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Isolation and Identification of Microbial Species Found in Cocoa Fermentation as Microbial Starter Culture Candidates for Cocoa Bean Fermentation in Colombia

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Abstract

Microbial activity involved in the cocoa beans fermentation process is essential to maintain and improve the organoleptic and nutritional qualities of chocolate; therefore, the aim of this investigation was to search and select microbial isolates with the potential to improve the quality of cocoa beans. Fermentation experiments were conducted on farms located in Maceo (Antioquia), San Vicente de Chucurí (Santander), and Rivera and Algeciras (Huila), Colombia. Yeast, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and mesophilic aerobic microorganisms were obtained from different fermentation batches. The growth of these microorganisms was tested in six treatments as follows: 50% cocoa pulp agar (CPA), high concentrations of glucose (10%), ethanol (5%), and acetic acid (7%), an acidic pH of 3.0, and a high temperature of 50°C for 24 h. The isolates with the highest growth were identified by 18S and 16S rRNA gene analysis, revealing a high diversity of species associated with cocoa fermentation, including eight species of yeasts (*Debaryomyces hansenii, Meyerozyma guillermondii, Wickerhanomyces anomalus, Pichia guillermondii, Pichia kudriavzevii, Trichosporon asahii, Candida parapsilosis*, and *Pichia manshurica*), six species of LAB (*Pediococcus acidilactici, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus farraginis, Lactobacillus rhamnosus,* and *Leuconostoc mesenteroides*), four species of AAB (*Gluconobacter japonicus, Acetobacter tropicalis, Acetobacter pasteurianus*, and *Acetobacter malorum/tropicalis*), and three species of *Bacillus* spp. (*Bacillus aryabhattai /megaterium, Bacillus subtilis,* and *Bacillus coagulans*). In general, microbial populations increased in cocoa batches after 12 h of fermentation and decreased after 84-96 h. All the yeast isolates grew in 10% glucose and CPA, 85.7% in 5% ethanol, and 95% at a pH of 3.0. All the yeast isolates were affected by 7% acetic acid and incubation at 50°C for 24 h. Eighty-five percent of the LAB grew in 10% glucose, 100% in 5% ethanol, 42.8% in CPA, 64% at a pH of 3.0, and 35.7% grew after being exposed to 50°C for 24 h; all were affected by 7% acetic acid. As for the AAB, 100% grew in 10% glucose, 71% in 7% ethanol, 100% grew in CPA, in 7% acetic acid, and at a pH of 3.0, while 100% were affected by incubation at 50°C. Three yeast isolates, *W. anomalus, D. hansenii* and *M. guillermondii,* three LAB isolates, *P. acidilactici*, *L. brevis*, and *L. plantarum*, and three AAB isolates, *A. tropicalis*, *A. pasteurianus* and *G. japonicus*, were selected as promising strains to be used in a microbial starter culture for cocoa bean fermentation to improve the organoleptic quality of cocoa.

Keywords: Cocoa bean, fermentation, *Theobroma*, microorganisms

INTRODUCTION

Cocoa fermentation is an important process for grain quality. In addition to the microbial population, other factors influence this process, such as environmental conditions, management practices, type of material, and environmental pollution (air, dust, and insects), which make fermentation outcome highly variable (De Vuyst & Weckx, 2016). In the fermentation of grains, many microorganisms in growth and processing environments intervene (Schwan & Wheals, 2004). Proper fermentation is directed by a particular succession of microbial activities, where four groups of microbes have been characterized, including yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and some species of the *Bacillus* genus, which are synonymous of a good fermentation (De Vuyst & Weckx, 2016; Lima *et al*., 2011). The cocoa pulp is composed mainly of water (82-87%), sugars (10-13%), pentosan (2-3%), citric acid (1-2%), and salts (8-10%), which stimulate the growth of microorganisms populations (Schwan & Wheals, 2004). During the first 48 hours, the fermentation conditions are low in oxygen, and the grains are colonized by yeasts and LAB that degrade the seed pulp; this, in turn, favors the entry of oxygen into the fermentation mass. Both yeasts and LAB consume pulp sugars and organic acids, producing ethanol and lactate. The increase in oxygen in the fermentation mass favors the colonization of AAB. The oxidation of ethanol by AAB produces CO_2 and heat, which increases the temperature of the mass that can reach a temperature of 51°C (Schwan & Wheals, 2004).

The LAB contributes to the fermentation of cocoa, controlling bacterial growth and pH, and, therefore, the desired metabolism conditions of microorganisms and beans (De Vuyst *et al*., 2010). Ethanol is oxidized to acetic acid by AAB. Besides, volatile substances produced during fermentation, such as alcohols, aldehydes, esters, and ketones, contribute to the generation of essential flavor and aroma precursors in grains (Schwan & Wheals, 2004; Lima *et al*., 2011; Ho *et al*., 2014).

