Cladosporium cladosporioides starter culture can positively influence flavour and bioactive compounds of fermented and dried cocoa beans from the Amazon biome

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Summary

The objective of this study was to investigate the influence of *Cladosporium cladosporioides* during cocoa fermentation on the physico-chemical characteristics, phenolic compounds, free bioactive amines and volatile compounds. Two fermentation treatments were carried out: a spontaneous, control process and another one with the addition of *C. cladosporioides* starter culture. The use of this starter culture did not affect significantly the temperature and acidity ($p > 0.05$) of cocoa throughout fermentation. It led to decreased levels of total phenolic compounds, catechin and epicatechin. In addition, it caused an increase in the levels of reducing sugars (glucose and fructose), an increase in bioactive amines (cadaverine, putrescine, phenylethylamine and spermidine) and an increase in the levels and diversity of volatile compounds (2-phenethylacetate, benzaldehyde and acetophenone, responsible for floral, fruity and candy notes) in fermented and dried cocoa. Multivariate analysis (principal component analysis and hierarchical cluster analysis) emphasized the differences between the two treatments. Based on these results, the use of *C. cladosporioides* as a starter culture during cocoa fermentation seems to be promising. However, further studies are needed to ascertain their impact on the sensorial characteristic and acceptance of chocolate.

Keywords

phenylethylamine; spermidine; benzaldehyde

In 2020, Brazil produced 757 thousand tons of chocolate. The country is the 7th largest producer of cocoa beans, with 270 thousand tons in 2020 [1]. The chocolate production process encompasses several steps including harvesting of the fruit, opening it, selecting the seeds with pulp, fermentation, drying, roasting, refining and conching. Every step is of great importance within the process but fermentation in particular [2, 3].

It is during the fermentation step that, through metabolic, physico-chemical and biochemical reactions, the desirable sensory characteristics of chocolate are achieved. The main actors are microorganisms belonging to three groups: yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The yeasts degrade and convert sugars from the mucilaginous pulp into ethanol, whereas LAB convert sugars into lactic acid and AAB metabolize ethanol produced by the yeasts into acetic acid [4]. Other microorganisms recently isolated from cocoa fermentations in the Brazilian Amazon have other properties that are still being ascertained by the food industry. Here belong filamentous fungi that are good producers of pec-

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tinolytic enzymes, such as *Cladosporium cladosporioides* [5, 6].

The use of yeast starter cultures for cocoa fermentations in the Amazonian biome has already been shown to be effective in reducing the fermentation time (by up to 24 h). In addition, it increased antioxidant activities [7]. However, there are still no reports on the use of filamentous fungi as a starter culture during cocoa fermentation.

Cladosporium cladosporioides is a filamentous fungus that commonly colonizes plants and the soil [8]. It is a microorganism that has already been molecularly identified during cocoa fermentation [5, 6]. It can excrete enzymes important for obtaining well-fermented cocoa, e.g. polygalacturonase [6, 9–11] and can also be beneficial by activities against toxinogenic microorganisms [12]. It has been associated with grain quality in fermented coffee [13].

Spontaneous fermentation usually results in cocoa beans with different quality among batches, as many biological and environmental factors can directly affect the fermentation process [4]. In this context, it is likely that, by adding desirable starter cultures, it would be of biotechnological potential to improve Amazonian cocoa fermentation, through the release of relevant enzymes [10, 14]. This topic deserves further investigation.

Thus, this work reports, for the first time, the use of *C. cladosporioides* as a starter culture in the fermentation of cocoa seeds and its impact on the process taking into account the physico-chemical characteristics together with the profile and levels of bioactive and flavouring compounds.

MATERIALS AND METHODS

Starter culture

Cladosporium cladosporioides strains were isolated and identified during natural (spontaneous) on-farm cocoa fermentation in Tucumã, Brazilian Amazon (06°51'44"S 51°09'40"W), as previously reported [5]. The strains were stored under freezing temperatures (–18 °C) in the Bank of Microorganisms of the Laboratory of Biotechnology Processes (Federal University of Pará, Belém, Pará, Brazil). The fungus was reactivated in potato dextrose agar (PDA, pH 5.6, Himedia, Mumbai, India) at 30 °C for 10 days. An aliquot of *C. cladosporioides* was transferred to inclined tubes containing PDA and, after incubation for 10 days at 30 °C, saline solution consisting of 0.5 ml·l^{-1} Tween 80 (Neon Química, Suzano, Brazil) and 0.8 g·l^{-1} sodium chloride (Dinâmica, São Paulo, Brazil) was added to the tube and homogenized to suspend the spores. A Neubauer chamber (Kasvi, São José dos Pinhais, Brazil) was used to count and adjust the final concentration of 500 ml suspension with 10^7 spores per millilitre [15, 16].

Cocoa fermentation

The cocoa fruits, Forastero variety, were harvested and processed (fermentation and sun-drying) on a farm in Tomé-Açu city (Brazil, 2°28'41.3"S 48°16'50.7"W) in 2019. After manual opening with stainless steel knives, the seeds with pulp (90 kg) were transferred to wooden boxes (1.0 m length × 0.40 m width × 0.3 m height) where they remained until the end of fermentation for seven days (168 h), defined according to the producer's guidelines.

