Determination of bacterial isolates from cocoa almonds during fermentation in "saf's" agroforestry system in the Amazon

ABSTRACT

Agroforestry systems (SAFs) are an alternative for sustainable development as they enable the recovery of degraded areas and reduce deforestation, contributing to breaking the cycle of traditional family farming, so common in the Amazon region. The significant appearance of endophytic microorganisms, such as bacteria in cocoa almonds, can benefit its production commonly with fermentative bacteria. The aim of the study was to characterize the microbiota of cocoa beans during the fermentation process. The isolation of bacteria was performed from the collected samples; one of the applied procedures was the scraping of the dried and fermented cocoa almonds. Afterward, aliquots were subcultured in a Petri dish with a culture medium containing Blood agar and MacConkey agar to verify bacteria. Cultures were analyzed by counting colony-forming units (CFU/mL). Molecular analyses and sequencing were utilized to describe the microbial diversity**.** DNA sequencing and phylogenetic analyzes were performed to emphasize microbial morphology characterization. Gram-negative bacilli (*Enterobacter* spp. and *Citrobacter* spp.) and Gram-positive bacilli (*Bacillus* spp.) were found in cocoa beans after 72 h of fermentation. This work contributed to the characterization of endophytic bacteria in cocoa seeds, enabling in-depth studies of *in vitro* verification of the potential for biocontrol of these endophytic bacteria in cocoa cultivation.

Keywords: Cocoa almonds, endophytic bacteria, molecular analysis

INTRODUCTION

"Cocoa almonds are a very important crop as it provides food, income, employment, industrial raw materials, and resources for poverty alleviation". (Prazeres and Lucas, 2020). "The state's cocoa-growing industry has become one of the most competitive in the world due to its high average productivity and low production costs. These factors are related to the preservation characteristics of cocoa production in the agroforestry system, manufacturing the cultivation of cocoa in Pará an interesting alternative for sustainable rural development" (Brainer, 2021; Gateau-Rey *et al.*, 2018).

"The agroforestry system is multifunctional in which forest and agricultural species (such as cocoa) are intentionally used in the same area. The main advantages of agroforestry systems include more efficient land use, soil protection, higher yields and income, and biodiversity

conservation. In addition, it helps the farmer with wood, fruit, and natural fertilization. This agroforestry system preserves biodiversity and traditional knowledge" (Paraense *et al.,* 2013). "To obtain a quality almond, cocoa processing is extremely important, and it must be well obtained from harvesting, fermentation, drying, and storage" (De Oliveira *et al.*, 2018). Spontaneous fermentation is carried out by microorganisms transferred to the kernels of workers' hands, surfaces, and tools used to cut fruit and containers during fermentation. Yeasts are the first microorganisms present in the fermentation process, and soon after, the bacteria, among them the acetic bacteria (Souza, *et al*.,2016; Gateau-Rey *et al.,* 2018).

The microorganisms that are part of the fermentation process are denominated endophytic microbiota, a commonly used term (Macedo, 2009). "The cocoa almonds contain microorganisms that act beneficially on the bean in the stage of fermentation, as is the case of some enterobacteria, lactic acid bacteria, and yeasts and acetic bacteria; their participation occurs in 48 h to 120 h of the fermentation process, that grow in acidic pH" (Souza *et al*., 2017).

"In some cases, during the process of contamination by pathogenic microorganisms, fungi can produce mycotoxins and aflatoxins, which are products that generate fungi with toxic and harmful effects on health. Aflatoxins occur naturally in nuts, cereals, and rice under conditions of humidity and high temperatures, especially during the storage process. This mycotoxin is harmful and could cause liver disease" (Souza *et al*., 2017; Reece, 2015).

The few studies on the fermentation of cocoa grown in the Amazon region in Brazil make it possible to know the species that participate in spontaneous fermentation in agroforestry systems and thus genetically characterize them to know their biotechnological potential for future ventures in the region. The aim of the study was to characterize the bacterial microbiota of cocoa almonds from fermentation and drying process using a molecular approach.