Many studies have reported the diversity of microorganism species present in cocoa fermentation; however, it is crucial to identify the most beneficial strains for improving grain quality (Thompson *et al*., 2013). Due to the high microbial diversity, the use of molecular tools for the identification of interspecific and intraspecific diversity has increased significantly (Guerber *et al*., 2003). The analysis of the DNA of each cell provides the highest amount of information on the genotypic and phenotypic identities of each organism. The PCR technique using specific primers identifies and characterizes microorganisms. With the help of molecular markers for the identification of species and populations, the restrictive fragment analysis of PCR products has proven to be suitable for the study of different bacteria and yeast species by identifying them at the genus level.

Accordingly, the aim of this study was to improve the knowledge of the microbial population dynamics in cocoa fermentation on producer farms in different Colombian cocoa-producing regions and to identify isolates with potential characteristics for improving the quality of cocoa beans.

MATERIALS AND METHODS

Monitoring and Isolation in Specific Culture Media

Isolation of microorganisms

Cocoa fermentation was monitored in three municipalities on farms selected due

to the good quality characteristics of their cocoa beans. Three hundred grams of beans were aseptically scooped from the center of the fermenting mass at 12 h intervals to isolate microorganisms. Forty grams of the cocoa beans were mixed with sterile saline solution (0.85% sodium chloride (Merck KGaA) at a rate of 1:1.25 using a vortex (IKA MS3 digital) for 10 min until obtaining a cocoa pulp solution. Serial 10-fold dilutions were made until 10^{-14} in sterile saline solution followed by spread platting of 0.1 mL agar media specific to each class of organism for isolation and enumeration. Values of colony-forming units per milliliter (CFU/mL) were transformed to log10 CFU/mL. Specific media for each microbial class were employed as follows: Yeast [(Freitas, 1998), TYGKCC-0.5% casein peptone, 0.5% yeast extract, 0.1% D-glucose, 0.1% K_2HPO_4, 0.1% $CaCO_3$, 1% cocoa pulp (Freitas, 1998), 500 mg/L chloramphenicol, and 1.5% agar]; acetic acid bacteria [(Ohmori, 1982), 1% yeast extract, 0.4% pectone, 6% glucose, 1% calcium carbonate, 2% agar, and 100 mg/L nystatin, at pH 7.4]; lactic acid bacteria [1% peptone, 0.5% yeast extract, 2% D-glucose, 0.5% K_2HPO_4, 0.5% $CH_3COONa.3H_2O$, 0.05% $MgSO_4.7H_2O$, 0.02% $MnSO_4.4H_2O$, 1 mL Tween 80, 1.8% agar, and 10% tomato juice (Ardhana & Fleet, 2003). Cycloheximide was replaced by nystatin (3,000 IU that correspond to 1 mg of nystatin), at pH 6.5]. All media were autoclaved at 121°C for 15 min. Incubations were performed at 30°C from 48 to 72 h (Lefeber *et al*., 2012), and the numbers of log10 CFU/mL in each culture medium were recorded.

Physicochemical characteristics of the cocoa beans during fermentation

To determine the pH of the beans and testas, 4 g of beans were weighed, and the testas were removed with a scalpel. The testas and beans were placed into separate mortars, each containing 20 mL of distilled water, macerated, and then the pH was measured in each. The titratable acidity was determined with 0.1 N NaOH (López *et al*., 2019). The rest of the sample was subjected to drying by solar radiation until reaching a constant weight. Dried samples were stored at 1±2°C. The fermentation index (FI) (FI = 460/530) through the analysis of condensed anthocyanins (460 nm and 530 nm) was determined by spectrophotometry (Thermo scientific, Helios zeta UV-VIS). Fermented cocoa beans (0.2 g) were macerated in distilled water and deposited in flat-bottom9 plastic crates; then, 20 mL of CH_3OH-HCl (97:3) were added. The mixture was homogenized and placed at 8°C for 19 h. Subsequently, the mixture was centrifuged for 20 min at 9,410 x g, and 4°C (Heal Force, Neofuge 23 R). The supernatant absorbance was calculated at 460 nm and 530 nm to determine the FI (Caporaso *et al*., 2018; Sunoj *et al*., 2016).

Screening of microorganisms

Six growth conditions were analyzed to select the most promising isolated candidates to be used in a fermentation starter culture that improves the quality of cocoa beans, considering the fermentation conditions of cocoa beans (i.e., high concentrations of sugars, high acidity, and high temperature). Forty-two isolates (21 yeast, 14 LAB, and 7 AAB) obtained from various cocoa fermentations were subjected to six growth treatments, as follows: high concentrations of 10% glucose, 5% ethanol, and 7% acetic acid, high acidic pH (3.0), high temperature (50°C) and 50% cocoa pulp agar (CPA). Ethanol was added to the culture media after autoclaving. The treatment with a pH of 3.0 was adjusted with 1 N HCL, which was added after autoclaving. For the 50% cocoa pulp treatment, the agar

and pulp were autoclaved separately and were then mixed in a laminar flow cabinet. Each microorganism suspension was adjusted in sterile saline solution to a McFarland 2 index, and then 10 µL of each suspension was placed at four points on the agar. The plates were incubated for 96 h at 28±2ºC, and then, the diameters of the colonies were measured. The data was analyzed using an ANOVA, and differences of means were established with an LSD all-pairwise comparisons test and Tukey's HSD all-pairwise comparisons test at $P < 0.05$.