Two different fermentations processes were performed in duplicate (n = 2). The first type of fermentation was undertaken without the addition of starter cultures, designated as spontaneous fermentation (CP). The second type of fermentation was performed with the addition of the starter culture *C. cladosporioides* (SP). For inoculation, 500 ml of the spore suspension (10^7 particles per millilitre) was sprayed on the surface of the seeds. Banana leaves were used to cover the boxes. After 48 h fermentation, the sample was mixed every 24 h for aeration. Fermentation lasted 144 h (six days). Afterwards, the fermented seeds were dried in large barges under natural sunlight until moisture was reduced to 60 g·kg^{-1} [7].

During fermentation, temperature of the cocoa mass was measured in 24 h intervals at five different points within the mass using HT-600 thermometer (Instrutherm, São Paulo, Brazil). Samples were collected throughout fermentation (in 24 h intervals) and analysed for pH, total phenolic compounds, catechin, epicatechin, sugars (glucose, fructose and saccharose), ethanol and acetic acid. In addition, samples were collected at 0, 96 and 144 h of fermentation and analysed for free bioactive amines. After drying the fermented cocoa, samples were analysed for the same parameters and also for moisture content as well as flavour compounds. Prior to analysis, the samples were ground using a mill A11 (IKA, Staufen, Germany),

Carbohydrates, ethanol and acetic acid

For the extraction of carbohydrates, ethanol and acetic acid, the cotyledons were manually ground and 1 g sample was transferred to a sterile microtube (2 ml). An aliquot (1 ml) of deionized water was added and the content of the microtube was vortexed for 5 min. The content was trans-

ferred to a new microtube and another 1 ml of deionized water was added to the precipitate of the previous step, which was vortexed under the same conditions. The microtubes containing 2 ml of the homogenate were centrifuged at 2 000 ×*g* for 10 min and the supernatant was stored in another tube. The precipitate was resuspended in 500 μl deionized water, vortexed and centrifuged as described above. The final volume of 2.5 ml was centrifuged once and the supernatant was filtered through a nylon syringe filter (pore size 0.22 μm) [17].

The analysis of sugars, ethanol and acetic acid was performed by high-performance liquid chromatography (HPLC) using Finnigan Surveyor (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a refractive index (RI) detector operating at 35 °C, using an ion-exchange column Aminex HPX-87H (300 mm × 7.8 mm, 9 μm particle size; Bio-Rad, Hercules, California, USA) at 30 °C [18]. Sulfuric acid (5 mmol·l^{-1}) was used as eluent at a flow rate of 0.6 ml·min^{-1} for 25 min. The injection volume was 20 μl [19]. The compounds were identified based on the retention time of authentic standards obtained from Sigma Aldrich (St. Louis, Missouri, USA). Quantification was made possible by external analytical curves of saccharose (from 0.005 g·l^{-1} to 3 g·l^{-1}, R^2 = 0.98, limit of detection (*LOD*) 0.30 g·kg^{-1}, limit of quantification (*LOQ*) 0.92 g·kg^{-1}), glucose and fructose (from 0.005 g·l^{-1} to 1 g·l^{-1}, R^2 > 0.99, *LOD* 0.03 g·kg^{-1}, *LOQ* 0.08 g·kg^{-1}), acetic acid (from 0.005 g·l^{-1} to 3 g·l^{-1}, R^2 > 0.99, *LOD* 0.03 g·kg^{-1}, *LOQ* 0.09 g·kg^{-1}) and ethanol (from 0.2 g·l^{-1} to 3 g·l^{-1}, R^2 > 0.99, *LOD* 0.04 g·kg^{-1}, *LOQ* 0.11 g·kg^{-1}).

Free bioactive amines

Free bioactive amines were extracted from 5 g samples with 0.007 g·l^{-1} trichloroacetic acid (Sigma Aldrich), homogenized in a shaker for 5 min, followed by centrifugation at 11 000 ×*g* for 10 min at 4 °C [20]. Extraction was repeated twice, the supernatants were poured into a 25 ml volumetric flask and filtered through qualitative celullose filter paper. Nine bioactive amines (spermidine, putrescine, agmatine, cadaverine, serotonin, histamine, tyramine, tryptamine and phenylethylamine) were determined by ion-pair reversed-phase HPLC [21]. The equipment used was the LC-10AD Shimadzu (Kyoto, Japan) with SIL-10AD VP automatic injector. The amines were separated using a Novapak C18 column (3.9 mm × 300 mm, particle size 4 μm, pore diameter 600 nm; Waters, Massachusetts, USA) and a gradient elution of A (0.2 mol l^{-1} sodium acetate (Sigma Aldrich) and 15 mmol·l^{-1} sodium octanesulfonate (Sigma Aldrich), pH 4.9) and B (acetonitrile, HPLC grade, 99 % (Sigma Aldrich)) [21]. The amines were identified by comparing the retention times with those of authentic standards (Sigma Aldrich). Quantification was performed fluorimetrically (excitation at 340 nm, emission at 445 nm) after post-column derivatization with *o*-phthalaldehyde (Sigma Aldrich), using external analytical curves for each amine (R^2 > 0.99). The results were expressed in milligrams per kilogram.

