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Identification of Lactic Acid Bacteria on Raw Material for Cocoa Bean Fermentation in the Brazilian Amazon

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Abstract: The prevalent microbiota during cocoa fermentation is one of the main factors responsible for the chemical and biochemical changes that lead to desirable chocolate characteristics. However, the microbiota can be affected by several factors, including the edaphoclimatic conditions, which are typical of the production region. The objective of this study was to identify LAB in Amazonian cocoa prior to fermentation. LAB were isolated using MRS agar. By sequencing the 16S region of isolated LAB in MRS agar, it was possible to identify five LAB species that were registered in the GenBank database (accessions from MT117900 to MT117915). They included *Pediococcus acidilactici*, which was the most prevalent, followed by *Lactobacillus farraginis*, *L. parafarraginis*, *L. zeae*, and *L. casei.* Studies are needed to ascertain their specific roles and impact on cocoa quality. It is likely that they can be available as starter cultures to enhance the quality characteristics of chocolate.

Keywords: *Theobroma cacao*; chocolate; *Lactobacillus*; *Pediococcus*

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1. Introduction

Chocolate is one of the most consumed and appreciated food around the world. Its production includes several steps with well-defined purposes, including fermentation, dehydration, conching, and tempering [1,2]. The fermentation of cocoa seeds is of utmost relevance as it prevents seed germination and also leads to the formation of compounds that are precursors of the compounds responsible for the chocolate flavor [3–5]. It is a spontaneous process that takes place at the farm immediately after harvesting the ripe fruit. It occurs naturally in wooden boxes covered with plantain leaves and burlap for five to seven days, depending on the cocoa variety and the traditions of each country [3].

During fermentation, there is a succession of microbial activity. In the first 48 h of fermentation (anaerobic phase), yeasts use the fermentable sugars of the mucilaginous pulp that surrounds the seed (glucose and fructose), converting them into ethanol and CO_2 [5,6]. Lactic acid bacteria (LAB) work together with yeasts and convert glucose and fructose into lactic acid and ethanol [7,8]. From the third day (72 h) on, the aerobic phase begins by revolving the cocoa seeds, when acetic acid bacteria (AAB) converts the ethanol into acetic acid, which is volatilized upon continuous revolving of the mass [2]. This reaction is highly exothermic, which raises the temperature (~50 °C), favoring physical and chemical reactions inside the seeds, e.g., degradation of phenolic compounds by polyphenoloxidase,

thus reducing bitterness and astringency; killing of the germ; formation of the internal compartments of the beans [2,5,9].

Due to the relevance of the microbial flora on the quality of the chocolate, studies are needed to identify the autochthonous flora that are typical of each region in order to determine their role in chocolate making. In addition, some can produce biogenic amines, which are relevant in the production of neuroactive compounds (e.g., phenylethylamine and tryptamine), but also putrefactive amines (putrescine and cadaverine) and amines associated with adverse effects to human health (histamine and tyramine) [10,11].

In this context, the objective of this study was to isolate and identify the LAB in cocoa beans used for the traditional cocoa fermentation in Tomé-Açu, Pará (PA), Brazil, a region with a significant production of cocoa in the Brazilian Amazon.

2. Materials and Methods

2.1. Cocoa Samples

Cocoa fruits, var. Forastero from Konagano Farm in Tomé-Açu city, PA, Brazil (02°28′41.3″ S and 48°16′50.7″ W) were used in this study. The fruits were harvested, opened manually with stainless knives, and the seeds with pulp were separated from placenta. Next, the seeds were placed in wooden boxes (90 kg/each) and covered with banana leaves and burlap.

Prior to fermentation, small portions of seeds and pulp (20 g) were removed from different parts within the fermentation box (total of 200 g). The samples were collected aseptically in sterile polyethylene bags and taken to the laboratory under refrigeration (4 ± 2 °C) [12].

