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Isolation of autochthonous microorganisms to formulate a defined inoculum for small-scale cocoa fermentation

Aislamiento de microorganismos autóctonos para formular un inoculo definido para la fermentación del cacao en pequeña escala

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Abstract

In cocoa fermentation exists a wide microbial diversity; the most important microorganisms are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Such diversity can result that fermented cocoa quality is not always the same. The use of microbial consortia can steer the process, allowing the production of fermented cocoa beans with homogeneous quality and safety. For that reason, it was proposed to use indigenous microorganisms to formulate defined inocula to conduct the small-fermentation of Mexican cocoa. A total of 54 strains were isolated from the spontaneous fermentation of cocoa. These included yeasts (*Candida*, *Rhodotorula*, *Saccharomyces* and *Yarrowia*), LAB (*Lactobacillus* and *Lactococcus*), and AAB (*Acetobacter* and *Gluconobacter*). The cocoa fermentations were inoculated with an inoculum composed (mixed) of lipolytic *Yarrowia*, *Lactococcus lactis*, and *Acetobacter aceti*; only varying inoculation way (mixed or microbial succession) and were compared with spontaneous fermentation. It was observed that fermentation conducted by succession inoculation showed similar behavior to the spontaneous process, obtaining well-fermented cocoa beans with homogeneous quality and safety.

Keywords: Cocoa bean fermentation, yeast, lactic acid bacteria, acetic acid bacteria, microbial successions, small- scale fermentation, defined inoculum.

Resumen

En la fermentación del cacao existe una gran diversidad microbiana siendo las levaduras, las bacterias ácido lácticas (BAL) y las bacterias ácido acéticas (BA) las más importantes. Esta diversidad puede provocar que el cacao fermentado no siempre presente la misma calidad. El uso de consorcios microbianos puede dirigir el proceso permitiendo la obtención de un grano de cacao fermentado con calidad homogénea e inocuo. Por esta razón, se propone la utilización de los microorganismos autóctonos para formular inóculos definidos para dirigir el proceso de fermentación del cacao mexicano en una pequeña escala. Se lograron aislar 54 cepas a partir de una fermentación espontánea del cacao. Dentro de las cuales se encontraron levaduras (*Candida*, *Rhodotorula*, *Saccharomyces* y *Yarrowia*), BAL (*Lactobacillus* y *Lactococcus*) y BAA (*Acetobacter* y *Gluconobacter*). Las fermentaciones del cacao fueron inoculadas con un inóculo conformado por *Yarrowia* lipolytica, *Lactococcus lactis* y *Acetobacter aceti*, variando solamente la forma de inoculación (mixta o sucesión microbiana), y fueron comparadas con una fermentación espontánea. Se observó que la fermentación conducida por un inóculo agregado en forma sucesiva presentó un comportamiento similar al proceso espontáneo, obteniéndose granos de cacao bien fermentados con calidad homogénea e inocuos.

Palabras clave: Fermentación del grano de cacao, levaduras, bacterias ácido lácticas, bacterias ácido acéticas, sucesiones microbianas, fermentación en pequeña escala, inóculo definido.

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1 Introduction

Fermentations have been traditionally used for food processing, increasing their nutritional and functional value due to the production of microbial metabolites (González-Olivares *et al*., 2011; Mendoza-Avendaño *et al*., 2019). The fermentation involves using a metabolic pathway to obtain organic compounds without an exogenous oxidizing agent (Bourdichon *et al*., 2012). Furthermore, if fermentation process is performed correctly, it will increase the microbiological stability and safety of perishable products such as milk, meat, cereals, and vegetables (Cocolin *et al*., 2016; Reyes *et al*., 2018). Besides, fermentation impacts significantly on the physicochemical, microbiological, sensory, and safety characteristics of the food product (Bortolini *et al*., 2016). For food production, there are three important types of fermentation, alcoholic, lactic, and acetic. During cocoa bean fermentation, these kinds of fermentation converged (De Vuyst and Weckx, 2016; Ho *et al*., 2015; Pereira *et al*., 2016; Schwan and Wheals, 2004; Schwan *et al*., 2015). The fermentation of cocoa beans is a complex process that involves several autochthonous microorganisms, which are inoculated from the utensils used in previous post-harvest operations, pod surfaces, worker's hands, and fermentation containers. The process has been extensively studied. However, although there is evidence about the microorganisms involved in the process (microbial succession), it has not yet been possible to industrialize it on a large scale with suitable starter cultures to obtain a homogeneous quality in the fermented cocoa beans. Yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) are the main microorganisms involved in this process (Adler *et al*., 2014; Camu *et al*., 2008a, 2007; De Vuyst and Weckx, 2016; Schwan *et al*., 2015).