Physico-chemical characterization

The physico-chemical analyses were performed according to the Association of Official Analytical Chemists [22], namely, analysis of moisture (method 963.15), pH (method 970.21) and total titratable acidity (*TTA*, method 31.06.06). All the analyses were performed in triplicate.

Total phenolic compounds

The samples were defatted with *n*-hexane (Synth, Diadema, Brazil) and the phenolic compounds were extracted using a solution containing 70.0 % acetone, 29.5 % water and 0.5 % acetic acid (v/v/v) following Brito et al. [20]. Quantification of total phenolic compounds (*TPC*) was performed by the Folin-Ciocalteu spectrophotometric method at 760 nm [23] using a UV-visible spectrophotometer EVO 60 (Thermo Fischer Scientific). The results were expressed in milligrams per kilogram of cocoa beans, based on the analytical catechin standard (Sigma Aldrich) with a curve from 20 mg·l^{-1} to 100 mg·l^{-1} ($R^2 \geq 0.99$).

Catechin and epicatechin

An aqueous ethanol solution (1 : 1, v/v) was used for the extraction of catechin and epicatechin from the samples following the method described by Chagas Junior et al. [7]. The compounds were separated and quantified by HPLC, using Model 1260 (Agilent Technologies, Santa Clara, California, USA) connected to a UV detector at 280 nm. A Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, particle size 5 μm; Agilent Technologies) was used at 25 °C. The mobile phases A (water-acetonitrile, 99.8 : 0.2, v/v) and B (methanol, HPLC grade, ≥ 99.9 % (Sigma Aldrich)) followed a linear gradient from 0 % to 50.0 % B over 0–12 min and from 50 % to 100 % B for 13–20 min, at a flow of 1.2 ml·min^{-1}. The injection volume was 20 μl [7, 24]. The phenolic compounds were identified based on retention times of the peaks in relation to the standards and quantified using external analytical curves of catechin (Sigma Aldrich), at concentra-

tions from 3.125 g·l^{-1} to 50 g·l^{-1} (R^2 > 0.99, *LOQ* 0.31 g·kg^{-1}, *LOD* 0.10 g·kg^{-1}) and of epicatechin (Sigma Aldrich), at concentrations from 3.125 g·l^{-1} to 100 g·l^{-1} (R^2 > 0.99, *LOQ* 0.11 g·kg^{-1}, *LOD* 0.04 g·kg^{-1}). The analyses were carried out in triplicate.

Volatile compounds

The fermented, dried and ground samples (15 g) were subjected to simultaneous distillation and extraction for 2 h using 4 ml of pentane (Sigma Aldrich). Samples (2 μl) of the pentanic concentrate were injected into a gas chromatograph coupled to a mass spectrometer (GC-MS), QP-2010 Plus system (Shimadzu). A splitless mode was used along with a DB-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies). Helium was used as the carrier gas at a flow of 1.2 ml·min^{-1}, and the temperatures of the injector and the interface were 250 °C. The oven temperature was adjusted from 60 °C to 250 °C, using a gradient of 3 °C·min^{-1} [25]. An electronic impact of the mass spectrometer was 70 eV and ion source temperature was 220 °C. Chemical identification was performed by comparing mass spectra with standard substances in the system data library and literature data [26]. Quantitative analysis of the chemical constituents was performed by peak-area normalization using a gas chromatograph with a flame ionization detector (QP 2010 system, Shimadzu) using the same conditions as used for GC-MS, except that hydrogen was the carrier gas. Chemical identification was carried out by calculating the retention indices (*RI*) using a homologous series of *n*-alkanes (C8–C24, Sigma Aldrich).

Statistical analysis

The results were subjected to analysis of variance (ANOVA) and the means were compared by the Duncan's test (p < 0.05). For principal component analysis (PCA), the values of pH, *TTA*, contents of *TPC*, epicatechin, catechin, sugars, ethanol, acetic acid, bioactive amines and the classes of the volatile compounds were considered as the active variables. For the hierarchical cluster analysis (HCA), all variables previously used for PCA were considered to form groups based on Euclidean distances (Ward's method). Statistical analysis was carried out using the Statistica 7.0 software (StatSoft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Changes during fermentation

The quality of the fermentation was followed by measuring temperature and pH of the fermenting mass. In both treatments, the temperature of the cocoa increased over time (Fig. 1A), reaching maximum levels at 120 h (approximately 45 °C), decreasing afterwards to approximately 42 °C (at 144 h). In a similar way, for both treatments, the increase in temperature followed two different rates, a faster one up to 72 h followed by a slower rate. This temperature change pattern has been reported to be usual during cocoa fermentation [7, 20]. The increase in temperature at the beginning of the process, up to 48 h, is said to be due to the action of yeasts, through the conversion of sugars from the seed pulp into ethanol, and also to citrate-positive LAB that convert citric acid into