Molecular Characterization

DNA extraction

DNA was extracted from pure cultures of yeasts, LAB, and AAB. Yeasts were cultured in yeast glucose chloramphenicol broth (YGC) at 30ºC, LAB in MRS broth at 35ºC, and AAB in YPGC broth at 35ºC. Two milliliters of each growth culture medium were transferred to microtubes. A quick DNA-Fungal/Bacterial kit for extraction (Zymo Research) was used following the recommended instructions of the manufacturers. The concentration and purity of the DNA were obtained using a Thermo Scientific NanoDrop 2000/2000C spectrophotometer, and then by electrophoresis on 1% agarose gel.

PCR amplification

Yeast 18S PCR amplification was performed with some initiators (NL1-F, LR6-R, ITS1-F, ITS1-R) previously used by Rodriguez *et al.* (2008), for the molecular identification and establishment of a barcode. These sequences are presented in Table 1. The PCR protocol used was performed with 21 µL of Taq Ready Mix (Sigma Chemical Company), 1 µL of LR6-R/ITS1-R primers, 1 µL of NL1-F/ITS1-F primers, and 2 µL of DNA; the process was carried with the following conditions: A denaturation stage that lasted 3 min at 94°C followed by 30 cycles at 94°C for 45 s, 63°C for 40 s, 72°C for 90 s, and 72°C for 10 min. PCR products were visualized after electrophoresis on a 1.5% agarose gel. Finally, products were sequenced by Corpogen (Bogotá, Colombia) in partnership with Macrogen Korea (export permit number for non-CITES specimens: 01753) (Table 1).

For LAB and AAB, 16S rRNA genes were amplified with the same reaction volumes used in the Taq Ready Mix, reverse primer, forward primer and genomic DNA, under the following conditions: 94°C for 3 min followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 65°C (1st cycle) to 55°C (10th cycle) for 1 min, and extension at 72°C for 1 min; subsequently, 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Finally, an extension to 10 min was carried out in the last cycle. PCR products were visualized after electrophoresis on a 1.5% agarose gel. Finally, products were sequenced by Corpogen (Bogotá, Colombia) in partnership with Macrogen Korea (export permit number for non-CITES specimens: 01753).

Table 1. Yeast 18S rDNA primers used for sequence identification

Primer	Sequence
NL1-F	5'-GCATATCAATAAGCGGAGGAAAAG-3'
LR6-R	5'-CGCCAGTTCTGCTTACC-3'
ITS1-F	5'-TCCGTAGGTGAACCTGCGG-3'
ITS1-R	5'-TCCTCCGCTTATTGATATGC-3'

RESULTS AND DISCUSSION

Isolation of Prevalent Species in the Cocoa Fermentation Process

This study showed an abundant microbial species diversity in cocoa fermentations

identifying some key strains for their tolerance to heat, high glucose or, ethanol concentration, and acid pH; this is crucial since the cocoa pulp is rich in fermentable sugars (10-15%) such as glucose, fructose, and sucrose (Duarte *et al*., 2010), and has a low pH of 3.0–3.5 mainly due to the presence of citric acid (Ardhana & Fleet, 2003). These conditions are selective for the initial growth of yeasts and lactic acid bacteria.

On the Rivera (Huila) farm 12 h after the beginning of the cocoa fermentation, the microbial populations began to increase. The presence of the three different groups reported, i.e., yeast, LAB, and AAB (Figure 1a, 1b, and 1c), were observed from the beginning of the fermentation. Between 48 and 108 h, the highest CFU values for the three groups were recorded with a gradual decrease after 120 h (Figure 1a). Regarding pH, the cotyledon, testa, and pulp solutions registered the following values at the end of the fermentation, the testas exhibited a pH of 3.8 at 0 h of fermentation and reached a pH of 4.4 at the end of the process, while the cotyledons registered an initial pH of 6.5 and finished with a pH of 4.2 (Figure 1b). For the municipality of San Vicente de Chucurí (Santander), CFU values at the beginning of the fermentation were higher than those registered in Rivera (Huila). Pulp CFU values were maximum between 60 and 72 h of fermentation; from this point onwards, the populations decreased (Figure 1c). The pH values of the testa and pulp solutions were similar; however, the pH of the cotyledon registered a value of 6.0 at 0 h and decreased as fermentation progressed to a pH of 4.1 at 132 h (Figure 1d). Similarly, microbial populations at the municipality of Maceo (Antioquia) showed high CFU values in the malt extract medium with a decrease after 120 h of fermentation; the most abundant populations were recorded between 84 and 96 h (Figure 1e). The behavior of the pH values for the pulp and cotyledon solutions showed similar values in the Rivera and San Vicente de Chucurí farms at the end of the fermentation, with average pH values between 4 and 4.5 (Figure 1f). Morphologies were highly diverse, signaling confirmed differences revealed by molecular identifications. Unique isolates passed to the second phase of selection and characterization by specific tests such as resistance to ethanol, acetic acid, temperature, and acidic pH.