In cocoa bean fermentation, there are three main phases. The first one comprises the yeast´s growth, which predominantly belongs to *Hanseniaspora*, *Saccharomyces*, *Kluyveromyces*, *Candida*, and Pichia genera. These yeast are favored by the high concentration of glucose and citric acid and the low availability of oxygen, yielding ethanol and other compounds as carbon dioxide, acetic and succinic acid, and glycerol (Ardhana and Fleet, 2003). Moreover, yeast produces a large number of aromatic compounds precursors, as higher alcohols and esters (Iñiguez-Muñoz *et al*., 2019; Nawaz *et al*., 2020), that contribute significantly to the chocolate aroma profile (Crafack *et al*., 2013; Ho *et al*., 2014). In the second phase LAB populations increases and yeast decrease. The relevance of LAB during cocoa bean fermentation is controversial. Some authors, as Ho *et al*. 2014 highlighted that LAB is not essential in cocoa fermentation, whereas others proved that the presence of limited diversity of LAB, as *Lactobacillus fermentum* can produce volatile compounds such diacetyl, acetoin, and 2,3-butanediol that support AAB growth (Adler *et al*., 2013; Papalexandratou *et al*., 2013). Furthermore, some LAB can convert the citric acid present in cocoa pulp into lactic acid, mannitol, and other compounds, which increase cocoa pulp pH (Lefeber *et al*., 2011). The third phase is characterized by an increase in the population of AAB and simultaneously decrease in LAB. AAB is responsible for the oxidation of ethanol produced by yeast, and the conversion of lactic acid produced by LAB to acetic acid (Adler *et al*., 2014; Moens *et al*., 2014). The rise in temperature, the decrease in pH, and penetration of acetic acid and ethanol to cocoa bean are the cause of the embryo death (Garcia-Armisen *et al*., 2010; Lefeber *et al*., 2012). These factors induce the damage to the internal structure of the cocoa bean and activate a large number of endogenous enzymes such as invertase, glucosidases, proteases, and polyphenol oxidase, which lead to the development of flavor precursors and the color change in the cotyledons (Camu *et al*., 2008a).

Concerning the cocoa bean fermentation, there are several methods to conduct this process, which vary according to the country where the process takes place. The most widely applied approaches are heap, box, tray, and platform (Arana-Sánchez *et al*., 2015; Daniel *et al*., 2009; Papalexandratou *et al*., 2013, 2011a). The fermentation in box is the most widely used cocoa bean fermentation method in the world. The amount of cocoa to be fermented ranges from 5 to 2000 kg, depending on the production capacity of the cocoa farm (Schwan *et al*., 2015). The microbial diversity present in the cocoa fermentation depends on the type of fermentation used, the geographical location of the process, and cocoa variety, among other factors. The most commonly reported microorganisms in this process around the world are *Pichia kudriavzevii*, *Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Acetobacter pasterianus* (Figueroa-Hernández *et al*., 2019; Meersman *et al*., 2013; Ozturk and Young, 2017; Papalexandratou *et al*., 2013). However, these microorganisms are not present in all cocoa bean