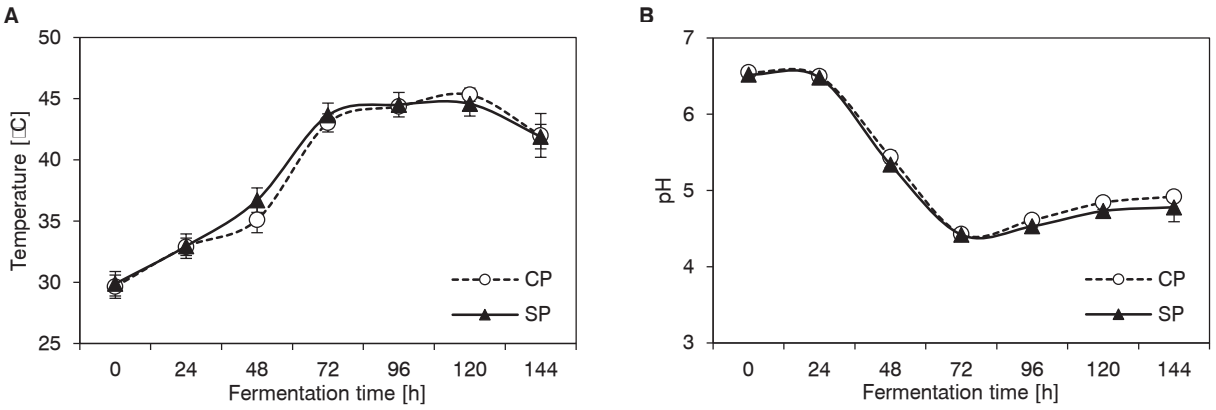

Fig. 1. Temperature and pH during fermentation of cocoa beans.

A – temperature, B – pH.
CP – spontaneous fermentation without the addition of starter cultures (control), SP – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.

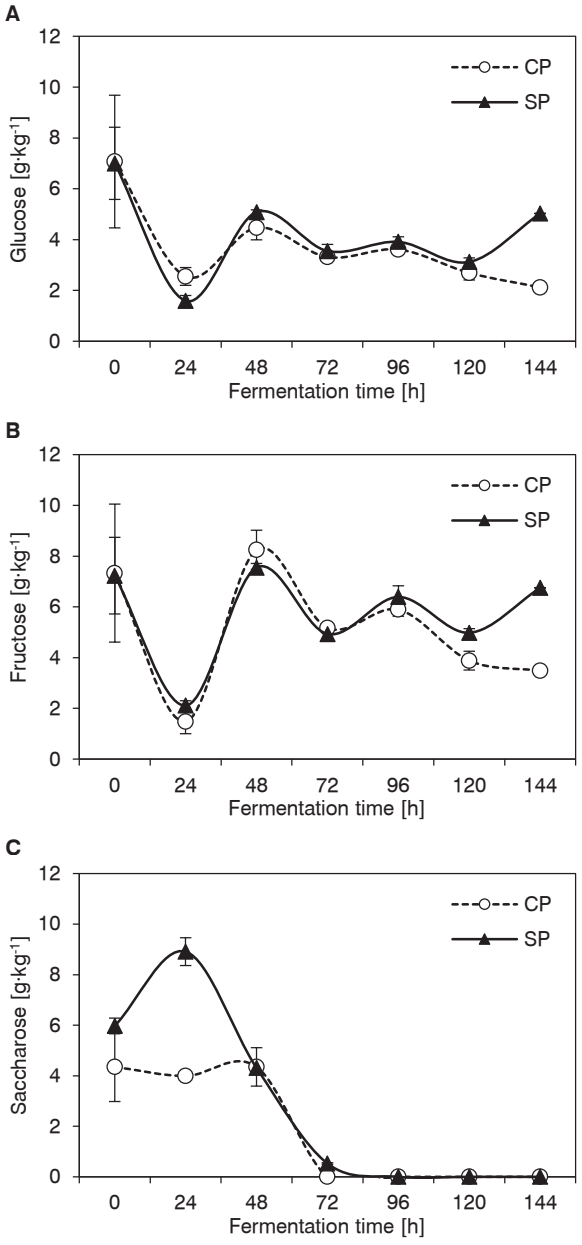

Fig. 2. Content of carbohydrates during fermentation of cocoa beans.

A – glucose, B – fructose, C – saccharose.
CP – spontaneous fermentation without the addition of starter cultures (control), SP – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.

lactic acid. After 48 h fermentation, the increase in temperature is mainly attributed to the action of bacteria, both LAB and AAB, the latter converting ethanol into acetic acid in an exothermic reaction [3, 27].

The pH changes in both treatments (Fig. 1B) followed a similar pattern with no change up to 24 h (approximately pH 6.5), a decrease from 24 h up to 72 h (approximately pH 4.4) and a slight increase up to the end of fermentation (approximately pH 4.8). Similar changes were described in the literature for successful fermentations [7, 20]. However, the pH of the cocoa with *C. cladosporioides* starter culture was lower ($p < 0.05$) compared to control at 96 h and 120 h of fermentation. The decrease in pH during fermentation is attributed to metabolites that are excreted by the microorganisms present in the medium, such as ethanol, lactic acid and acetic acid [4]. However, as fermentation goes on, after 96 h, acids may evaporate due to the increased temperature [2].