MATERIALS AND METHODS

Data Collection

The fermentation experiment was developed on the assets 'Fazenda Konagano", which is located in the district of the Municcipaly of Tomé-Açu, with the coordinates Lat. 2°28'41.58"S and Long. 48°16'49.05"W, Figure 1, is an international reference in Agroforestry System-SAF, having its plantation consortia of Pimenta do Reino, Cocoa, Cupuaçu, Pitaya, Banana, Chestnut from Pará, Orange, Coco, and Açaí.

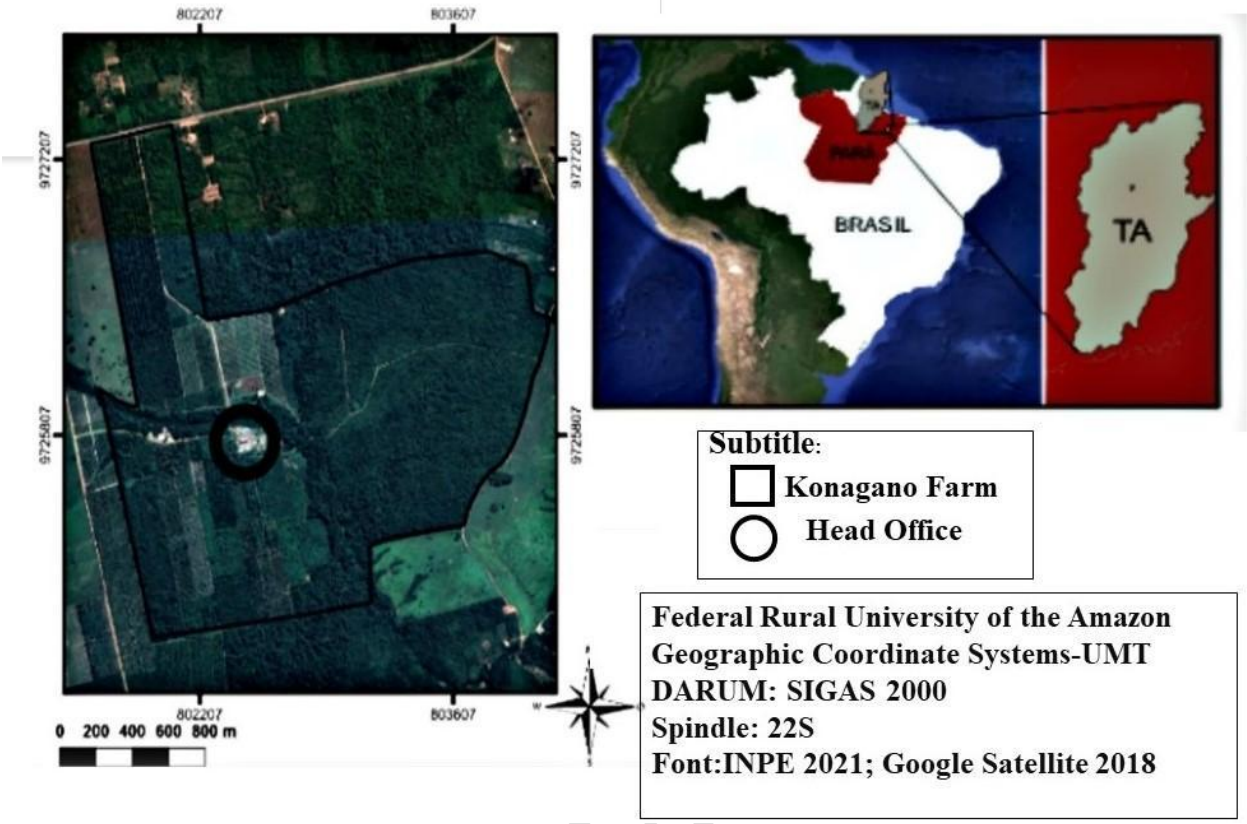

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Figure 1: Fazenda Konagano" located in the district of the Municcipaly of Tomé-Açu**.**

The almonds freshly harvested in the field were subjected to fermentation; the procedure occurred in an 80 cm to 1 m² wooden box containing the almonds over a period of 5 days (Oliveira, 2015 with adaptation) (Figure 2).