fermentation processes. There have been several studies have used well-defined microbial cultures to conduct the cocoa bean fermentation in order to guarantee a homogeneous quality (Lefeber *et al*., 2012; Pereira *et al*., 2012; Schwan, 1998). Schwan (1998) have demonstrated that the inoculation of Brazilian cocoa bean fermentation with mixed starter culture produced a better quality fermented cocoa bean. The starter culture used in this study was composed of one yeast (*S. cerevisiae* var. chevalieri), two lactic acid bacteria (*L. lactis* and *L. plantarum*), and two acetic acid bacteria (*A. aceti* and *G. oxydans* subsp. suboxydans), which are autochthonous microorganisms from the cocoa fermentation process. Another study conducted in Brazil by Lefeber *et al*. (2012) has tested two mixed starter cultures with two cocoa bean fermentations (box and heap). The microbial cultures used in the fermentation the first was composed of *S. cerevisiae*, *L. fermentum*, and *A. pasterianus*, and the second one consisted only by bacterial strains. It has been concluded that the inoculation of cocoa bean fermentation with bacterial culture accelerated this process due to the conversion of citric acid to lactic acid, whereas the inoculation of the fermentation with a mixed microbial culture, composed of yeast, lactic acid bacteria, and acetic acid bacteria it was essential to increase the quality of the fermented cocoa beans.

There have not been enough studies in Mexico to identify the microbial diversity associated with cocoa bean fermentation. Only in a study performed by Arana-Sánchez *et al*. (2015), identified cocoa yeast diversity during fermentation. Therefore, it is crucial to conduct more investigations in order to identify and characterize the cocoa bean microbiota during fermentation and apply these findings to the design defined inocula to obtain high-quality cocoa beans for the international market. For this reason, this work aims to isolate autochthonous microorganisms from Mexican cocoa to formulate defined inocula to conduct small cocoa bean fermentation.

2 Materials and methods

2.1 Biological material used in this study

The ripe cocoa pods of the Forastero variety used in this study were harvested in the region of Aldama in Tabasco, México. The microbial strains used in this study were isolated from a spontaneous Forastero cocoa bean fermentation performed at Aldama, Tabasco State. These microbial strains were later identified as *Yarrowia lipolytica*, *Lactococcus lactis*, and *Acetobacter aceti*. Each of the isolated strains was freeze-dried and stored at -18 °C.

2.2 Isolation of microorganism of cocoa bean fermentation

Samples of 200 g were taken from each of the fermentation boxes, every 24 hours. The sample of 20 g of cocoa was taken under sterile conditions and then diluted with 180 g of physiological salt solution (0.85%) in a dilution bottle. The dilution was shaken for 10 minutes. The decimal dilutions were performed up to the 1x104 dilution. An inoculum of 0.1 mL was taken from the last three dilutions for the inoculation of the Petri dishes that contained adequate media for microorganisms' growth. In the case of yeasts, 10% acidified PDA agar with 10% tartaric acid was used, MRS agar for LAB, and modified Carr agar for AAB were used. Inoculated Petri dishes were incubated for 48 hours at 28 °C. The colonies that presented different colonial morphology were selected for growth using the same conditions until pure strains were obtained (Pereira *et al*., 2012).

2.3 Biochemical identification of autochthonous microorganisms

For the biochemical identification of the yeast strains isolated from cocoa fermentation, morphological colonial (size, color, borders, elevation, surface, appearance, reflected light, consistency, and pigment production) and cellular (simple staining) characterization were carried out. The assimilation test for carbon compounds was determined using the API 20 AUX, Identification System (Biomérieux, France). Additionally, colonies that presented a cellular morphology similar to that reported for lactic acid bacteria were tested for catalase, oxidase, and mobility. The API 50 CH kit was used for the carbohydrate fermentation test of LAB strains. For the case of the colonies isolated in the CARR medium and which presented cellular morphology similar to that reported for acetic bacteria (cylindrical, ellipsoidal, or rod-shaped cells frequently grouped in pairs or chains), the following additional tests were conducted: oxidation of ethanol to acetic acid, flagella staining, growth at acid pH, and mobility test (Boone *et al*., 2001; Kurtzman *et al*., 2010). The API tests were exploited with APIWEB^{TM} software.