In both treatments, saccharose, glucose and fructose contents decreased during fermentation (Fig. 2). In the first 24 h, saccharose levels increased significantly in the treatment with the fungus starter culture releasing more saccharose into the medium. Saccharose levels decreased at higher rates to non-detectable levels at 72 h and 96 h fermentation in control treatment and *C. cladosporioides* starter culture treatment, respectively. The levels of glucose and fructose decreased at lower rates, varying throughout fermentation, which was probably associated with saccharose hydrolysis to glucose and fructose by invertase excreted by the yeasts [7] and also due to *C. cladosporioides* cellulase activity, liberating glucose, which can be isomerized to fructose [5]. At the end of fermentation, glucose and fructose levels were higher (twice as much) in the treatment with starter culture compared to control. This can be advantageous due to the role of these reducing sugars in Maillard reaction during roasting, generating compounds responsible for the desired colour and the roasted flavour typical for cocoa beans [4, 28].

Both ethanol and acetic acid are fermentation products and, therefore, they are not present at the beginning of the fermentation (Fig. 3), being detected only after 24 h. The levels of both of them differed between treatments throughout fermentation, in particular at 24 h and 48 h for both of them, but also at 120 h and 144 h for acetic acid. Ethanol results from sugar metabolism by the yeasts [18]. However, it is metabolized by AAB into acetic acid [2, 3].

Acetic acid levels increased from 24 h, reaching highest levels at 72 h fermentation and decreasing afterwards. The decrease in acid concentration is mainly due to evaporation, as the temperature increases, and also due to aeration (due to daily stirrings) of the fermentation mass [3]. At the end of fermentation, the cocoa fermented with *C. cladosporioides* starter culture had higher levels of acetic acid than the control treatment.

With respect to free bioactive amines (Fig. 4), none of the nine investigated were detected,

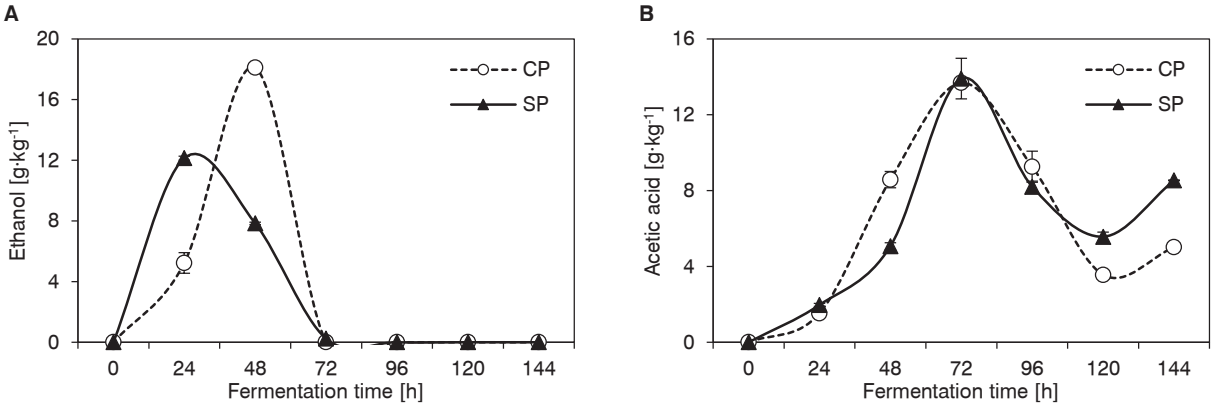

Fig. 3. Contents of ethanol and acetic acid during fermentation of cocoa beans.

A – ethanol, B – acetic acid.
CP – spontaneous fermentation without the addition of starter cultures (control), SP – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.

which means that their content was ≤ 0.4 mg·kg^{-1} in cocoa prior to fermentation. At 96 h fermentation, four amines were detected: spermidine, tryptamine, tyramine and phenylethylamine, at total contents of 7.72 mg·kg^{-1} and 58.93 mg·kg^{-1}, for control and treatment with starter culture, respectively. Two additional amines were detected at 144 h, putrescine and cadaverine, at total contents of 48.16 mg·kg^{-1} and 67.43 mg·kg^{-1}, for control and treatment with starter culture, respectively.

The low occurrence of free bioactive amines in cocoa prior to fermentation was previously reported in the literature [20]. However, fermentation can induce the formation of amines due to liberation of amino acids from proteolysis, followed by amino acid decarboxylase activity from microorganisms [20]. The use of *C. cladosporioides* as a starter culture enhanced the types and levels of amines formed, with higher levels ($p < 0.05$) of spermidine, putrescine, cadaverine and phenylethylamine compared to the control. Higher levels of spermidine are desirable as they have health promoting properties and also, they can prolong shelf life of chocolate due to its antioxidant activity [29, 30]. However, the higher levels of putrescine and cadaverine (from ornithine and lysine decarboxylation, respectively) may be undesirable as these amines can impart a putrid flavour to the chocolate and the consumption in high levels of tyramine can lead to headaches [7, 31]. Phenyethylamine has been suggested to have mood modulation properties, although scien-