2.2. Lactic Acid Bacteria (LAB) Isolation and Purification

The seeds were mixed thoroughly and a 20 g sample was aseptically macerated and homogenized with 180 mL of peptone water (pH 7.20, Kasvi, São José dos Pinhais, PR, Brazil) (dilution 10^{-1}), followed by serial dilutions down to 10^{-8}. For LAB counting, aliquots of 1 mL of each dilution were transferred to sterile Petri dishes (*pour plate* technique) and added to around 30 mL of De Man, Rogosa & Sharpe agar (MRS agar, pH 6.20, Kasvi) containing 0.2% nystatin (Prati-Donaduzzi, Toledo, PR, Brazil). The Petri dishes were incubated at 37 °C for 96 h and the LAB counts were expressed in $\log_{10}$ CFU/g [12,13].

After incubation, 35% of the LAB colonies were isolated [14] and submitted to catalase and Gram tests for LAB confirmation. Catalase-negative and Gram (+) colonies were isolated and enriched in Petri dishes containing MRS agar by the streaking technique and incubated at 37 °C for 48 h. This procedure was repeated twice to obtain pure colonies.

2.3. DNA Extraction

Genomic DNA was extracted following the procedures of Sambrook and Russell [15]. Isolated and purified colonies were transferred to microtubes containing 100 µL of Tris-EDTA buffer (TE—Tris-HCl 1 M pH 7.5, EDTA 0.5 M pH 8.0), 300 µL of *lysis buffer* (Tris-HCl 1M pH 9.0 and 300 mM in solution, 100 mM EDTA in solution, 50% sucrose, 10% SDS), 300 µL of *homogenization buffer* (Tris-HCl 1 M, NaCl 1 M, EDTA 0.5 M and Sucrose), and 10 µL of proteinase K (10 mg/mL, Promega, Madison, WI, USA) and subjected to heat treatment (56 °C for 12 h).

Subsequently, the microtubes were cooled to room temperature and 600 µL of phenol-chloroform-isoamyl alcohol (25:24:1 [*v*/*v*/*v*], Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were added. After homogenization and centrifugation (1500× *g*/10 min), the upper layer was transferred to a new microtube containing 600 µL of chloroform-isoamyl alcohol (24:1 [*v*/*v*]). The aqueous fraction was incorporated into 100 µL of cold sodium acetate (3M, pH 8.0) and 500 µL of cold isopropanol for DNA precipitation, and after centrifugation at 1500× *g* for 10 min, the supernatant was carefully discarded. An additional 500 µL of 70% cold ethanol was added and the solution was centrifuged again (1500× *g* for 10 min.). The supernatant was carefully discarded so as not to lose the precipitate. The residue (DNA)

was oven-dried at 37 °C and subsequently resuspended in 100 µL of TE buffer (pH 6.0) and frozen (−18 °C) until further analysis.

2.4. Polymerase Chain Reaction (PCR) and Sequencing

The 16S region was amplified through the PCR technique using 2 µL of the extracted DNA. For each sample, the DNA was mixed in microtubes containing 11.4 µL of ultrapure water (Ambion, Carlsbad, CA, USA), 5 µL of Q-solution (Qiagen, Düsseldorf, Germany), 2.5 µL of 10× buffer solution (Invitrogen, Waltham, MA, USA), 1 µL of 10 mM dNTP mix (Bioron, Römemberg, Germany), 2 µL of 50 mM $MgCl_2$ (Invitrogen), 0.1 µL of 5 U/µL Taq DNA polymerase (Invitrogen), and 0.5 µL of (10 pmol) of each primer: 616F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [16,17]. PCR analysis was carried out in a Labtrace thermocyler (model K960, Hangzhou, Zhejiang, China) programmed under the conditions: 94 °C/4 min, 30 denaturation cycles (94 °C/15 sec), annealing (55 °C/2 min), extension (72 °C/3 min), final extension (72 °C/10 min), and cooling (4 °C) [16].