2.4 *Molecular identification of autochthonous yeast*

2.4.1 *Yeast DNA Extraction*

The yeast was grown on PDA Agar and incubated at 37 °C for 24 hours, then suspended in sterile water until a scale 2 of MacFarland standard was achieved. To obtain the DNA samples, the DNeasy Blood and Tissue kit® (Qiagen, Hilden, Germany) was used. The DNA samples were stored at -20 °C (Nielsen *et al*., 2005).

2.4.2 *Yeast DNA Amplification*

The ITS regions were amplified using the primers BMB-CR (5'- GTACACACCGCCCGTCG-3') (Lane *et al*., 1985); 5.8s (5'-CGCTGCGTTCTTCATCG-3'), 5.8S-R (5'-TCGATGAAGAACGCAGCG-3'), and LR0 (5'-GCTTAAGTCAGGGT-3') (Hopple and Vilgalys, 1994). PCR was performed within a final volume of 25 μL containing 50 ng of extracted DNA, Taq 1 X buffer, 2.5 mM $MgCl_2$, 0.25 mM of the dNTPs mixture, 25 pM primers (forward and reverse), and 1.5 U Taq DNA Polymerase (Promega, USA). The amplification was carried out as follows: Initial denaturation cycle at 94 °C for 1 minute, 45 s at 50 °C, 30 cycles of 1 minute at 72 °C and a final extension at 72 °C for 7 minutes in a Mastercycler (Eppendorf AG, Germany). The PCR products were analyzed by 1.8% agarose gel electrophoresis in TAE 1X buffer and stained with ethidium bromide.

2.4.3 *DNA sequencing and molecular identification*

The PCR products were sequenced in the automated sequencing system (Perkin Elmer/Applied Biosystems Model 3730) at the Instituto de Biotecnología de la Universidad Nacional Autónoma de México (IBT-UNAM) using the Taq FS Dye Terminator Cycle Sequencing Fluorescence-Based Sequencing method. After sequencing, the sequence was aligned and compared to the GeneBank database using the BLASTn program of the National Center of Biotechnology Information (NCBI).

2.5 *Formulation of defined inoculum for cocoa bean fermentation*

Strains of *Yarrowia lipolytica*, *Lactococcus lactis*, and *Acetobacter aceti*, previously isolated from cocoa bean fermentation, were grown in Petri dishes with a specific medium for each of them (PDA for *Yarrowia*, MRS for *Lactococcus* and Carr for *Acetobacter*), the dishes were incubated at 28 + 2 °C for 48 h or until microbial growth of approximately 10^9 CFU/mL was observed. This cell concentration was determined by plate count and optical density. A 1 mL inoculum was taken under sterile conditions from a culture to be placed in a tube with 5 mL of specific medium for each microorganism during 24 h at 28 + 2 °C (primary culture). Subsequently, 5 mL were taken from each of these primary cultures and inoculated into a 250 mL screw-capped bottle with 45 mL of the appropriate sterile culture medium. The bottles were incubated at 30 °C until 10^9 CFU/mL were obtained. One mL was taken from each of the cultures to inoculate 1 kg of cocoa, to obtain a cell concentration of 10^6 CFU kg^{-1} cocoa (Schwan, 1998).

2.6 *Small-scale cocoa bean fermentation protocols*

The cocoa pods were transported to university facilities, 24 hours after they had been cut. The pods were checked for any physical damage, fungal attack, or other diseases. Subsequently, the cocoa pods were washed with a 2% sodium hypochlorite solution to eliminate the superficial microbial load, and then they were opened manually with a sterile scalpel (for controlled fermentation) and with knives for spontaneous fermentation and washed cocoa. Four fermentation protocols were conducted, as shown in Table 1, which were based on the experimental protocols reported by Schwan (1998).