Figure 2: Fermentation processing Method in cocoa almonds.

The 20 samples of cocoa almonds were collected at the initial and final stages of each process (fermentation). The samples (n=05) were collected at the initial moment (0 h), 48 h (n=10), and after 5 days (120 h, n=05) of fermentation which is the final phase of this process. Then the samples were sent to the Microscopy laboratory at the University Federal Rural of Amazon to identify the bacteria and their incidence for each fermentation, according to Paranhos, 2011.

Isolation of bacteria for Morphological identification

To obtain the isolation of the bacteria, the study was conducted as follows, from the collected samples, the fermented and dried cocoa beans were ground, then the enriched and differential culture medium, blood agar (BA), and MacConKey agar (MC) (Laborclin-USA). The agar (BA), is enriched culture medium for bacteria, was prepared at a concentration of 40 g of blood agar base, 950 mL of distilled water in the preparation of 1000 mL of BA, sterilized in an autoclave for 15 min at a temperature of 121°C. After the autoclaved, 50 mL of blood was added to the selective growth inhibitor medium of Gram-positive bacteria. The MacConKey agar – (MC) (Laborclin-USA), a selective culture medium for Gram-negative fermenting bacteria, was elaborated at a concentration of 50 g of MC diluted in 1000 mL of distilled water, sterilized in an autoclave for 15 min at 121ºC. At the end of the sample and culture media preparation process, the samples were inoculated for the “Spread Plate" technique and incubated in bacteriological incubators at 37±1°C/48 h, with plate readings performed for 24 to 48 h (De Jesus and Bastos, 2018).

For bacterial colonies where there was a mixed culture, with the presence of more than one colony on the same plate, the culture on agar 523 (Phyto Casein Hydrolysate Sucrose) was replicated, at a concentration of 10 g of sucrose, 4 g of yeast extract, 8 g of sucrose, 2 g of K2HPO4, 0.8 g of $MgSO_4$ and 17 g of water diluted in 1000 mL of distilled water, autoclaved at 121ºC for 20 min, then applied to the "Spread Plate" technique to obtain isolated colonies (Paulus, 2017).

For Gram staining, the protocol reported by Thiele, 2019 was used.

For the biochemical tests, TSI (Triple Sugar Iron Agar) was used at a concentration of 64.52g for 1000 mL, SIM (Indol Sulfide Motility) at a concentration of 30 g for 1000 mL, Urea at a concentration of 38.7 g for 1000 mL, Citrate at a concentration of 24.28 g for 1000 mL and Lysine at a concentration of 34.56 g for 1000 mL. After the biochemical reagents were prepared, the bacteria were inoculated and incubated in bacteriological incubators at 37±1ºC/48h. The biochemical tests evolved from 24 to 48 h (Rodulfo et al., 2016).

Extraction of Bacterial DNA

DNA from bacteria was extracted using CTAB (cetyl trimethyl ammonium bromide) (Mariano and Souza, 2016). For the DNA extraction, the bacterium was initially cultivated in an enriched and differential solid medium, blood agar (BA), with growth for 36 h. After cultivation, between one and three colonies of bacterial growth were added to microtubes with 567 µL of 1X TE and homogenized. The cell wall was physically broken, and the DNA that was exposed and extracted, 30 µL of 10% SDS, 3 µL of proteinase K were used and incubated around 1 h to 1 h and 30 min at 37°C. Then 100 µL of 5M NaCl were added with 80 IU from CT 80 µL of