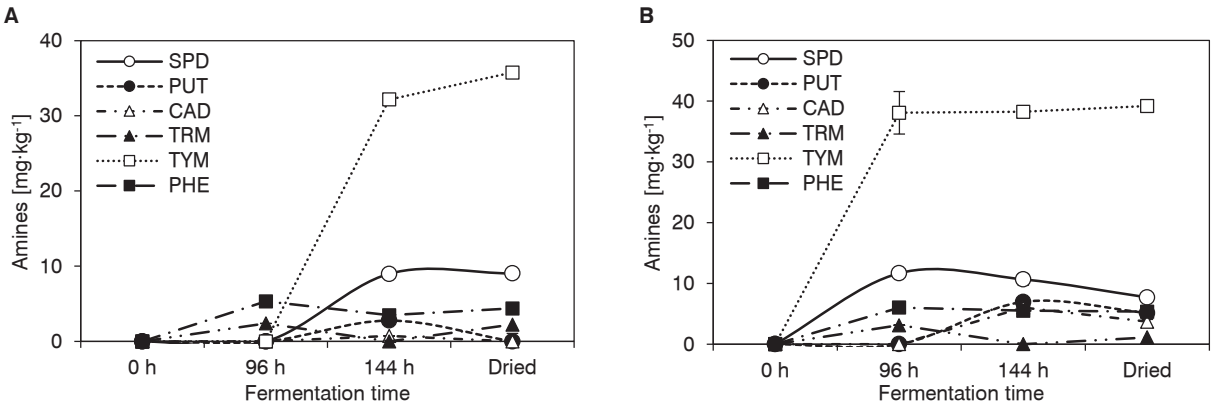

Fig. 4. Content of bioactive amines in fermented and dried cocoa beans.

A – spontaneous fermentation without the addition of starter cultures (control), B – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.
SPD – spermidine, PUT – putrescine, CAD – cadaverine, TRM – tryptamine, TYM – tyramine, PHE – phenylethylamine.

Tab. 1. Physico-chemical characteristics of dried fermented cocoa beans.

Parameter	CP	SP
Moisture content [%]	4.68 ± 0.14 [a]	4.89 ± 0.09 [a]
pH	5.30 ± 0.03 [a]	5.31 ± 0.04 [a]
Total titratable acidity [meq·kg^{-1}]	0.20 ± 0.34 [a]	0.20 ± 0.20 [a]
Phenolic compounds content		
Catechin [g·kg^{-1}]	1.84 ± 0.31 [a]	1.13 ± 0.04 [b]
Epicatechin [g·kg^{-1}]	3.24 ± 0.37 [a]	2.28 ± 0.07 [b]
Total phenolic compounds [g·kg^{-1}]	78.35 ± 3.12 [a]	59.14 ± 1.07 [b]
Bioactive amines content		
Spermidine [mg·kg^{-1}]	9.04 ± 0.00 [a]	7.71 ± 0.28 [b]
Putrescine [mg·kg^{-1}]	0.00 ± 0.00 [b]	5.15 ± 0.32 [a]
Cadaverine [mg·kg^{-1}]	0.00 ± 0.00 [b]	3.70 ± 0.29 [a]
Tryptamine [mg·kg^{-1}]	2.23 ± 0.60 [a]	1.13 ± 0.10 [b]
Tyramine [mg·kg^{-1}]	35.78 ± 0.93 [b]	39.21 ± 1.54 [a]
Phenylethylamine [mg·kg^{-1}]	4.38 ± 0.01 [b]	5.36 ± 0.15 [a]
Total bioactive amines [mg·kg^{-1}]	51.43 ± 1.29 [b]	62.26 ± 2.49 [a]

Values represent mean ± standard deviation. Different letters in superscript in the same line indicate statistically different values ($p \leq 0.05$). Total titratable acidity is expressed as milliequivalents of NaOH.
CP – spontaneous fermentation without the addition of starter cultures (control), SP – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.

tific studies on its relevance in human nutrition are still incomplete, and its presence in chocolate has been valued [7, 30, 31]. Contents of tryptamine and tyramine, which result from decarboxylation of tryptophan and tyrosine, respectively [31], were not significantly affected by *C. cladosporioides*.

Impact of *C. cladosporioides* on dried fermented cocoa

According to Tab. 1, the dried fermented cocoa obtained with the *C. cladosporioides* starter culture (SP) did not differ from control (CP) with respect to moisture content, pH and total titratable acidity. However, SP differed from CP regarding the levels of total phenolic compounds, catechin and epicatechin which were significantly lower. *C. cladosporioides* can excrete enzymes into the fermenting media, especially laccase, which catalyses oxidation of diphenols and polyphenols, thus contributing to final colour, reduced bitterness as well as to improved flavour and aroma of the chocolate [32, 33].

Fermented cocoa from the Brazilian Amazon region presents various profiles of bioactive amines because of the prevalent microflora during the fermentation process. In this study, the levels of total bioactive amines were different from previous studies in the same region [7]. The treatment with the culture of *C. cladosporioides* led to significantly higher contents of the bioactive amines spermidine, putrescine, cadaverine, tyramine and phenylethylamine, but no difference was observed for tryptamine ($p < 0.05$). The levels found are below the no adverse effect levels (NOAEL) and should not represent a risk to human health. However, individuals that are under monoamine-oxidase inhibitor (MAOI) drugs treatment, should limit the ingestion of 6 mg of tyramine per meal to prevent migraines and hypertension crises [31, 34].