For mixed or succession inoculation fermentations protocols, 4 kg cocoa beans with mucilage were placed in 5 kg styrofoam boxes, previously disinfected with a 70% ethanol solution. Mixed fermentation protocol was conducted with the inoculation at the beginning of the process with the defined inoculum composed by *Yarrowia lipolytica*, *Lactococcus lactis*, and *Acetobacter aceti*. In contrast the succession, fermentation protocol had three inoculation times: at the beginning with yeast strain, 24 hours with LAB strain, and 48 hours with AAB strain. All fermentation boxes were then covered with a sterile blanket and placed in a laminar flow hood to avoid contamination were then covered with a sterile blanket and placed in a laminar flow hood to avoid contamination. For spontaneous fermentations, 4 kg of cocoa mucilage were taken and placed in a 5 kg styrofoam box and fermented with natural microbiota. The "washed" cocoa was placed on a drying bed and exposed to the sun for drying (average 32-35 °C) until a 7% humidity of was obtained. All the small-scale fermentation protocols were performed in duplicate.

Table 1. Small-scale cocoa fermentation protocols.

Protocol name	Cocoa pod conditions	Fermentation inoculum
Spontaneous (SF)	No washed, with 2 % sodium hypochlorite solution	Natural microbiota
Washed cocoa (WC)	Washed, with 2 % sodium hypochlorite solution	None
Mixed inoculation form fermentation (MIF)	Washed, with 2% sodium hypochlorite solution	Mixed microorganism's inoculum added at the start of fermentation
Succession inoculation form fermentation (SIF)	Washed, with 2 % sodium hypochlorite solution	Microorganisms added in succession t=0; yeast t=24 LAB t=48 AAB

2.7 Microbial enumeration during small-scale cocoa bean fermentation protocols

Cocoa bean samples were aseptically collected at every 12 h interval during fermentation. A standard microbiological spread plate was used for plating on PDA for yeast, MRS for LAB, and Carr for AAB. The inoculated Petri dishes were incubated for 48 hours at 28 ± 2 °C, and the colonies observed were expressed as the number of colonies forming units in terms of log10 CFU/g (Camu *et al*., 2007).

2.8 Temperature and pH during small-scale cocoa bean fermentation protocols

At different time intervals during fermentation protocols, the temperature was recorded using a thermocouple system (ICPDAS® brand) with software program Ezdatalogger. The pH of the cocoa beans was determined using a digital pH meter (Orion 420-A) following the protocol of AOAC (1996).

2.9 Quality assessment of fermented cocoa beans

At the end of each of the fermentation protocols, samples of 200 beans were taken to evaluate the fermentation index of cocoa beans by using a cut test. These beans were cut longitudinally and categorized under the following groups: entirely brown- well fermented, partly brown and partly purple- partially fermented, purple- under fermented, insect damaged, moldy, or germinated (Schwan, 1998).

2.10 Statistical analysis

All the fermentation protocols were performed in duplicate. The mean value and standard deviation were calculated using a statistical program. The resultant data were evaluated by One-way ANOVA with $\alpha < 0.05$, and the difference between mean values was compared by the Tukey-Kramer Multiple Comparison test using NCSS 11 Statistical Software (NCSS, LLC, Kaysville, Utah, USA).

3 Results and discussion

3.1 Isolation and biochemical and molecular identification of microorganisms from spontaneous cocoa bean fermentation

During cocoa bean fermentation, three main microbial groups are involved: yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). However, sporulated bacteria, mainly *Bacillus*, and filamentous fungi have also been detected, especially at the end of fermentation (Ardhana and Fleet, 2003; Schwan and Wheals, 2004).

Table 2. Biochemical identification and biotechnological application of main microorganisms isolated during spontaneous Mexican cocoa bean fermentation in the present work.