CTAB/NaCl and placed in a water bath at 65°C for 10 min in addition to 780 µL of chloroform/isoamyl alcohol and manually shaken for 10 min and centrifuged for 5 min at 14.000 rpm after cooled.
"The supernatant was transferred to new tubes, and 360 µL of ice-cold isopropanol was added. Then, the microtube was inverted until the DNA became visible, and it was centrifuged at 14000 rpm for 25 min. However, immediately afterward, the supernatant was discarded, the DNA washed with 1 mL of 70% ethanol and centrifuged for 3 min at 14000 rpm, and the DNA was allowed to dry for 20 min in a laminar flow chamber. The DNA was resuspended in 50 µL of 0.1X TE added with ribonuclease (10 mg mL -1) and incubated at laboratory temperature 25° C. After completion of extraction, the samples were quantified in Qubit™. The amplicons were analyzed in 2% agarose gel (2% w/v). Then, the samples were diluted to concentrations of 10/µL and stored at -20°C" [Barros et al. 2022].

Polymerase chain reaction (PCR)
For the amplification of the genetic material (the DNA), DNA concentration was performed using the Biodrop µLite spectrophotometer (Thermo Fisher Scientific, USA). For identification, the 16S rDNA gene was used, with the aid of primers ITS-1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') with approximately 600 base pairs of amplification.
PCR was performed with a total volume of 25 µL of a reaction mixture consisting of 12.5 µL of 2X PCR Master Mix (Promega GoTaq® Master Mix, Wisconsin, USA), 20 pmol of the primers, and 100 ng/µL of bacterial DNA. PCR was performed on a thermal cycler apparatus (Mastercycler, Eppendorf, Germany).
PCR was performed with the following parameters:
Initial denaturation 3 min at 95ºC, followed by 25 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute and 30 seconds and final extension at 72ºC for 10 min. PCR amplification was analyzed by agarose gel electrophoresis (2% w/v). The run was performed in 0.5X TBE buffer at 120V for 40 min. Amplicons runs were performed using a UV transilluminator, and the images were captured using UPV software (BioDoc-it TM).
The PCR's products were purified using the Exo + SAP enzyme mixture containing two hydrolytic enzymes: recombinant Shrimp Alkaline Phosphatase (rSAP) and Exonuclease I (Exo I), following the manufacturer's recommendations.

Sequencing of Bacterial DNA
For bacteria, DNA plates were used with the help of ITS-1 (5' CTTGGTCATTTAGAGGAAGTAA-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The sequencing of the samples was performed at the company ACTGene Analyses Moleculares Ltda. (Biotechnology Center, UFRGS, Porto Alegre, RS) using the AB 3500 Genetic Analyzer automatic sequencer equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems). The DNA templates were purified using ExoSAP-IT™ PCR Product Cleanup reagent (Applied Biosystems) and quantified in the Qubit™. Afterward, they were labeled using 2.5 pmol of specific primer and 0.5 µL of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) reagent in a final volume of 10 µL. Labeling reactions were performed in an LGC XP Cycler thermocycler with an initial denaturation step at 96 ºC for 3 min followed by 25 cycles of 96 ºC for 10 sec, 55 ºC for 5 sec, and 60 ºC for 4 min. Once labeled, samples were purified by precipitation with 75% isopropanol and washed with 60%

ethanol. The precipitated products were diluted in 10 µL of Hi-Di™ formamide (Applied Biosystems), denatured at 95 ºC for 5 min, cooled on ice for 5 min and electro-injected in the automatic sequencer. Sequencing data were collected using the Data Collection 3 program (Applied Biosystems) with Dye parameters Dye Set "Z"; Mobility File "KB_3500_POP7_BDTv3.mob"; BioLIMS Project "3500_Project1"; Run Module1"FastSeq50_POP7_50cm_cfv_100" and Analysis Module 1 "BC-3500SR_Seq_FASTA.saz".
The resulting Data Collection files (ab1; electropherograms) were converted to FASTA files (seq; text) by Sequence Analysis Software v. 6 (Applied Biosystems) under standard parameters. These sequences were compared to 16S rDNA gene sequences of bacteria available in the GenBank database.

