWORDS
benzaldehyde, chocolate, epicatechin, fermentation, GC-MS

Practical Application: The addition of starter cultures was able to produce cocoa beans with good quality. Yeasts species isolated and identified in Amazonian cocoa fermentation can improve the profiles of aromatic compounds. Catechin and epicatechin contents are higher in inoculated cocoa beans fermentations.

1 | INTRODUCTION

Originating in the Amazon biome, cacao (*Theobroma cacao* L.) is a fruit of great economic importance in the globe, as its seeds are the raw material for the production of one of the most appreciated food product worldwide: chocolate. In 2020, the Brazilian market registered US$100.6 million in chocolate exports (ABICAB, 2021; Lima et al., 2011; Ozturk & Young, 2017).

On the international background, according to the International Cocoa Organization (ICCO), Brazil occupies the sixth largest world production of cacao seeds, being surpassed only by countries such as Ivory Coast, Ghana, Ecuador, Cameroon, and Nigeria (ICCO, 2021). In Brazil, Pará State was responsible for the largest production of fermented and dried cocoa beans in 2020 with 144,216 tons produced, which corresponds to about 53% of total Brazilian production (Agência Pará, 2021).

During cacao processing, fermentation can be seen as one of the essential steps since many of the physical and chemical transformations that take place ultimately impact chocolate flavor. It is a natural and spontaneous biological process characterized by the participation of a microbial consortium composed by groups of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (De Vuyst & Leroy, 2020; Schwan & Wheals, 2004). Each microbial group grows when conditions for it are favorable, being impacted by characteristics such as length of fermentation, temperature, oxygenation due to cocoa turning, pH, total acidity, and presence of other microorganisms (Camu et al., 2007; Crafack et al., 2013; Ramos et al., 2014).

The key role that some yeast species play during fermentation processes is well known. The species *Saccharomyces cerevisiae* and some of the genus *Pichia* spp. have been identified as being responsible for the significant production of certain aroma compounds important for chocolate flavor, like aldehydes, esters, and ketones (Bastos et al., 2018; Batista et al., 2015; Chagas Junior, Ferreira, Andrade, et al., 2021; Koné et al., 2016; Visintin et al., 2017). Furthermore, food products such as chocolate and beverages made with cocoa beans fermented with starter cultures of selected yeast species were reported to be better accepted by consumers in sensory analysis (Visintin et al., 2017).

In previous studies carried out by our research group, yeast species isolated and identified during fermentation of cocoa in several cities in the Pará State (Northern Brazil) provided fermented beans with distinct chemical characteristics concerning the total acidity, amount of antioxidant compounds and aromas (Almeida et al., 2019; Chagas Junior, do Espírito-Santo, et al., 2020; Chagas Junior, Ferreira, Andrade, et al., 2021; Gaspar et al., 2021; Serra et al., 2019), which may explain the current prominence of Pará fermented and dried cocoa beans in the market and in international chocolate festivals (CEPLAC, 2021).

The addition of starter cultures in the cocoa fermentation has been subject of scientific researches in several countries, as reviewed elsewhere (Figueroa-Hernández et al., 2019), as it provides a standardization of this process. The use of specific microorganisms as starter cultures can reduce the fermentation time, produce selected aromatic compounds, and inhibit putrefactive compounds (Chagas Junior, Ferreira, Andrade, et al., 2021).

The aim of this study was to evaluate the physical and chemical changes ascribed to cocoa beans during fermentation by starting cultures made up of *S. cerevisiae* or *P. manshurica*. Three fermentations (one without starting culture, one with *S. cerevisiae* inoculum, and one with *P. manshurica* inoculum) were evaluated for quality metrics such as pH, total acidity, sugar and phenolic component concentrations, acetic acid, ethanol, and aromatic profile.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Yeast extract was purchased from BD (Franklin Lakes, NJ, USA), saline peptone water, and bacteriological peptone purchased from Neogen (Lansing, MI, USA). Analytical Grade reagents and solvents: sodium hydroxide, acetone, acetic acid, sulfuric acid HPLC grade, methanol HPLC grade, and acetonitrile HPLC grade were purchased from Dinâmica (Indaiatuba, SP, Brazil). Glucose, pentane, Folin-Ciocalteu Reagent, Standards of catechin, epicatechin, acetic acid, and ethanol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ethanol and

n-hexane, both from analytical grade, were from J.T. Baker-Avantor (Radnor, PA, USA) and Synth (Diadema, SP, Brazil), respectively.

The extracts for the HPLC analysis were filtered through 0.45-μm membranes (Analítica, São Paulo, SP, Brazil), and the ultrapure water was obtained by the MilliQ system (Millipore Corp., Milford, MO, USA). The ultrapure water and solvents used in the analysis were filtered twice using 0.22-μm membranes (Sartourius Stedim Biotech GmbH, Göttingen, Germany).

2.2 | Reactivation of yeast strains and preparation of starter cultures

Strains of *Pichia manshurica* (PF, GenBank Access KU316788.1) and *Saccharomyces cerevisiae* (GenBank Access EU441887.1) used in this research were isolated by our research group during natural cocoa fermentation in Medicilância city and were chosen because they were prevalent in cocoa fermentation in Medicilância city (Almeida et al., 2019). The fermented and dried cocoa beans from this city is recognized by the good quality, and this can be associated with the fermentation's microbiota. All the strains were stored under freezing (−18°C) in the Bank of Microorganisms of the Laboratory of Biotechnological Processes of Federal University of Pará (LABIOTEC, UFPA, Brazil) and registered in Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen, A38D99F and A98D922, *P. manshurica* and *S. cerevisiae*, respectively).

In aseptic conditions, the strains were reactivated in Erlenmeyers flasks containing 100 ml of sterile yeast-extract-peptone-dextrose (YPD) broth (20 g/L glucose, 20 g/L bacteriological peptone, 10 g/L yeast extract, 1000 ml distilled water), and incubated in an orbital shaker at 150 rpm, 30°C/12 h (Baffi et al., 2011). For each culture, the reactivated strains were transferred to a bench bioreactor (FerMac 320, Electrolab Biotech, Tewkesbury, UK) containing 900 ml of sterile YPD broth under constant agitation at 150 rpm, at 30°C until counting 10^8 cells/ml (Chagas Junior, Ferreira, Gloria, et al., 2021; Ramos et al., 2014). After growing in the bench bioreactor, the cells were centrifuged (1700*g*, 4°C, 10 min), and the biomass was diluted in 1 L of sterile saline peptone water and stored up to 24 h under refrigeration (4°C) until use.