The PCR products were purified with the PCR clean-up kit (AP-PCR-250, Axygen, AR, USA), following the manufacturer's instructions. After purification, ≈20 ng of the amplified and purified DNA was prepared for sequencing using the Big Dye Terminator Kit v. 3.1 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Bidirectional reactions were sequenced and analyzed using an AB-3500 DNA Analyser (Applied Biosystems, Foster City, CA, USA) with the same pair of primers. The sequences obtained were edited with the BioEdit software and submitted to the GenBank database (http://ncbi.nlm.nih.gov/genbank, accessed on 3 March 2020).

3. Results and Discussion

The LAB count of the cocoa prior to fermentation was 7.60 $\log_{10}$ CFU/g. This count is similar to values reported in cocoa in previous studies in Tomé-Açu, Pará [12], and also in samples from Bahia, Brazil [18]. These initial LAB counts suggest that even in different regions of Brazil, there was similarity in LAB counts at the beginning of the fermentation process.

Fifteen colonies were isolated from the MRS and enriched. They all tested negative for catalase and were Gram (+). Five different LAB species (Figure 1) were active in the cocoa samples: *Pediococcus acidilactici*, *Lactobacillus farraginis*, *Lactobacillus parafarraginis*, *Lactobacillus zeae*, and *Lactobacillus casei*. *Pediococcus acidilactici* was the prevalent species (66.5%), followed by *Lactobacillus farraginis* (13.4%), whereas *Lactobacillus parafarraginis*, *Lactobacillus zeae*, and *Lactobacillus casei* were identified in 6.7% of the isolates. The registration in Genbank database is available in Table 1.

Pediococcus acidilactici has been reported in cocoa during on-farm fermentation in Igrapiúna, Bahia, but in small counts (19.5% of the total count) [19,20]. However, it was the predominant LAB during laboratory cocoa fermentation in Ilhéus, Bahia [21]. Even though it was reported in cocoa from Bahia, this was the first time that this species was reported as a raw material for the fermentation of Amazonian cocoa.

The *Lactobacillus* genus is widely reported in cocoa fermentations in several locations, especially the species *L. fermentum* and *L. plantarum* [3,5]. In this study, for the first time, different *Lactobacillus* were identified, e.g., *L. farraginis*, *L. parafarraginis*, *L. zeae*, and *L. casei*.

The presence of these LAB species identified in Amazonian cocoa could be relevant during fermentation as they can play roles in the inhibition of the growth of undesirable pathogenic or deteriorating microorganisms [22,23].