Phenylethylamine, which has been reported to be a neurotransmitter and mood modulator, was present at higher ($p < 0.05$) contents in cocoa fermented with *C. cladosporioides* starter culture compared to spontaneous fermentation [7, 31, 35]. Therefore, the use of this starter culture is promising, as it enhances formation and accumulation of this appreciated compound in cocoa.

As indicated in Tab. 2, 14 volatile compounds were detected in the fermented and dried cocoa. These compounds were grouped into four main chemical classes: aldehydes, ketones, esters and pyrazines. Aldehydes were among the essential volatile compounds in cocoa beans, representing 30.9 % and 32.9 % of the total in cocoa from control and treatment with the culture of *C. cladosporioides*, respectively. Among them, phenylacetaldehyde is relevant in chocolate, due to its floral and honey notes [17, 36, 37]. Benzaldehyde is also relevant due to the roasted cocoa and sugary notes [37]. These compounds result from degradation of phenylalanine through Strecker degradation [3], but also through the metabolism of this amino acid by LAB present in the fermentation medium [38, 39]. In the ketones group, acetophenone has

a floral and sweet aroma and, like most aldehydes, it also results from Strecker degradation of phenylalanine [3]. Esters are also relevant in cocoa and chocolate (in particular 2-phenetyl acetate) as they contribute to its complex aroma with fruity, sweet and floral notes [17]. They result from the metabolism of yeasts and acetic acid bacteria during fermentation through production of ethanol and acetic acid, respectively, which undergo esterification, resulting in compounds with floral notes [17]. Aldehydes and pyrazines are considered the most crucial flavour components in cocoa beans, due to their low molecular weight and high volatility [37]. Tetramethylpyrazine can be obtained either from the metabolism of *Bacillus subtilis* during fermentation, from Strecker degradation or from the oxidation of aminoketones during drying [3, 11, 36, 37]. Other volatile compounds were identified, namely, ethylbenzene, styrene, *n*-dodecane and *n*-tridecane. The presence of styrene stands out, which has a balsamic aroma [38], present in samples from both fermentation processes.

When comparing the two treatments, all 14 compounds were detected in the control, whereas only 13 were detected in the treatment with *C. cladosporioides*, due to the absence of *n*-dodecane in the latter. Another difference between the treatments was the prevalence, based on peak areas, of benzaldehyde (4.1 % vs 2.7 %), acetophenone (6.2 % vs 4.0 %) and 2-phenetyl acetate (6.5 % vs 4.0 %) in the treatment with *C. cladosporioides* compared to control. However, lower percentages of tetramethylpyrazine were found in cocoa beans treated with the culture of *C. cladosporioides* (40.2 % vs 43.0 %).

Differentiation by multivariate analysis

Previous studies on cocoa for chocolate production and manipueira fermentation to obtain tucupi in the Brazilian Amazon have successfully used multivariate analysis (PCA and HCA) to characterize and distinguish treatments [7, 40]. In a similar way, we were able to differentiate the treatments – control (CP) and inoculated with

Tab. 2. Volatile profile of fermented and dried cocoa beans.

Compound	Retention index	Proportion of area [%]		Odour description [11, 17, 35, 37-39]
		CP	SP	
Aldehydes				
Benzaldehyde	953	2.7	4.1	Roasted almonds, candy, burnt sugar
Phenylacetaldehyde	1 036	28.2	28.8	Fruity, floral, honey
Total		30.9	32.9	
Ketones				
Acetophenone	1 061	4.0	6.2	Flower, almond, sweet
Total		4.0	6.2	
Esters				
Isopenthyl acetate	867	4.1	3.9	
Isoamyl acetoacetate	880	0.5	0.2	
Ethyl octanoate	1 196	0.6	0.5	Fruity, floral
2-phenethyl acetate	1 255	4.0	6.5	Fruity, floral, candy
Isoamyl benzoate	1 393	1.4	1.3	Balsam, candy
Ethyl decanoate	1 395	0.2	–	Fruity, floral
Total		10.8	12.4	
Pyrazines				
Tetramethylpyrazine	1 085	45.0	40.2	Roasted cocoa, chocolate
Total		45.0	40.2	
Hydrocarbons				
Ethylbenzene	853	1.5	0.9	
Styrene	887	7.1	6.5	Balsam
n-Dodecane	1 199	0.5	–	
n-Tridecane	1 299	0.2	0.2	
Total		9.3	7.6	
Overall total		100.0	99.3	

CP – spontaneous fermentation without the addition of starter cultures (control), SP – fermentation with the addition of the starter culture of *Cladosporium cladosporioides*.