2.3 | Cocoa fruits and fermentation of cocoa seeds

The cocoa fruits (Forastero variety) were harvested in February 2018 at *Fazenda Cataiandeua*, in Igarapé-miri city, Pará, Brazil (latitude 01°58′30″S and longitude 48°57′35″W), manually opened with a stainless knife, and the seeds with pulp were separated from the endocarp and transferred to fermentation boxes (1.0 m length × 0.4 m width × 0.3 m height). The fermentation process was carried out in wooden boxes, in triplicate ($n = 3$), with approximately 45 kg of seeds in each box.

Three fermentation experiments were carried out at the same farm: CF—Control fermentation (spontaneous) without any starter culture addition; SF—fermentation with inoculum of *S. cerevisiae* (10^6 cells/g of cocoa), and PF—fermentation with inoculum of *P. manshurica* (10^6 cells/g of cocoa) (Visintin et al., 2017).

The previously prepared inoculum composed by each microorganism was sprayed (approximately 333 ml per box × 3 boxes with different starter culture) and distributed evenly in three layers on the cocoa seeds, reaching a concentration of approximately 10^6 cells/g of cocoa (Visintin et al., 2017). The wooden boxes containing the cocoa seeds remained covered with banana leaves for 7 days (168 h), with mass turning every 24 h, according to the methodology carried out by the producer.

Approximately 300 g of cocoa seeds were collected (composed of portions taken from different places in the fermentation boxes) at times 0, 48, 96, and 168 h (days 1, 2, 4, and 7 of the fermentation). The collected samples were stored in sterile polyethylene bags, frozen (−18°C), and transported in coolers with reusable ice sheets until arrival at the laboratory and stored (−18°C) until analysis. After 7 days of fermentation, the fermented cocoa beans were subjected to a natural drying process on wooden platforms in the open for 72 h with direct sunlight, and it was turned every 12 h. This process aimed to reduce the moisture content reached the range of 6–7% (Efraim et al., 2010).

2.4 | Physical and physicochemical analyses of cocoa beans during fermentation

The temperature during the fermentation processes was measured in the middle of the fermentation mass at three different points of the fermentative mass ($n = 3$).

The cocoa beans were manually peeled, and the embryo was removed (also in fermented and dried samples) for subsequent grinding of the cotyledons in an analytical mill (model A11, IKA, Staufen, Germany) for pH (method 970.21), moisture content (method 931.04), and total titratable acidity (TA, method 31.06.06), as per the Association of Official Analytical Chemists guidelines (Horwitz & Latimer, 2006). All of the analyses were carried out in duplicate.

2.5 | Determination of catechin and epicatechin during fermentation

Catechin and epicatechin were determined in the cocoa beans after extraction with ethanol/water solution (1:1, v/v) in an ultrasonic bath (model Q3.0/40A, Ultronique, Indaiatuba, SP, Brazil) for 10 min at 25°C, followed by centrifugation 1500*g* for 10 min, and the supernatants were filtered through 0.22-μm syringe filters before the injection into the HPLC-DAD system (Chagas Junior, Ferreira, Gloria, et al., 2021).

The extracts (20 μl) containing the phenolic compounds were analyzed by HPLC (mod. 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) equipped with a C18 column (20 cm; 4.6 mm×150 mm, 5 μm, Agilent Technologies) at 25°C, and the compounds were monitored by a diode array detector (DAD) set at 280 nm. The mobile phase consisted of an aqueous solution of water/acetonitrile (99.8:0.2, eluent 1) and methanol (eluent 2). The compounds were separated at a flow rate of 1.2 ml/min by a linear gradient from 0% to 50% over the first 12 min, and then increased from 50% to 100% over 13–20 min (Chagas Junior, Ferreira, Gloria, et al., 2021; He et al., 2010). Catechin and epicatechin were identified based on the co-elution with authentic standards and quantified by analytical curves of catechin (3.125–50 μg/ml, $r^2 > 0.99$, LOQ = 0.31 mg/g, LOD = 0.10 mg/g) and epicatechin (3.125–100 μg/ml, $r^2 > 0.99$, LOQ = 0.11 mg/g, LOD = 0.04 mg/g) (Table 1). The results were expressed in mg/g.

2.6 | Determination of carbohydrates (glucose, fructose, and sucrose) ethanol, and acetic acid

The cocoa seeds collected during fermentation and after drying (10 g) were homogenized in Falcon tubes containing 10 ml of sterile ultrapure water, vortexed twice for 5 min. At each step, the liquid supernatant was transferred to another test tube and centrifuged at 4400*g* for 10 min at 4°C. The precipitate was discarded, the supernatant was diluted with sterile ultrapure water, and aliquots were filtered before injection into the HPLC for the quantification of carbohydrates, acetic acid, and ethanol (Ramos et al., 2014; Rodriguez-Campos et al., 2011).

Filtered aliquots (20 μl) were injected into an HPLC system (Finnigan Surveyor model, Thermo-FisherScientific, Waltham, MA, USA) equipped with a refractive index detector (RID) at 30°C and ion exchange column (300 × 7.8 mm, Aminex HPX-87H, Bio-Rad Laboratories Inc., Hercules, CA, USA), and the compounds were separated with sulfuric acid 5-mM solution as mobile phase at a flow rate of 0.6 ml/min (Martins et al., 2015).

The compounds were identified by co-elution with authentic standards under the same conditions and quantified by external analytical curves for each compound (Table 1). All the analyses were performed in triplicate, and the results were expressed in mg/g (wet or dry basis).

2.7 | Extraction and identification of volatile compounds in fermented and dried cocoa beans

The fermented and dried cocoa beans were crushed in an Ika mill (model A11B), and 15 g was added in a balloon with distilled water and exposed to a 2-h simultaneous distillation–extraction process using pentane as solvent. The volatile concentrate was injected into a gas chromatograph linked to a mass spectrometer (GC-MS, Shimadzu Corp., Kyoto, Japan), which was fitted with a DB-5MS column (30 m × 0.25 mm × 0.25 μm) (Chagas Junior, Ferreira, Andrade, et al., 2021; Gaspar et al., 2021).