The fermentation index (FI) registered at each sampling moment exhibited a similar trend throughout the three regions. Since the FI is a rate between the absorbance at 460 and 530 nm, this relationship was mainly affected by the absorbance values at 530 nm, which were high at the beginning of the fermentation and decreased as the process progressed; on the contrary, the values at 460 nm remained relatively constant. This trend was observed in the three regions under study. The beans obtained from the Santander region recorded the lowest absorbance values in the two wavelengths under study, which may indicate a lower presence of anthocyanins in the beans of this region (Figure 2). Cotyledon staining is a typical genetic characteristic associated with the cocoa variety. Usually, the anthocyanin content is high at the beginning of the fermentation and decreases as it progresses (Pelaez *et al*., 2016).

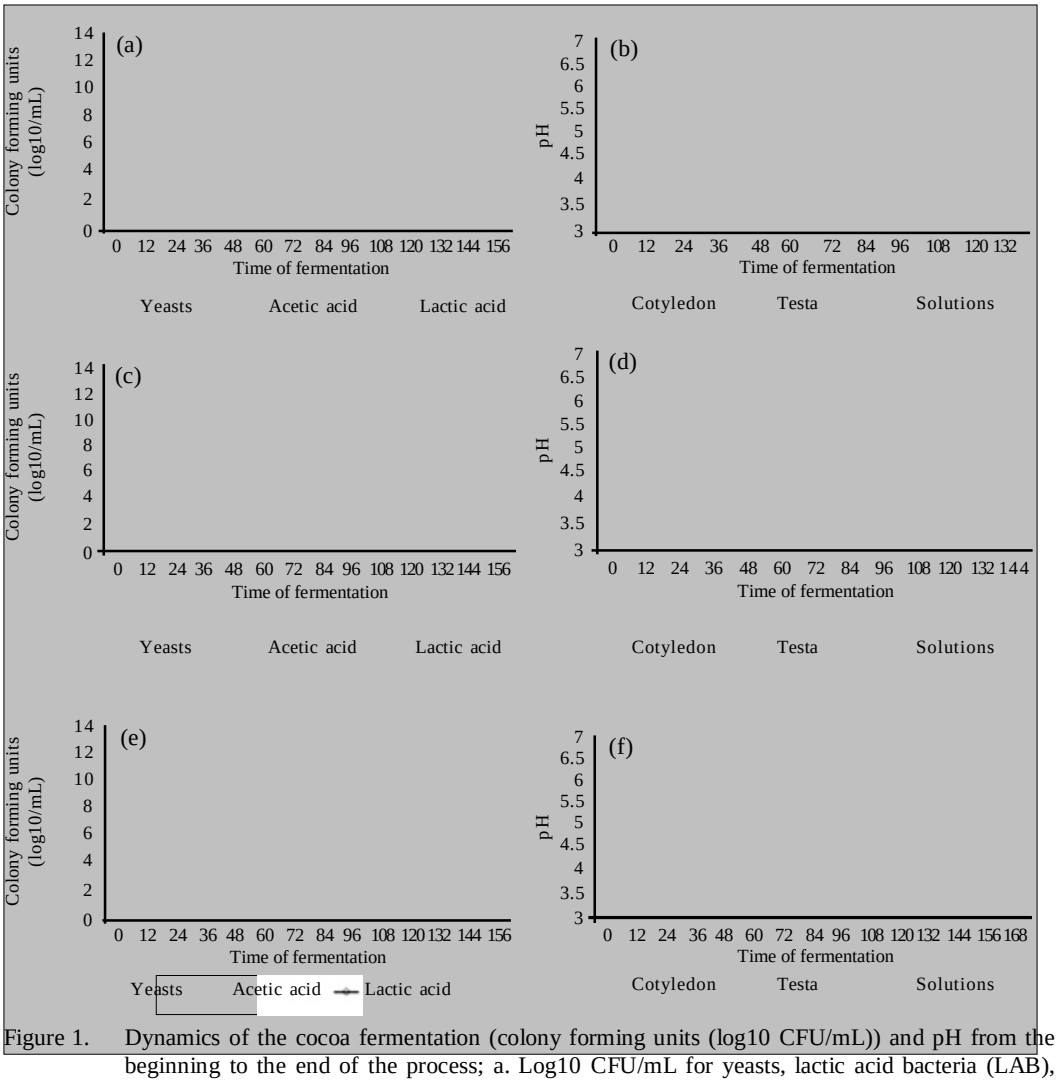

Figure 1. Dynamics of the cocoa fermentation (colony forming units (log10 CFU/mL)) and pH from the beginning to the end of the process; a. Log10 CFU/mL for yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (municipality of Rivera, Huila), b. Culture pH for cotyledon, testa, and pulp solutions (municipality of Rivera, Huila), c. Log10 CFU/mL for yeasts, LAB, and AAB (San Vicente de Chucurí, Santander), d. Culture pH for cotyledon, testa, and pulp solutions (San Vicente de Chucurí, Santander), e. Log10 CFU/mL for yeasts, LAB, and AAB (Maceo, Antioquia), and f. Culture pH for cotyledon, testa and pulp solutions (Maceo, Antioquia).