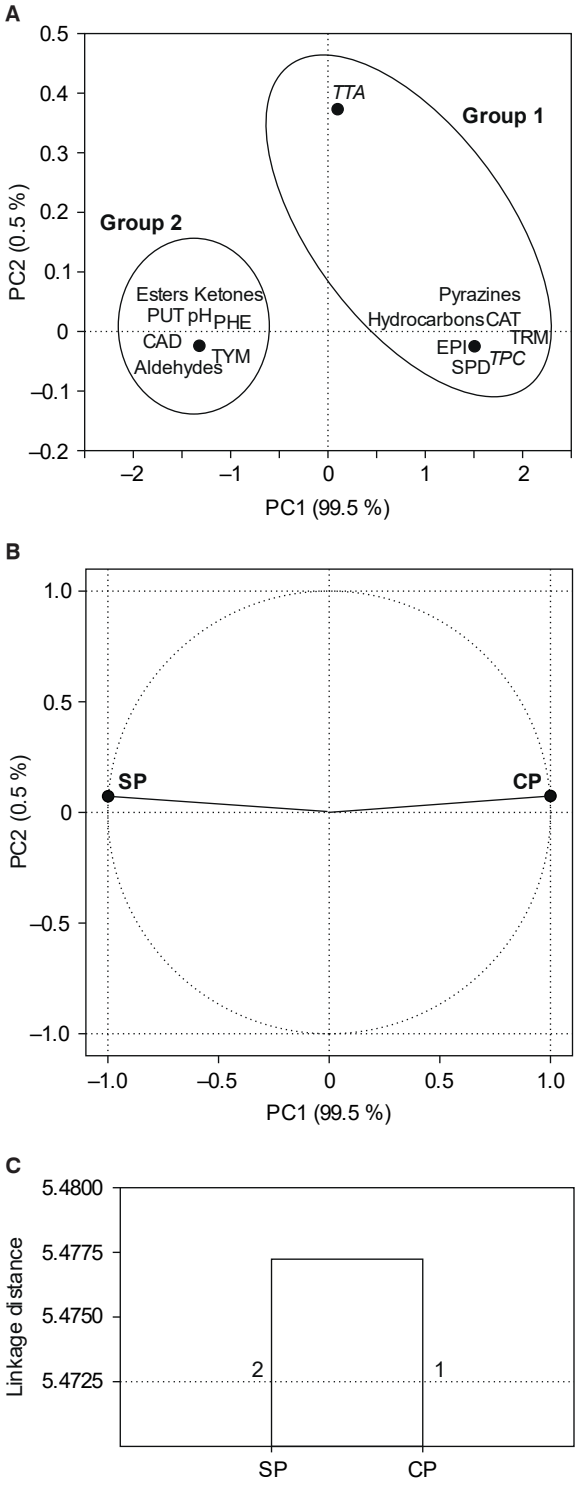

Fig. 5. Multivariate analysis of fermented and dried cocoa.

A, B – principal component analysis (PCA), C – hierarchical cluster analysis (HCA).
TTA – titratable total acidity.
Phenolic compounds: *TPC* – total phenolic compounds, CAT – catechin, EPI – epicatechin.
Bioactive amines: CAD – cadaverine, PHE – phenylethylamine, PUT – putrescine, SPD – spermidine, TRM – tryptamine, TYM – tyramine.

C. cladosporioides (SP) (Fig. 5). PCA (Fig. 5A and Fig. 5B) was able to explain 100 % (PC1 + PC2) of the parameters analysed in the treatments, which was confirmed by HCA (Fig. 5C). *C. cladosporioides* affected the processes, dividing them into two distinct groups. The first (Group 1) represented the dried cocoa submitted to spontaneous fermentation, which was characterized by high acidity (*TTA*) as well as high contents of total phenolic compounds (*TPC*), catechin and epicatechin. These results should be monitored because at high levels, phenolic compounds can lead to cocoa beans with bitter and astringent flavours. In addition, there were lower levels of spermidine, putrescine, cadaverine and phenylethylamine and higher levels of undesirable volatile compounds in cocoa beans (hydrocarbons) in this group.

The second group (Group 2) was the treatment with inoculation of *C. cladosporioides.* It was characterized by higher levels of putrescine and cadaverine, which could be detrimental to the sensory characteristics of the product. However, there was a wider variation of volatile components in this group, which concerned aldehydes, ketones and esters that are responsible for the fruity, floral and other desirable aromas of chocolate. In addition, it showed lower TTA and higher concentration of phenylethylamine indicating that these cocoa beans can be a raw material for the manufacture of chocolates with desirable characteristics.

Multivariate analysis showed that the addition of a starter culture of *C. cladosporioides* was beneficial with respect to phenolic compounds, bioactive amines, acidity and volatile compounds. However, further studies are needed regarding biotechnological innovations and techniques, as well as studies on the impact on sensory properties and acceptance of the chocolate.

CONCLUSIONS

C. cladosporioides was used for the first time as a starter culture for cocoa fermentation in the Brazilian Amazon. It proved to be effective in promoting well-executed fermentations with satisfactory levels of bioactive compounds (catechin and epicatechin) considered as antioxidants. However, these levels need to be monitored because, in very high amounts, they can provide bitter and astringent cocoa beans. A greater variety of volatile compound was also observed, which are attributed to good quality chocolates with fruity, floral and well-fermented roasted cocoa notes. On the other hand, levels of bioactive amines, such as putrescine and cadaverine, were higher in the fermentation

with starter culture, which may be an indication of activities of undesirable microorganisms. Multivariate analysis was effective in defining fermentation phases as well as in identifying fermented and dried cocoa beans. Additional studies are needed to define the real role of *C. cladosporioides* during the cocoa fermentation in the Brazilian Amazon.

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