The oven temperature was adjusted from 60°C to 250°C, using a ramp of 3°C/min, and helium was used as a carrier gas, with a flow of 1.2 ml/min. A mass spectrometer with an electron ionization source (model GC-2010A, Shimadzu Corp.) at 70 eV with the temperature of the ion source at 220°C was used. The quantification of the volatile compounds was carried out by peak-area normalization using flame ionization detector (FID, QP 2010 system, Shimadzu Corp.) and the same conditions used in the GC-MS, except for the carrier gas, which was hydrogen (da Silva Júnior et al., 2021). The identification was carried out by the comparison of the mass spectra and retention index (RI) with those of standard compounds existing in the system library and with data from literature (ADAMS, 2007). Retention index was obtained using a homologous series of *n*-alkanes (C8-C24) (Adams, 2007; Mondello, 2015; Stein et al., 2011).

2.8 | Cut test

About 100 fermented and dried cocoa beans from each treatment were submitted to the cut test, which consisted of cutting each cocoa bean longitudinally and evaluating the formation of the internal compartments and color. The cocoa beans were classified according recent studies (Koné et al., 2020; Papalexandratou et al., 2019): well fermented, semifermented, unfermented, slate-like cocoa bean, moldy cocoa bean, and infested by insects. All the analyses were performed in triplicate.

TABLE 1 Analytical curves, limits of quantification (LOQ), and detection (LOD) for the compounds analyzed by HPLC

Compound	Standard curve: Concentration range (g/L)	Linear equation (R^2)	LOQ (mg/g)	LOD (mg/g)
Carbohydrates				
Sucrose	0.005–3	$y = 0.0000003x + 0.11$ (> 0.98)	0.92	0.30
Glucose	0.005–3	$y = 0.0000005x +$ 0.0192 (> 0.999)	0.08	0.03
Fructose	0.005–3	$y = 0.0000005x +$ 0.0209 (> 0.999)	0.08	0.03
Phenolic compounds				
Catechin	3.125–50	$y = 0.000001x +$ 0.5831 (> 0.999)	0.31	0.10
Epicatechin	3.125–100	$y = 0.0000004x +$ 0.9615 (> 0.999)	0.11	0.04
Other compounds				
Acetic acid	0.1–1	$y = 0.000001x - 4926$ (> 0.999)	0.05	0.02
Ethanol	0.2–3	$y = 0.000001x$ +0 .05		
(≥0.999)	0.11 mg/g	0.04 mg/g		

Note: R^2—Determination coefficient of linear regression.

2.9 | Statistical analysis

The means obtained were compared by analysis of variance (ANOVA) and compared by Duncan's test (5% significance, $p \leq 0.05$) using the Statistica 7.0 software (STATSOFT INC., 2004).

3 | RESULTS AND DISCUSSION

3.1 | Physicochemical characteristics of cocoa beans during fermentations

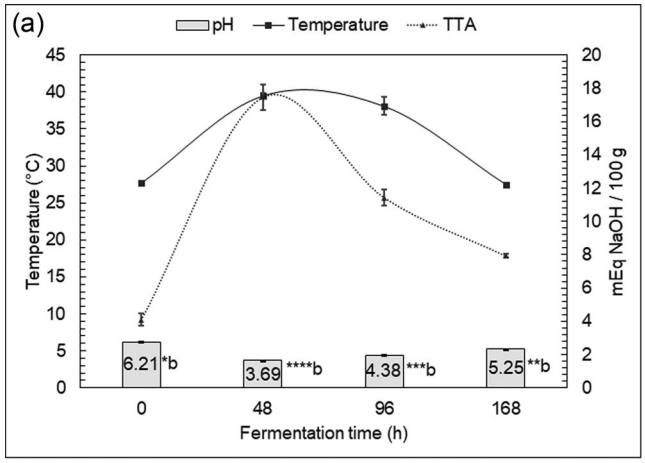
The physicochemical parameters found for the cocoa beans during fermentation can be found in Figure 1. As in previous studies on cocoa fermentation (Brito et al., 2017; Chagas Junior, Ferreira, Gloria, et al., 2021), our results characterized all the treatments (CF, PF, and SF) as typical: temperature and total acidity (TA) increased throughout the fermentation until reaching their maximum values and started to decrease; the pH values decreased until intermediate fermentation times followed by an increase thereafter.

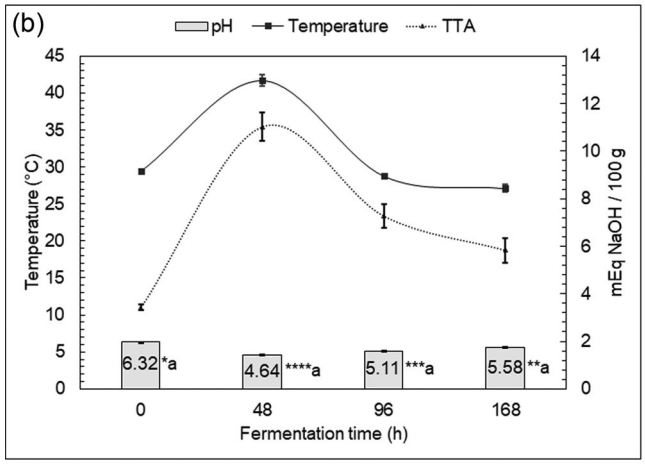
In all the treatments, the temperature reached its maximum values within 48 h of fermentation: 39.5°C (CF), 41.73°C (PF), and 39.47°C (SF), differing statistically ($p < 0.05$, Duncan test). The highest temperature was found in the fermentation with *P. manshurica* (PF, ≈41°C). According to the literature, in treatments with the addition of starter cultures with some species of yeasts such as *P. kudriavzevii*, temperatures reach their peak due to the microbial load added to the medium, which can provide an increase in metabolic speed (Chagas Junior, Ferreira, & Lopes, 2020).