Phylogenetic Analyses

"Phylogenetic relationships among the bacteria strains were performed by the alignment of sequences using the Clustal X 2.0 software" (Larkin *et al*., 2007). "Phylogenetic trees were constructed using the software Mega 6.0 and Neighbor-joining methods" (Tamura, 2011).

RESULTS AND DISCUSSION

Morphology Analyses

The morphologically identified and biochemical analyses were Gram-positive bacilli from the *Bacillus* spp. group isolated on blood agar and catalase staining and Gram-negative bacilli, enterobacteria by isolation on Mac Conkey agar, fermenting lactose, producing glucose and non-urease.
Bacteria were found during the 120 h of fermentation of cocoa almonds. Gram-negative bacteria, Enterobacteria (n=21) were found in 24 h until 48 h, these are endophytic bacteria belonging to the microbiota of cocoa (Lima *et al*., 2011). Lactic acid bacteria (n=04) were found witin 48 h, and several species of *Bacillus* spp.(n=19), endophytic bacteria, were found until 120 h.

Molecular Taxonomy

DNA was extracted from 21 Gram-negative colonies morphologically identified as Enterobacteriaceae and 19 Gram-positive as *Bacillus* spp. However, taxonomic identification was only possible for 07 clones of Enterobacteria spp and 05 clones of *Bacillus* spp. Colonies of lactic acid bacteria were not identified by molecular taxonomy.
The Gram-negative bacteria DNA were found: *Citrobacter sp.*, *Enterobacter hormaechei*, and Gram-positive bacteria DNA *Bacillus cereus* strain and *Bacillus thuringiensis*. (Table 1).
Table 1: Identity of bacteria morphologically found in the cocoa bean fermentation process.

Bacteria Identification	Colonies Morphology	Taxonomic Identification-Clones	
Gram negative	Enterobacteriaceae (n=21)	Enterobacteria spp	
Gram positive	Bacillus sp.(n=19)	*Bacillus* spp	

	lactic acid bacteria (n=4)	-	

Table 2: Identity of bacteria found in the cocoa bean fermentation process. Isolates internal code B1 to B4**.**

	nº (clones quantity, n=12)	Identity	16S rDNA Identity %	Accession No
BACTERIA	B1(4)	*Citrobacter sp.*	89,7%	EU675855
	B2(1)	*Bacillus cereus*	81.99%	JF772081
	B3(3)	*Enterobacter hormaechei*	76.47%	MK806684
	B4(4)	*Bacillus thuringiensis*	92.50%	MW578838

The bacteria identified are endophytic bacteria of cocoa seeds in the present study; they participate in the fermentation process in the first h (48 h) but have potential contaminants because they are enterobacteria that are bioindicators of environmental quality (Enterobacter and Citrobacter). *Bacillus thuringiensis* found between 72 and 120 h are also endophytic bacteria that protect the fermentative microbiota of cocoa seeds against phytopathogens, while Bacillus cereus presents pathogenic characteristics in almonds and cereals.

Gram-negative bacilli were possibly present in almonds belonging to the Enterobacteriaceae family, characterized by being glucose fermenters with or without gas production. Enterobacter is a genus that belongs to this family and is considered an owner responsible for a range of health infections (Tullio,2019). Gram-negative bacteria of the Pseudomonas genus are part of the plant growth-promoting rhizobacteria (RPCP) group, along with the Bacillus genus, where they promote growth.

Citrobacter spp. and *Enterobacter* spp., are Gram-negative bacteria belonging to the family Enterobacteriaceae. These bacteria belong to the total coliform groups that are indicators of environmental contamination (Madigan, 2016).

The Gram bacilli identified morphologically belonged to the *Bacillus* spp. The microorganisms found in fermentation, such as *Bacillus* spp., are used as probiotics in animals, in which they live in the intestines, helping to prevent and combat pathogens, ale, aid in digestion (Monnerat *et al*., 2018).