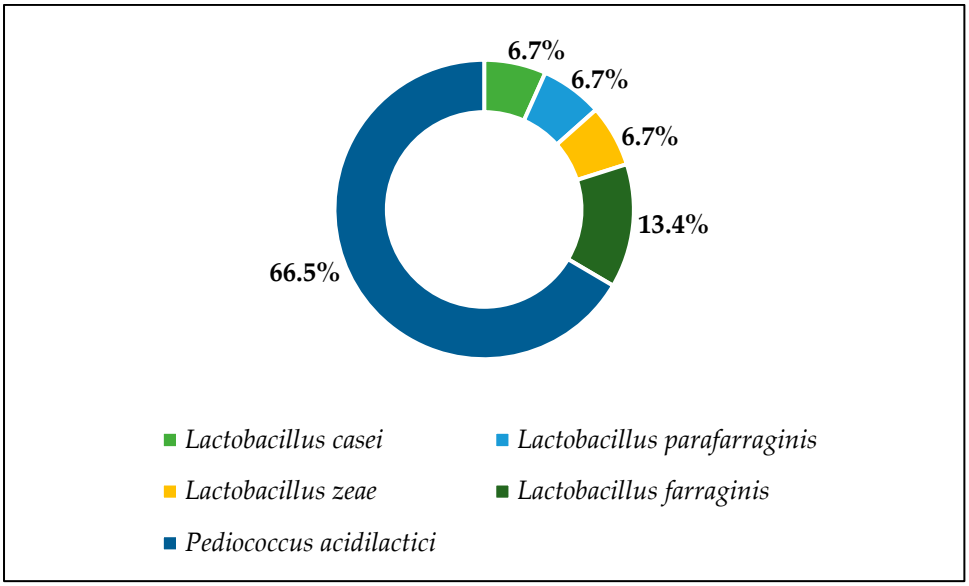

Figure 1. Distribution of LAB species at the beginning of the on-farm cocoa bean fermentation, Tomé-Açu, PA, Brazil, 2017.

Table 1. Lactic acid bacteria identified at the beginning of the on-farm cocoa bean fermentation, Tomé-Açu, PA, Brazil, 2017.

Isolate (Sample)	GenBank Accession	Score (%)	E-Value	Pairwise Identity (%)
Pediococcus acidilactici (3)	MT117910	100	0	100
Pediococcus acidilactici (6)	MT117913	100	0	100
Pediococcus acidilactici (7)	MT117914	100	0	100
Pediococcus acidilactici (8)	MT117915	100	0	100
Lactobacillus parafarraginis (10)	MT117900	100	0	100
Lactobacillus farraginis (15)	MT117901	100	0	100
Lactobacillus farraginis (16)	MT117902	100	0	100
Pediococcus acidilactici (17)	MT117903	100	0	100
Pediococcus acidilactici (19)	MT117904	100	0	100
Pediococcus acidilactici (20)	MT117905	100	0	100
Pediococcus acidilactici (25)	MT117906	100	0	100
Pediococcus acidilactici (35)	MT117908	100	0	100
Lactobacillus zeae (37)	MT117909	100	0	100
Lactobacillus casei (40)	MT117911	100	0	100
Pediococcus acidilactici (45)	MT117912	100	0	100

For example, *Pediococcus acidilactici* is a LAB capable of producing antimicrobial compounds (different organic acids, phenyl-lactic acid, oxygen peroxide, and becteriocins), which has already been reported in studies involving the fermentation of wine and cocoa, inhibiting the production of undesirable compounds in food storage such as excess bioactive amines and the proliferation of spoilage bacteria such as *Salmonella thyphimurium* and *Pseudomonas aeruginosa* [21,24–26] in addition to its antioxidant capacity [27].

Lactobacillus farraginis and *L. parafarraginis* have been identified in corn silages and have been reported to provide increased aerobic stability due to carbohydrate degradation (e.g., glucose, lactose, and fructose) and to favor the conversion of lactic to acetic acid [28,29]. These properties could be advantageous during cocoa fermentation as increased acetic acid concentrations can have antifungal activity, thus preventing mycotoxin production by filamentous fungi. In addition, it may accelerate fermentation by initiating the aerobic phase earlier.

It is likely that *L. zeae* and *L. casei* can play inhibitory functions in relation to some pathogenic microorganisms, such as *Staphylococcus aureus* and *Escherichia coli*, thus increasing the preservation as reported in the literature for yogurt, cheese, and fermented beverages [22,23]. The presence of these species in the cocoa fermentation may prevent the growth of pathogenic organisms [20,30].

Knowledge regarding the roles of the LAB species identified in cocoa in this study is still lacking. However, they play interesting roles during the fermentation of other products [3,31]. Studies are needed to ascertain the role of these LAB during cocoa fermentation and their impact on chocolate quality.

4. Conclusions

For the first time, four species of lactic acid bacteria (LAB) were isolated and identified in the raw material for cocoa fermentation in the Brazilian Amazon. Some (*Pediococcus acidilactici)* were also found in cocoa from Bahia, whereas the *Lactobacillus* species identified were not. The role of the identified species during cocoa fermentation must be ascertained, as well as their impact on chocolate quality.

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