The pH and TA values are related to the acids that are produced by the metabolism of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) found during cocoa fermentation (Chagas Junior, Ferreira, & Lopes, 2020). Some LABs species use the glucose and fructose (fructophilic LAB species) found in the pulp surrounding the seeds and convert them into lactic acid, acetic acid, ethanol, manniton, and CO_2(De Vuyst & Leroy, 2020). In turn, AAB use ethanol produced by yeasts in the anaerobic phase to generate acetic acid (first 48 h of fermentation). This reaction is highly exothermic and increases the fermentation temperature to values above 40°C, making it possible to trigger various chemical and structural reactions in cocoa seeds, which in turn, lose their germination capacity (De Vuyst & Leroy, 2020).

These reactions are essential for the promotion of good quality fermented cocoa beans accepted by the market, as they visibly indicate whether a fermentation was well conducted or not by evaluating the formation of characteristic aromas, good interior compartmentalization of the seed, and brown coloration features.

Regarding the phenolic compounds found in the cocoa beans during fermentation, the contents of catechin and epicatechin are indicated in Figure 2. Epicatechin is the major phenolic compound found naturally in cocoa beans and undergoes oxidative reaction by the enzyme polyphenoloxidase (PPO), which starts its activity when fermentation reaches temperatures equal or greater than





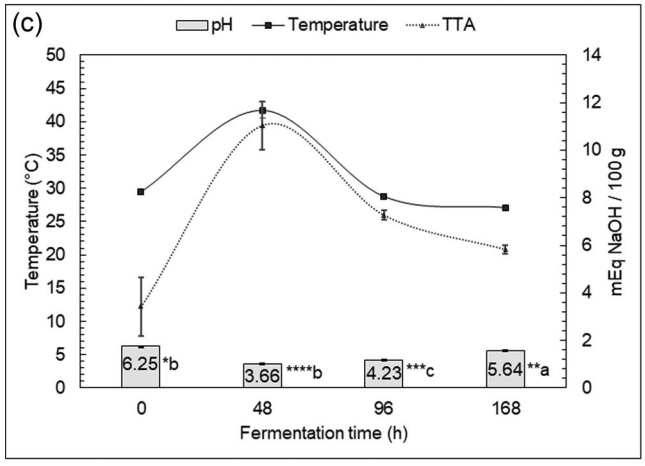
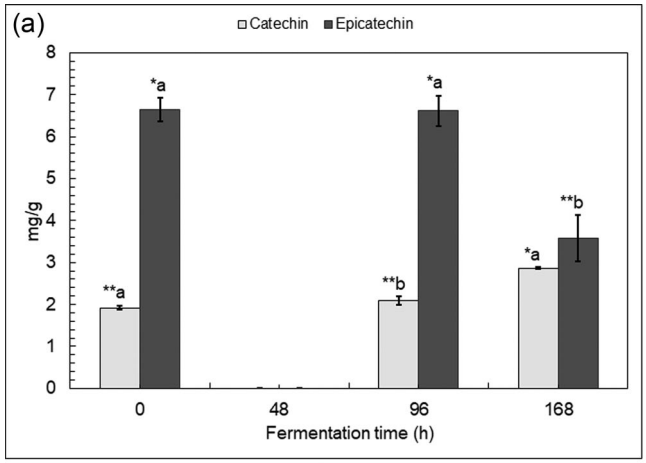
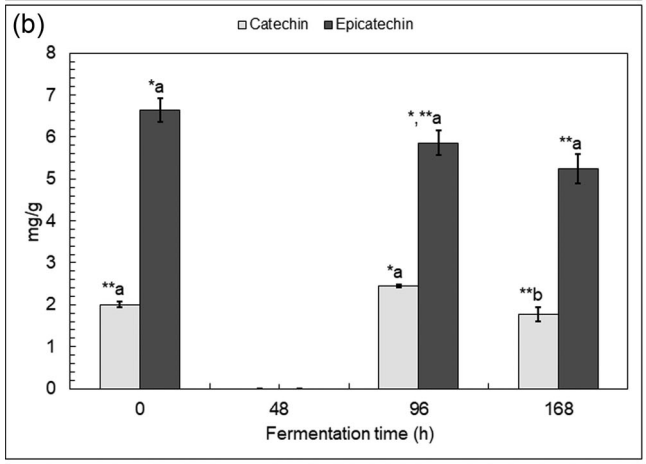


FIGURE 1 Temperature, pH, and total titratable acidity (TTA) during cacao fermentation: control treatment (CF, a); with *Pichia manshurica* inoculum (PF, b); with *Saccharomyces cerevisiae* inoculum (SF, c) inoculum. *Note*: Equal (*) are not statistically different (Duncan test, $p > 0.05$) between the fermentation times of each treatment; different lowercase letters are statistically different (Duncan test, $p < 0.05$) between fermentation treatments





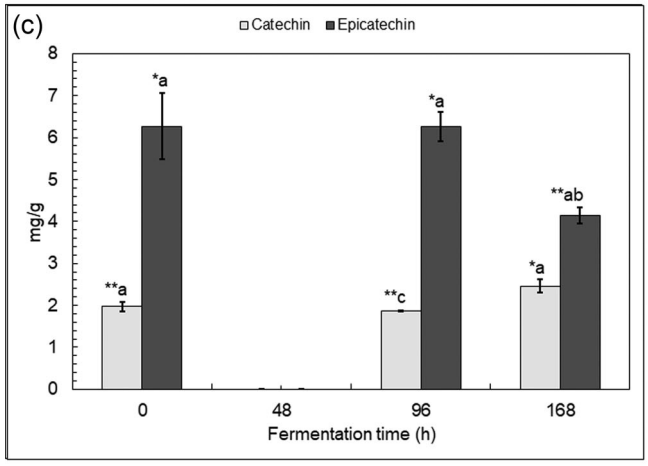


FIGURE 2 Results[a] of catechin and epicatechin during cacao fermentations: control treatment (CF, a), treatment using *Pichia manshurica* inoculum (PF, b); treatment using *Saccharomyces cerevisiae* inoculum (SF, c). *Note*: [a]In wet basis. Equal (*) are not statistically different (Duncan test, $p > 0.05$) between the fermentation times of each treatment; different lowercase letters are statistically different (Duncan test, $p < 0.05$) between fermentation treatments

42°C (Camu et al., 2008; De Vuyst & Weckx, 2016; Efraim et al., 2011; Gaspar et al., 2021; Nazarauddin et al., 2006). The PF and SF fermentations presented higher levels of epicatechin than CF; however, this aspect should be monitored, as high concentrations of phenolic compounds in cocoa beans can provide astringent and bitter flavors (Chagas Junior, Ferreira, Gloria, et al., 2021; Leite et al., 2013). The Cocoa Quality Index—CQI establishes that the levels of catechin and epicatechin should be higher than 0.083 mg/g and 2.23 mg/g, respectively (Araújo et al., 2014).