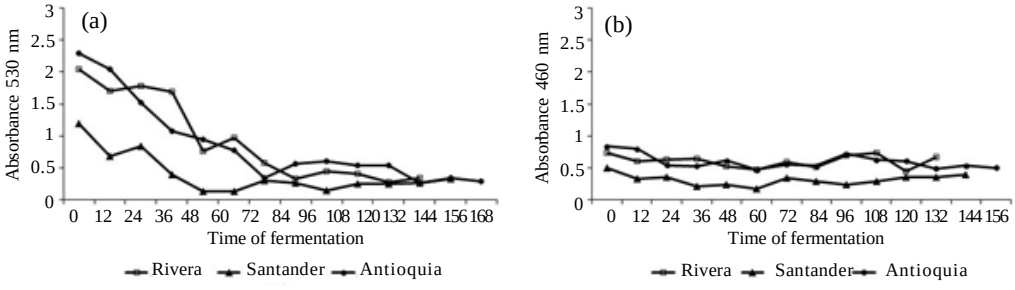

Figure 2. Absorbance values 530 nm (a), and 460 nm (b) from supernatants obtained from fermented and dried cocoa beans in three sampled regions of Colombia during the entire fermentation process

Selection of Microorganisms by Physicochemical Parameters

Resistance testing is the most widely accepted practice for the selection of the group of microorganisms that comprise a microbiological initiator culture, guaranteeing in this way its permanence during the fermentation phase. This is the reason why each of the conditions surrounding this process must be evaluated to select the best strains.

Twenty-one isolates obtained from different cocoa fermentation processes in the three regions identified as yeasts were subjected to various growth analysis tests (Table 2). All the yeast isolates (100%) grew on agar medium supplemented with 10% glucose. Isolates 77, 88, 90, and 93 showed the largest colonies ($F_{20,83} = 8.63$; $p = 0.000$) on 10% glucose medium. The growth of isolates 63, 73, 77, and 87 on 5% ethanol-supplemented agar was superior ($F_{20,83} = 130.43$; $p = 0.000$) compared to the other isolates; however, 85.7% of the yeast isolates grew on this medium. All the yeast isolates were inhibited by 7% acetic acid, and 100% of the yeast isolates grew on CPA; however, in descending order, isolates 85, 92, and 97 grew best in this medium ($F_{20,83} = 7.37$; $p = 0.000$). Growth was stimulated ($F_{20,83} = 9.10$; $p = 0.000$) in isolates 94, 97, and 99 when cultured in a medium with an acidic pH of 3.0; on the contrary, isolate 88 exhibited no growth at this pH (Table 2).

In medium selective for LAB, 14 identified isolates were subjected to growth analysis tests (Table 3). Isolates 6, 13, 24, 46, and 314 showed high growth ($F_{13,55} = 3.79$; $p = 0.0005$) in 10% glucose medium, whereas isolates 56 and 315 showed no growth. All isolates grew in 5% ethanol medium, while isolates 12, 24, 34, 308, and 309 showed numerically higher values but with no statistical differences. Six isolates, 12, 13, 24, 34, 44 and 56, showed growth in CPA ($F_{13,55} = 61.46$); $p = 0.000$), while nine isolates, 7, 13, 24, 34, 44, 56, 308, 314 and 315, showed growth at a pH of 3.0 ($F_{13,55} = 23.73$); $p = 0.000$). Further, five isolates, 6, 12, 13, 34 and 44, subjected to 50°C for 24 h were tolerant to this temperature ($F_{13,55} = 120.05$; $p = 0.000$).

Table 2. Yeast colony diameters[1)] of isolates obtained from several cocoa fermentation processes in three regions of Colombia cultured on agar media subjected to different treatments