Bacteria of the genus Bacillus spp. were cells isolated from the fermentation process, having characteristics of antibiosis, through the phytopathogenesis of the production of hydrolytic enzymes in the wall of other microorganisms. *Bacillus subtilis*, Gram-positive, is an example of a growth inhibitor of Fusarium unglutinous fungi (Melo, 2019).

The genus Bacillus includes species beneficial and food contaminants. *Bacillus cereus* is contaminant of cereals and cereal-based products, which cacao, therefore, *Bacillus thuringiensis* and other *Bacillus* sp. are Gram-positive benefits for antibiosis in the environment of soil and cereals.

To be considered an effective percentage with the reported species, this identity needs to be above 80%. This information was verified in all species except for B3, which was 76%, Table 1.

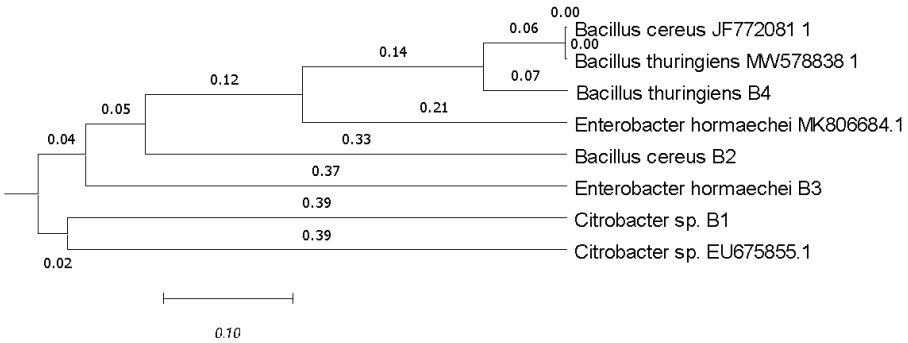

Figure 3. Phylogenetics analyses into the bacterial isolates studied and accession numbers in Genbank. Phylogenetic trees were constructed using the software Mega 6.0 and Neighbor-joining methods, bootstrap- 0.00-1.0. Genetics distance and accession numbers GENBANK were calculated for species Enterobacter *Hormaechei; Citrobacter sp; Bacillus cereus; Bacillus thuringiens*.

In the phylogenetic analysis and alignment sequences in Clustal W were analysed, and a cluster resulted from the genetic identity of the studied species (Figure 3). Citrobacter sequences (B1 and EU675855.1) were inserted in the same single clade, while *Enterobacter Hormaechei*(B3 and MK806684.1) and B*acillus cereus sequences* B2 and *Bacillus thuringiensis* (B4 and MW578838.1 species) and *Bacillus cereus* JF772081.1 were in a separate clade. A genetic distance was high into the clades.
In this study, the sequences (B1 to B3) were near the phylogenetic tree. The studied sequences (B2) presentation slow indented in *Bacillus cereus* JF772081.1 in compaction another sequences (B4) presentation in the same cluster that *Bacillus thuringiensis*, the four clones/ species are of the same genera.
Enterobacter hormaechei was first described by Hormaeche and Edwards around 1986 (Hoffman, 2005). According to Machado 2015, this bacterium is endophytic and commonly found in seeds, having antibiosis characteristics, aiding in the biological control of pathogenic fungi through the synthesis of indoleacetic acid; therefore *Bacillus thuringiensis were* identified *Bacillus* spp. diversity in cocoa beans in the Northeast of Brazil during fermentation processing methods (Bastos, 2016)

Several studies emphasize the importance of replacing the use of chemical fertilizers for more sustainable production using bacteria of the *Enterobacter and Bacillus* genus, among others, which are being widely used in agricultural biotechnology as growth promoters, biofertilizers providing some nutrients for plants and as biocontrol agents (Sharma V, Kaur, and Sharma, 2021).

CONCLUSION

This study provides new data about bacterial microbiota in the cocoa crop grown in soils of the Amazon region. Further research is essential to know the more about the active metabolites of endophytic bacterial isolates from cocoa to explore them for potential biotechnological applications

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