3.2 | Contents of carbohydrates, acetic acid, and ethanol during fermentation of cocoa beans with and without starter culture addition

The evaluation of the contents of ethanol, acetic acid, sucrose, glucose, and fructose were statistically equal in all fermentation tests at the beginning of fermentations ($p > 0.05$, Duncan test). The performance of the CF, PF, and SF treatments can be visualized in Figure 3.

During the first 48 h, glucose was consumed more quickly in all the treatments, differing statistically ($p < 0.05$, Duncan test) only from fermentation with *S. cerevisiae* (SF) inoculum, which showed a decrease by 87% of its initial content. The glucose contents decreased about 40% and 35% in the first 48 h for the spontaneous fermentation (CF) and in the inoculated process with *P. manshurica* (PF), respectively. There was an expressive reduction ($p < 0.05$, Duncan test) of 57% of fructose in the first 48 h in the CF treatment.

Sugars are converted to ethanol and CO_2 during the first 48 h of fermentation due to the metabolism of yeasts occurring naturally in cocoa fermentations (Schwan & Wheals, 2004). Some species, including *Hanseniaspora opuntiae*, *Pichia kluyveri*, and *S. cerevisiae*, have a high capacity for producing pectinolytic enzymes, which hydrolyze the pectin chain in cocoa pulps, releasing sugars for chemical reactions (Batista et al., 2015; De Vuyst & Leroy, 2020).

Despite the high consumption of analyzed carbohydrates (Figure 3a–c), the CF fermentation showed stability in the ethanol content, unlike the other treatments, which show the production of this compound in the first 48 h of fermentation, with higher values in the PF fermentation (5.26 mg/g) being statistically equal ($p > 0.05$, Duncan test) to SF fermentation (4.84 mg/g). *Saccharomyces cerevisiae* is defined as one of the most important yeast species involved in cocoa bean fermentation, as it has a high fermentative capacity characterized by a high ethanol production (Ardhana & Fleet, 2003; Batista et al., 2015).

The highest amount of ethanol was identified at 48 h time in the treatments PF and SF (5.26 and 4.84 mg/g, respectively) without no statistical difference ($p > 0.05$). Some yeasts in the genus *Pichia* and the species *S. cerevisiae* are highly efficient in the production of ethanol due to their metabolic capacity (De Vuyst & Weckx, 2016) and so may be good alternatives for the production of starter cultures.

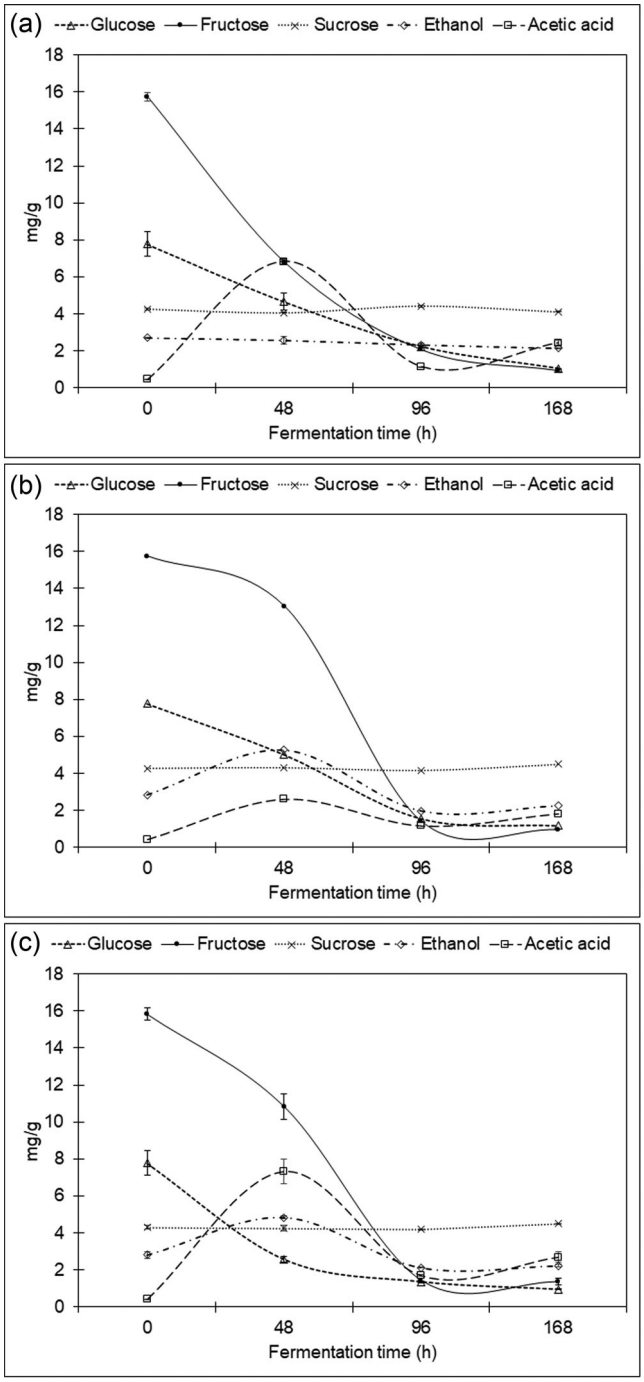


FIGURE 3 Analysis of organic compounds[a] (carbohydrates, acetic acid and ethanol) during cacao fermentations: control treatment (CF, a), treatment using *Pichia manshurica* inoculum (PF, b); treatment using *Saccharomyces cerevisiae* inoculum (SF, c). Note: [a]In wet basis

TABLE 2 Physicochemical analysis[a] in fermented and dried cacao inoculated with different starter cultures

	CF	PF	SF
Parameters			
Moisture content (%)	4.34 ± 0.16a	4.44 ± 0.06a	4.44 ± 0.01a
Sugars (mg/g)			
Glucose	0.95 ± 0.03a	0.99 ± 0.06a	0.88 ± 0.02a
Fructose	1.82 ± 0.04a	1.10 ± 0.08c	1.27 ± 0.01b
Sucrose	4.15 ± 0.05a	4.20 ± 0.03a	3.92 ± 0.10b
Phenolic compounds (mg/g)			
Catechin	3.55 ± 0.05a	3.72 ± 0.33a	1.95 ± 0.01b
Epicatechin	2.86 ± 0.58c	4.30 ± 0.24a	3.61 ± 0.06b
Other compounds (mg/g)			
Ethanol	2.14 ± 0.08a	2.18 ± 0.04a	1.86 ± 0.01b
Acetic acid	0.56 ± 0.04c	1.87 ± 0.15b	3.59 ± 0.15a

Abbreviations: CF, control; PF, *Pichia manshurica* inoculum; SF, *Saccharomyces cerevisiae* inoculum in Igarapé-Miri, PA, Brazil, 2018.