Number	Isolate	Glucose (10%)	Ethanol (5%)	Acetic acid (7%)	CPA	pH 3	50°C
62	*Wickerhamomyces anomalus*	11.20 fg	0.0 j	0	6.16 i	11.13 de	0
63	*Wickerhamomyces anomalus strain* ADJ2	9.32 g	8.28 bc	0	7.65 cdefg	8.31 efg	0
65	*Wickerhamomyces anomalus*	9.34 g	0.0 j	0	7.93 cdefg	5.27 g	0
66	*Wickerhamomyces anomalus*	10.67 fg	8.09 bc	0	7.27 fgh	11.50 cde	0
67	*Debaryomyces hansenii strain* YE3	13.95 def	6.65 hi	0	8.38 bcde	10.43 de	0
73	*Meyerozyma guilliermondii*	11.17 fg	8.73 ab	0	8.60 bcd	10.40 de	0
77	*Pichia manshurica strain* CBS 11610	20.46 bc	9.19 a	0	7.68 cdfg	9.94 de	0
78	*Debaryomyces hansenii*	11.96 fg	7.80 cde	0	7.54 defgh	10.17 de	0
81	*Meyerozyma guilliermondii*	18.79 bc	0.0 j	0	6.84 ghi	10.35 de	0
84	*Candida stellimalicola strain* NRRL	19.87 bc	7.10 fgh	0	7.39 efgh	6.13 fg	0
85	*Candida parapsilosis strain* CDC317	19.08 bc	7.77 cdef	0	10.85 a	13.33 bcd	0
87	*Meyerozyma guilliermondii*	17.13 cde	8.22 bc	0	8.58 bcd	10.45 de	0
88	*Meyerozyma guilliermondii*	22.05 ab	6.25 i	0	7.18 fghi	0.0 h	0
89	*Meyerozyma guilliermondii*	10.04 fg	6.82 ghi	0	7.56 defgh	9.17 ef	0
90	*Meyerozyma guilliermondii*	20.58 bc	8.03 cd	0	8.63 bcd	11.30 de	0
92	*Meyerozyma guilliermondii*	11.77 fg	7.97 cd	0	9.47 b	10.73 de	0
93	*Meyerozyma guilliermondii*	25.14 a	7.13 efgh	0	6.92 ghi	7.91 efg	0
94	*Pichia guilliermondii*	13.37 efg	6.97 gh	0	6.48 hi	15.23 bc	0
95	*Meyerozyma guilliermondii*	18.45 bcd	7.37 defg	0	8.24 cdef	13.55 bcd	0
97	*Meyerozyma guilliermondii*	16.56 cde	7.82 cde	0	8.70 bc	19.07 a	0
99	*Meyerozyma guilliermondii*	13.59 efg	7.38 defg	0	7.61 cdefg	16.89 ab	0

Notes: [1)]Mean diameter (mm), n = 4 (growth of four colonies) and Means in columns and with the same superscript are not significantly different ($P > 0.05$).

Seven isolates were characterized as acetic acid bacteria, with isolates 310, 312, and 313 exhibiting significant growth ($F_{6,27}$ = 14.72; p = 0.000) in the 10% glucose medium (Table 4). In the 5% ethanol medium, five isolates showed some growth, while two were inhibited ($F_{6,27}$ = 75.27; p = 0.000). In the 7% acetic acid medium, all isolates showed growth; however, isolates 287 and 307 presented the largest colonies ($F_{6,27}$ = 5.30; p = 0.0018). Isolate 287 showed the largest growth (P<0.05) in CPA medium ($F_{6,27}$ = 19.57; p = 0.000), while isolate 312 exhibited the largest growth in an acidic medium with a pH of 3.0 ($F_{6,27}$ = 9.03; p = 0.0001). No isolates grew when subjected to 50°C.

The fermentation of cocoa pulp increases the temperature during the exothermic production of acetic acid, helping to decrease the pulp cells of the cocoa beans (Nielsen *et al*., 2007). Consequently, enzymes responsible for the degradation of sugars, amino acids, and peptides contribute to the release of volatile compounds related to flavor and aroma precursors. Some studies (of Meersman *et al*., 2015a,b) carried out with tolerant yeasts at high temperatures indicate that they are suitable for starter cultures as they can suppress the growth of the native cocoa microbiota and improve the quality of the final product. Some authors denote that thermotolerance is a decisive factor for the effectiveness of a microbiological initiator culture (Meersman *et al*., 2015a).

Table 3. Lactic acid bacteria colony diameters[1] of isolates obtained from several cocoa fermentation processes in three regions of Colombia cultured on agar media subjected to different treatments

Number	Isolate	Glucose (10%)	Ethanol (5%)	Acetic acid (7%)	CPA	pH 3	50°C
6	*Pediococcus acidilactici*	26.60 a	14.30 a	0	0.0 c	0.0 e	16.34 c
7	*Pediococcus acidilactici*	18.95 ab	17.59 a	0	0.0 c	18.89 bcd	0.0 d
12	*Pediococcus acidilactici*	24.26 ab	29.93 a	0	7.45 b	0.0 e	32.29 a
13	*Pediococcus acidilactici*	30.83 a	24.59 a	0	9.04 ab	29.70 ab	28.05 ab
24	*Lactobacillus brevis*	25.80 a	26.17 a	0	11.75 a	4.44 e	0.0 d
34	*Leuconostoc mesenteroides*	17.18 ab	25.12 a	0	7.49 b	25.39 ab	24.15 b
44	*Pediococcus acidilactici*	21.97 ab	24.47 a	0	8.83 ab	23.39 ab	28.00 ab
46	*Lactobacillus farraqinis*	28.34 a	16.63 a	0	0.0 c	0.0 e	0.0 d
56	*Lactobacillus plantarum*	0.0 b	12.62 a	0	11.18 a	9.53 cde	0.0 d
209	*Lactobacillus rhamnosus*	20.50 ab	13.02 a	0	0.0 c	0.0 e	0.0 d
308	*Lactobacillus plantarum*	23.85 ab	30.50 a	0	0.0 c	21.22 bc	0.0 d
309	*Lactobacillus plantarum*	19.22 ab	27.15 a	0	0.0 c	0.0 e	0.0 d
314	*Lactobacillus plantarum*	30.53 a	22.09 a	0	0.0 c	35.38 a	0.0 d
315	*Lactobacillus plantarum*	0.0 b	10.05 a	0	0.0 c	7.44 de	0.0 d