[a]Means ± standard deviation with different lower case letters in the same line (treatments) are statistically different (Duncan test, $p < 0.05$). Results in dry basis.

In the PF treatment, there is a great decrease in the ethanol contents at 96 h, which may show a slower microbial succession, a fact also observed in the amount of acetic acid throughout all the studied periods. The low amount of acetic acid found in the treatment with PF may indicate the low performance of AAB, which are responsible for oxidizing ethanol to acetic acid, and thus triggering different physical and chemical reactions inside cocoa seeds. The practice of turning the seeds during fermentation can facilitate the evaporation of acetic acid, decreasing its contents, as suggested between times 48 h and 168 h (Chagas Junior, Ferreira, & Lopes, 2020; De Vuyst & Leroy, 2020).

The acetic acid production was statistically equal ($p > 0.05$, Duncan test) at the beginning of the fermentations with values around 0.45 mg/g. At 48 h, acetic acid values were maximum and statistically different ($p < 0.05$, Duncan test). The CF and SF fermentations showed greater amounts, suggesting that there was a more expressive conversion of ethanol to acetic acid by the AAB.

3.3 | Physicochemical characteristics of fermented and dried cocoa beans

The results concerning the physicochemical analysis in both the fermented and dried cocoa beans are indicated in Table 2.

For marketing purposes, cocoa beans must present a maximum moisture content of 8% (Types 1 and 2) or 9% (Type 3 and Out of Type) after drying (Brazil, 2008), because if they are dried in excess the husk becomes brittle, while if the moisture content is above 8%, fungi development are expected to be observed. In our research, the moisture contents indicated that the cocoa beans were properly dried and included in the Type 1 classification.

The contents of glucose and fructose were lower compared to sucrose, which remained stable even after the drying process. It is suggested that there was no proliferation of invertase-producing yeasts and that there was no hydrolysis of glycosidic bonds through the action of lactic and acetic acids that are formed during cocoa fermentation, which in turn would release new glucose and fructose molecules to the quite to the fermentation mass for the metabolism for the other groups of microorganisms. Microbiological analysis is suggested for future studies to correlate microbial dynamics with the findings of physicochemical analyses.

Catechin and epicatechin were quantified in both the fermented and dried cocoa beans (Table 2). Epicatechin showed statistical differences ($p < 0.05$, Duncan test) between all three treatments, with higher values being observed in the treatments PF and SF (3.72 and 1.95 mg/g, respectively). This finding may be seen as an alternative to the use of *Pichia* species starter cultures in cocoa fermentations as a tool to obtain cocoa beans with higher antioxidant potential, as previously in another study for cocoa fermentations in the biome Amazonia (Chagas Junior, Ferreira, Gloria, et al., 2021). Some studies report the ability of yeast species of the genus *Pichia* to hydrolyze sugars linked to phenolic compounds naturally found in cocoa beans, converting them into simple and free phenols being absorbed, thus increasing the content of these substances (Chagas Junior, Ferreira, Gloria, et al., 2021; Ooi & Siow, 2020).

The amount of acetic acid in fermented and dried cocoa is a parameter to be evaluated, as it can reflect the characteristic aromas of vinegar. The amount of this in the SF

TABLE 3 Evaluation of cut test[a] in fermented and dried cacao inoculated with different starter cultures

	CF	PF	SF
Well fermented (%)	87.66 ± 3.06a	72.66 ± 9.29b	86.00 ± 3.46a
Semi fermented (%)	6.67 ± 1.53b	10.67 ± 2.08a	4.00 ± 1.73b
Under fermented (%)	5.67 ± 1.53c	16.67 ± 5.77a	10.00 ± 2.00ab
Slate-like (%)	0 ± 0	0 ± 0	0 ± 0
Moldy (%)	0 ± 0	0 ± 0	0 ± 0
Infested by insects (%)	0 ± 0	0 ± 0	0 ± 0
Total (%)	100 ± 1.25	100 ± 3.87	100 ± 1.44

Abbreviations: CF, control; PF, *Pichia manshurica* inoculum; SF, *Saccharomyces cerevisiae* inoculum in Igarapé-Miri, PA, Brazil, 2018.
[a]Means ± standard deviation with different lower case letters in the same line (treatments) are statistically different (Duncan test, $p < 0.05$).

treatment (3.5 mg/g) was higher than the other treatments, reinforcing that the presence of *P. manshurica*, as a starter culture, can reduce or accelerate the AAB performance during the cacao fermentation. The roasting step can be an alternative to reduce the acetic acid amounts by 70% (Rodriguez-Campos et al., 2012).

However, as explained above, these parameters must be monitored to determine maximum thresholds for these compounds before the sensory characteristics of chocolates are compromised.

Color variations in cotyledons are commonly used to determine the taste potential of seeds and their quality for chocolate production. Because the cut test (Table 3) is based on color changes in the cotyledons during the fermentation process, it reveals the fermentation index.

After cutting the fermented and dried beans, it was discovered that the control and *S. cerevisiae*-inoculated fermentations (CF and SF, respectively) had higher fermentation rates, with no significant difference ($p > 0.05$, Duncan test).

The contents of semifermented (light purple color) and unfermented cocoa beans were higher after the fermentation with PF(10.67% and 16.67%, respectively). This is a less than fully objective method based on the observation of the fissures formed inside the cotyledon, the color, and size of the cocoa beans, which is still commonly assessed on cocoa farms by the cocoa bean producers. Based on these parameters, the spontaneous fermentation (CF) showed better fermentation rates, but it is important to consider the cut-test results in relation to the volatile aroma compounds profiles (section 3.4) and other chemical and physical data discussed above.