Notes: [1)]Mean diameter (mm), n = 4 (growth of four colonies); Means in columns and with the same superscript are not significantly different (P > 0.05).

Table 4. Acetic acid bacteria colony diameters[1)] of isolates obtained from several cocoa fermentation processes in three regions of Colombia cultured on agar media subjected to different treatments

Number	Isolate	Glucose (10%)	Ethanol (5%)	Acetic acid (7%)	CPA	pH 3	50°C
286	*Gluconobacter sp.* LBN175	14.28 c	0.0 c	11.87 abc	8.84 b	10.87 b	0
287	*Gluconobacter japonicus*	19.60 bc	0.0 c	12.43 a	17.67 a	12.59 b	0
307	*Acetobacter tropicalis*	24.54 bc	20.95 a	12.56 a	8.14 b	10.91 b	0
310	*Acetobacter tropicalis*	37.88 a	14.60 b	9.44 c	10.36 b	14.95 b	0
311	*Acetobacter pasteurianus/pomorum*	19.79 bc	14.83 b	10.68 abc	8.14 b	12.35 b	0
312	*Acetobacter tropicalis*	35.83 a	19.91 a	9.80 bc	8.05 b	24.41 a	0
313	*Acetobacter malorum/tropicalis*	29.71 ab	14.54 b	12.08 ab	7.97 b	11.67 b	0

Notes: [1)]Mean diameter (mm), n = 4 (growth of four colonies). Means in columns and with the same superscript are not significantly different (P > 0.05).

Molecular Characterization

The sequence analysis of the yeast isolates showed that six correspond to the species *C. parapsilosis* (68, 72, 76, 83, 85, and 98), three are *D. hansenii* (67, 75, and 78), 13 are *M. guillermondii* (73, 81, 87, 88, 89, 90, 91, 92, 93, 94, 95, 97, and 99), four are *W. anomalus* (62, 63, 65, and 66), one is *P. manshurica* (77), one is *C. stellimalicota* (84), one is *P. guillermondii* (94), and three are *T. asahii* (64, 66, and 71). The characterization of LAB also registered high diversity. Sequence identification revealed that some isolates correspond to the species *P. acidilactici* (6, 7, 13, and 44), *L. brevis* (24), *L. plantarum* (56, 308, 309, 314, and 315), *L. farraginis* (46), *L. rhamnosus* (209), and *L. mesenteroides* (34). In the AAB group, some isolates belong to the species *Gluconobacter* sp. (286), *G. japonicus* (287), *A. tropicalis* (307, 310, and 312), *A. pasteurianus/pomorum* (311), and *A. malorum/tropicalis* (313). Some isolates were identified as *B. aryabhattai/megaterium* (319), *B. subtilis* (320, 321, and 322) and *B. coagulans* (316).

About 40% of the isolates obtained have been reported in previous studies of population identification in cocoa fermentation. The presence of all these species in the cocoa fermentation mixtures is of great importance because they are internationally referenced as beneficial organisms that produce prebiotics, antibiotics, and B12 vitamin, as is the case with *B. megaterium* (Tortoló & Bell, 2015). *Pediococcus acidilactici* is commonly found in fermented vegetables, dairy products and meat (Barros *et al*., 2001); it is classified as probiotic (Standen *et al*., 2013; Merrifield, 2013) and its bacteriocins have been evaluated for the control of *Listeria monocytogenes* (García *et al*., 2017). *L. brevis* is a fermentative bacterium that produces CO_2 and lactic acid, and can be found in numerous and different environments such as fermented foods; it has anti-inflammatory effects in periodontal illnesses (Della Riccia *et al*., 2007), probiotic properties (Ronka *et al*., 2003; Mancini & Fava, 2016), and has been reported as being able to control the growth of *Escherichia coli* and *Salmonella* (Syukur *et al*., 2013). Furthermore, it has been detected in cocoa fermentation studies. *L. plantarum* exhibits antifungal activity (Lavermicocca *et al*., 2000; Strom *et al*., 2002) and has been used in the treatment of illnesses produced by *Clostridium difficile* (Wullt *et al*., 2003) and colitis (Schultz *et al*., 2002). *L. plantarum* has probiotic potential and reduces the effects of cholesterol (Nguyen *et al*., 2007). This bacterium was previously reported by Lefeber *et al*. (2011) as being associated with cocoa fermentation. *D. hansenii* that has beneficial effects as it participates in maturation processes producing ethanol, CO_2, and a great variety of volatile compounds such as alcohols, esters, ketones, aldehydes and sulphuretted compounds that promote flavor and final aroma (Boekhout & Pfaff, 2003). *D. hansenii* can be found in products with high sugar content, and as it is osmotolerant, it is incredibly advantageous for some biotechnical applications (Breuer & Harms, 2006); further, it has been registered in several cocoa fermentation mixtures (Camu *et al*., 2008). *W. anomalus* is a non-*Saccharomyces* yeast found in wine fermentation that contributes to the aroma through the production of volatile compounds. In recent years, this yeast has been used as a biological control agent against fungi due to its capacity to produce mycotoxins (Coda *et al*., 2011). *Meyerozyma guillermondii* has been characterized as having antifungal features, as a phosphate solubilizer in soils (Nakayan *et al*., 2013), and it has also been associated with cocoa fermentations (de Melo *et al*., 2013). The yeast *M. guilliermondii* was identified in two of the monitored regions, Maceo (Antioquia) and San Vicente de Chucurí (Santander). Most of the isolates