3.4 | Volatile profile of the fermented and dried cocoa beans

Twenty-nine aromatic compounds were identified in fermented and dried cocoa beans (Table 4). The volatile compounds were grouped into six classes, including alcohols, aldehydes, ketones, esters, pyrazines, and other compounds (Figure 4). Twenty-three compounds were identified in the fermentation inoculated with PF, while 20 were identified in the other fermentations (CF and SF). The PF fermentation showed a greater variety of ester and ketone compounds, which can provide cocoa beans with more floral, fruity, and sweet aromas, as also observed in the fermentations conducted with *P. kudriavzevii* (Chagas Junior, Ferreira, Andrade, et al., 2021).

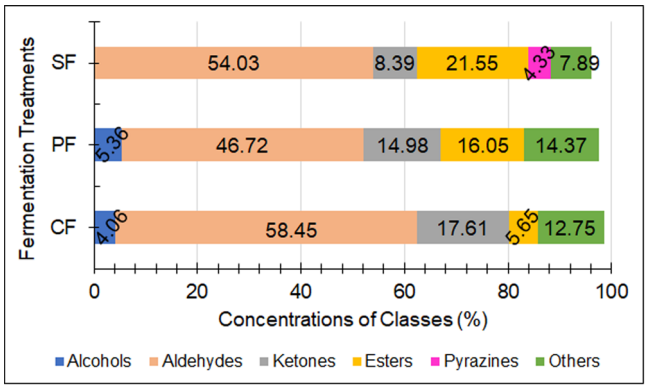


FIGURE 4 Distribution of volatile compound classes identified by GC-MS analysis in fermented and dried cacao beans inoculated with different inocula: control treatment (CF), treatment using *Pichia manshurica* inoculum (PF); treatment using *Saccharomyces cerevisiae* inoculum (SF)

For each compound, a descriptive odor based on the literature was given. Our findings suggested desirable flavor to the cocoa beans for chocolate production: fruity, floral, roasted, and chocolate-like notes. Flavor is an important quality attribute for cocoa beans and their derived products (chocolate), which is obtained by the volatile fraction of a variety of compounds (Bastos et al., 2019).

Alcohols are compounds of great importance for the development of desirable and pleasant flavors and aroma in cocoa beans (Tuenter et al., 2020). These compounds come from the metabolism of yeasts by the consumption of sugars during the anaerobic phase of fermentation (De Vuyst & Leroy, 2020). However, the alcohol content

TABLE 4 Percentage of volatile compounds identified and quantified by GC-MS analysis in fermented and dried cacao inoculated with different starter cultures

Retention index (RI)	Compound	Treatment			Odor description[a]
		CF (%)	PF (%)	SF (%)	
Alcohols					
900	2-heptanol	4.06	4.90	ND	Fruity, citrus, herbal
1113	Phenylethyl alcohol	ND	0.46	ND	Floral, sweet
Aldehydes					
953	Benzaldehyde	8.28	10.41	12.32	Roasted almonds, candy, burnt sugar
1036	Phenylacetaldehyde	49.47	35.35	39.59	Floral, fruity, honey
1272	2-phenylcrotonaldehyde	0.32	0.96	1.67	Poignant
1492	5-methyl-2-phenyl-2-hexenal	0.38	ND	0.45	Fruity, chocolate
Ketones					
888	2-heptanone	1.38	2.96	2.00	Fruity, banana;
1061	Acetophenone	2.31	5.09	3.49	Flower, almonds, sweet.
1090	2-nonanone	13.92	6.65	2.90	Fruity, sweet, waxy, green herbaceous
1293	2-undecanone	ND	0.28	ND	Fruity
Esters					
835	Sec-amyl acetate	0.54	1.45	2.17	Fruity, banana
879	Isoamyl acetate	1.02	ND	4.99	Fruity
898	Isopenthyl acetate	1ND	ND	3.80	Fruity
1165	4-ethyl phenol	0.11	1.45	ND	Smoke
1200	Ethyl octanoate	ND	0.52	1.22	Floral, fruity
1246	Ethyl phenylacetate	0.24	0.47	0.49	Sweet, waxy
1257	2-phenethyl acetate	0.58	2.15	1.66	Fruity, Sweet, roses, floral, honey
1394	Isoamyl benzoate	3.16	8.88	6.83	Balsam, sweet
1469	γ-decalactone	ND	0.14	ND	Fruity
1596	Ethyl dodecanoate	ND	0.30	0.39	Floral, fruity
1680	γ- dodecalactone	ND	0.69	ND	Fruity
Pyrazines					
1085	Tetramethylpyrazine	ND	ND	4.33	Roasted cocoa, chocolate
Others					
983	Myrcene	0.84	0.54	ND	Off-flavor
1030	Cis-β-Ocimene	0.43	ND	0.38	Floral
1100	Linalool	10.90	13.26	7.05	Floral
1129	Allo-ocimene	0.32	ND	0.29	Floral, sweet
1203	*n*-Dodecane	ND	0.22	0.17	Off-flavor
1291	Pyrrole	0.17	0.12	ND	Floral
1400	*n*-Tetradecane	0.09	0.23	ND	Off-flavor
	Overall (%)	98.52	97.48	96.19	

Abbreviations: CF, control fermentation without inoculum; ND: not detected; PF, fermentation with *Pichia manshurica* inoculum; SF, fermentation with *Saccharomyces cerevisiae* inoculum.

[a]Characteristics found in the literature: Bastos et al. (2019); Dzialo et al. (2017); Moreira et al. (2018); Rodriguez-Campos et al. (2011, 2012); Tuenter et al. (2020), Utrilla-Vázquez et al. (2020).

reduces with drying and roasting by volatilization. In this research, only two alcohols were found: phenylethyl alcohol and 2-heptanol, the latter being highlighted by high relative percentages.

Fermentation inoculated with PF as starter culture produced a higher content (4.9%) of 2-heptanol than SF fermentation (4.06%). *Saccharomyces cerevisiae* is reported to contribute to a large production of desirable aromatic

compounds in cocoa beans (Batista et al., 2016). Both yeasts were able to provide a fruity aroma for the cocoa beans. It is noteworthy that 2-heptanol is related to fine cocoa beans (Cevallos-Cevallos et al., 2018), which is used for making cocoa-derived products of greater commercial value. Concerning the phenylethyl alcohol, which contributes to floral notes to cocoa-derived products, its content was relatively low (0.46%), being found only in the fermentation inoculated with PF.