of this genus showed good growth in high concentrations of 10% glucose. *D. hansenii* was reported in Rivera (Huila) and San Vicente de Chucurí (Santander). The two isolates of this genus grew well in the growth tests and were, therefore, candidates to be included in a cocoa fermentation starter culture. *W. anomalus* isolates were detected only in Rivera (Huila), two of which had acceptable development in growth tests, being equally selected as candidates for a microbial starter culture for cocoa bean fermentation.

Regarding the LAB, isolates of *P. acidilactici* were detected at the three fermentation locations. Some LAB isolates showed good growth in all the evaluation media, including 50°C for 24 h, showing to be resistant to this high temperature. *L. plantarum* was present in fermentation mixtures from Algeciras (Huila) and Maceo (Antioquia). Species such as *L. rhamnosus* and *L. farraginis* have been reported as probiotics in the cocoa fermentation process (Moreira *et al*., 2013). Some isolates of this species showed good growth in 10% glucose, 5% ethanol, and at a pH of 3.0. *L. brevis* was isolated only at San Vicente de Chucurí (Santander); the isolate grew well in 10% glucose, 5% ethanol, 50% CPA and at a pH of 3.0, and, therefore, the three isolates belonging to this species were selected as promising to be included in a microbial starter culture for cocoa bean fermentation.

The AAB isolates showed good growth in 10% glucose. Isolates of *A. tropicalis, A. malorum*, and *A. pasteurianus* grew well in all media except for 50°C for 24 h, whereas isolates of the *Gluconobacter* genus did not grow in 5% ethanol or after exposure to 50°C for 24 h. The isolates of this group were the only ones that grew in 7% acetic acid medium. The AAB *A. tropicalis, A. pasteurianus/pomorum*, and *A. malorum* are found in sugar-rich fruit, flower, and vegetable substrates. These bacteria are found in vinegar production and have been reported in wine and cocoa fermentations (Navarro *et al*., 2013; Moens *et al*., 2014). A representative of each species was selected as promising to be included in a microbial starter culture.

CONCLUSIONS

The population dynamics of microorganisms from the three farms were similar. At all times during the monitoring, populations of yeasts, lactic acid bacteria, and acetic acid bacteria were recorded. High species diversity of microorganisms with internationally registered beneficial characteristics were found, such as *P. acidilactici, L. brevis, L. plantarum, L. farraginis, L. rhamnosus, Gluconobacter* sp., *G. japonicus, A. tropicalis, A. pasteurianus/pomorum, A. malorum/tropicalis, D. hansenii, M. guillermondii, W. anomalus, B. megaterium* and *B. subtilis*. However, microorganisms considered as pathogens were also found, such as *Penicillium* spp., *Aspergillus* spp., *E. hirae, E. hormaechei*, and *G. parapsilosis*. Therefore, it is necessary to establish standardization methodologies for cocoa fermentation to improve the nutritional safety and quality conditions of cocoa beans. The temperature of 50°C limited the growth of the isolates and inhibited the growth of yeasts and acetic acid bacteria completely; on the contrary, 28% of the lactic acid bacteria isolates showed resistance to this temperature. Under cocoa fermentation conditions, the tolerant lactic acid isolates corresponded to the species *P. acidilactici*. All acetic acid bacteria isolates grew in 7% acetic acid medium, while yeast isolates were completely inhibited by 7% acetic acid and exposure to 50°C for 24 h. The strains of the species *W. anomalus, D. hansenii, M. guillermondii, P. acidilactici, L. brevis, L. plantarum, A. tropicalis, A. pasteurianus* and *G. japonicus* are the most promising to be included in a microbial starter culture for cocoa bean fermentation to improve the organoleptic quality of cocoa.

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