Four aldehydes were identified: benzaldehyde, 2-phenyl crotonaldehyde, 5-methyl-2-phenyl-2-hexenal, and phenylacetaldehyde, the latter being the compound with the highest relative percentage (49.47% for the CF fermentation), followed by benzaldehyde, with the highest concentrations in fermentations with the starter cultures (SF = 12.32% and PF = 10.41%).

Regarding the aroma, benzaldehyde has a typical roasted almond odor and its production must be monitored to avoid bitter notes in the final product (Castro-Alayo et al., 2019). The other major compound in the class, phenylacetaldehyde, is reported to present its increased concentration during the fermentation process, becoming a positive attribute in the quality of the chocolates obtained, as it gives sweet and floral notes to the cocoa beans (Rodriguez-Campos et al., 2012).

Ketones are known as odorants in foods because they have a similar aroma to nuts. These compounds are formed through decarboxylation and oxidation of fatty acids (Rottiers et al., 2019). Among the identified ketones, 2-nonanone and acetophenone, with floral and fruity characteristics, respectively, they stood out among the other compounds of this class. Therefore, the starter culture composed by PF contributed considerably to the production of a greater variety of volatile compounds desirable to fermented and dried cocoa beans. Esters are considered compounds of great importance in the formation of foods flavor. However, the aroma will depend on its molecular structure and conformation and the odor becomes more sweet or metallic as the chain increases (Bastos et al., 2019). Eleven ester compounds were identified in this study, among them, sec-amyl acetate, isoamyl acetate, 2-phenethyl acetate, and isoamyl benzoate were highlighted with the highest relative percentage (Table 4). The PF treatment showed higher concentrations of isoamyl benzoate (8.88%), followed by the SF (6.83%) and CF (3.16%), respectively. In general, both of the starter cultures seem to have influenced positively the production of these compounds, which were reported as capable of contributing sweet, fruity, and balm notes (such as isoamyl benzoate) to cocoa beans (Chagas Junior, Ferreira, Andrade, et al., 2021).

Pyrazines are volatile heterocyclic compounds related to nutty, roasted, and green aromas. Among them, tetramethylpyrazine and trimethylpyrazine are the most important. Most are produced from α-aminoketones formed by the Strecker degradation as part of Maillard class of reactions during cocoa beans roasting (Serra et al., 2019; Utrilla-Vázquez et al., 2020). The parameters such as time and temperature of thermal reactions are considered critical factors that influence the concentration of pyrazines (Visintin et al., 2017). However, if cocoa fermentation occurs in the absence of yeast followed by subsequent roasting, there will be a reduction in pyrazine contents and, consequently, in the characteristic flavor of chocolates (Kongor et al., 2016). Tetramethylpyrazine was the only compound from the pyrazine group detected (only in the fermentation inoculated with *S. cerevisiae*), with a concentration of 4.33%, and it is reported as a very desirable compound in cocoa beans (coffee and chocolate notes) (Chagas Junior, Ferreira, Andrade, et al., 2021; Rodriguez-Campos et al., 2011).

Recent studies report the high occurrence of yeasts of the genus *Pichia* in Medicilândia and Tucumã cities (Almeida et al., 2019) and *S. cerevisiae* in Tomé-Açu city (Chagas Junior, do Espírito-Santo, et al., 2020), the latter having large amounts of tetramethylpyrazine when analyzed the fermented and dried cocoa beans (Chagas Junior, Ferreira, Andrade, et al., 2021). Our finding can be explained by differences in the local microbiota of Igarapé-Miri and that probably does not naturally dominate *S. cerevisiae* in the CF treatment, reinforcing the identification of tetratmethylpyrazine only in this treatment. Future studies of sensorial evaluation in chocolates produced in the region without the addition of starter cultures are suggested with the aim of verifying the profile of volatile compounds in this product.

Other volatile compounds also play important roles in the aroma of cocoa beans. Among them, seven distinct compounds were detected (myrcene, cis-β-ocimene, linalool, allo-ocimene, *n*-dodecane, pyrrole, and *n*-tetradecane). Linalool gained prominence and presented a higher concentration in the PF assay (13.26%) than in the other assays, CF (10.9%) and SF (7.05%). This compound has a positive contribution to the aroma of cocoa beans, and it is reported for being found in fine cocoa, contributing to a floral aroma (Cevallos-Cevallos et al., 2018). Regarding the compounds with off-flavor notes, myrcene, *n*-dodecane, and *n*-tetradecane, relatively low concentrations were detected, which can be negligible in the presence of the other quality compounds.

The amount and variety of aromatic compounds with fruity, floral, sweet, and typical aromas of fermented beans, as well as the low acidity and considerable amount of phenolic compounds, may suggest fermented and dried cocoa beans with a more adequate level, providing more competitive chocolates in the market.

4 | CONCLUSION

The starter cultures evaluated in this study will be a good alternative for use in cocoa fermentations in the Brazilian Amazon region. The use of the yeast species PF presented a superior performance compared to the culture of *S. cerevisisae*, in respect the quantity of desirable aromatic compounds for the production of chocolate (aldehydes, ketones, esters, and alcohols), low acidity level, and high content of phenolic compounds.

Our results, along with other studies developed on cocoa cultivation in the Amazon region, are indicative that greater investments in research and infrastructure on farms are needed in order to consolidate the region in the international cocoa and chocolate market.

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AUTHOR CONTRIBUTIONS

Osienne de Sousa Ferreira: conceptualization; data curation; formal analysis; investigation; visualization; writing – original draft. **Gilson C. A. Chagas Junior**: investigation; writing – original draft. **Renan Campos Chisté**: writing – review & editing. **Luiza Helena da Silva Martins**: investigation; methodology; visualization; writing – review & editing. **Lidiane Diniz do Nascimento**: formal analysis; methodology; visualization; writing – review & editing. **Alessandra Santos Lopes**: conceptualization; methodology; project administration; resources; supervision; visualization; writing – review & editing.

CONFLICT OF INTEREST

None to declare.

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