Microorganism isolated	Biochemical identification	Previously reported	Biotechnological applications of the microbial species
Y1	*Candida krusei (99 %)* *	During cocoa bean fermentation (Nielsen *et al*., 2005)	Ability to assimilate citric acid and lactic acid (Jespersen *et al*., 2005) and produced aromatic compounds as ethyl acetate and 2-phenylethanol (Mota-Gutierrez *et al*., 2019)
Y2	*Kloeckera sp. (99 %)**	During cocoa bean fermentation (Ardhana and Fleet, 2003)	Produces high concentration of relevant sensory compounds like acetic acid and ethyl acetate (Osborne, 2010)
Y3	*Saccharomyces cerevisiae (99 %)* *	During cocoa bean fermentation (Daniel *et al*., 2009; de Almeida *et al*., 2019; Illeghems *et al*., 2012; Jespersen *et al*., 2005; Nielsen *et al*., 2005; Papalexandratou *et al*., 2011b; Papalexandratou and Vuyst, 2011; Pereira *et al*., 2013; Pereira *et al*., 2012)	Produce high concentration of ethanol and tolerate high concentration of sugars and sugars in acid medium (de Almeida *et al*., 2019)
Y4	*Rhodotorula minuta (99 %)* *	During cocoa bean fermentation (Papalexandratou *et al*., 2011c)	Produces exopolysaccharides (Ramirez, 2016)
Y5	*Candida boidinii (99 %)* *	Isolated from soil of cocoa growing areas (Santana *et al*., 2018)	Lipase activity, ability to biofilm formation on fruit epidermis and co-aggregation with LAB species such as *Lactobacillus pentosus* (Camiolo *et al*., 2017)
LAB 1	*Lactococcus lactis (99 %)* **	During cocoa bean fermentation (Camu *et al*., 2007, 2008b; Passos *et al*., 1984)	Produces aromatic compounds as diacetyl, acetaldehyde and acetate (Song *et al*., 2017)
LAB 2	*Lactobacillus brevis (99 %)* **	During cocoa bean fermentation (Camu *et al*., 2008b; Kostinek *et al*., 2008; Lagunes- Gálvez *et al*., 2007; Nielsen *et al*., 2007)	Acid tolerant (Bosma *et al*., 2017) and potential probiotic properties (Fang *et al*., 2018)
LAB 3	*Lactobacillus plantarum (99 %)* **	During cocoa bean fermentation (Camu *et al*., 2008b; Garcia-Armisen *et al*., 2010; Kostinek *et al.*, 2008; Nielsen *et al*., 2007; Papalexandratou *et* al., 2013)	Citrate-fermenting, acid-tolerant, and ethanol-tolerant and antimicrobial compounds producer (Camu *et al.*, 2007) and probiotic potential (Melgar-Lalanne *et al*., 2019)
AAB 1	*Acetobacter aceti***	During cocoa bean fermentation (Ardhana and Fleet, 2003)	Oxidizes ethanol (Ardhana and Fleet, 2003) and produces short fatty acids (Krings and Berger, 1998)
AAB 2	*Gluconobacter sp.***	During cocoa bean (Schwan, 1998)	Produces off-flavors and late yeast development in cocoa fermentation due to production of gluconic acid (De Vuyst and Weckx, 2016)
B1	*Bacillus sp.* ****	During cocoa bean fermentation (Bortolini *et al*., 2016; Mota-Gutierrez *et al*., 2018)	Produces 2,3-butanediol, pyrazines, acetic acid, and lactic acid, under fermentative conditions, which may contribute to the acidity and perhaps, and off-flavors of fermented cocoa bean (Figueroa-Hernández *et al*., 2019)

*Percent of identification by the system API 20 C AUX, Biomereux. **Percent of identification by the system API 50 CH Biomereux. ***Identification by Gram and flagellum staining, mobility and catalase test and ethanol oxidation ****Identification by Gram and flagellum staining, mobility and catalase test.

In the spontaneous fermentation of Mexican cocoa beans, it has been possible to isolate 54 microbial strains. Among the autochthonous microorganisms found in this fermentation process were identified yeasts belonging to the genera *Candida, Rhodotorula, Saccharomyces*, and *Kloeckera*. Also, LAB species belonging to the genera *Lactococcus* and *Lactobacillus* have been identified, as well as species belonging to the genera *Acetobacter* and *Gluconobacter* (AAB) and sporulated bacilli of the genera *Bacillus* have been identified. From all this microbial diversity, ten microorganisms were biochemically identified as predominant in process, including five yeast, three LAB, and two AAB strains, which are shown in Table 2. It should be mentioned that the strain identified biochemically as *Kloeckera* sp. was further molecularly identified as *Yarrowia lipolytica*). Furthermore, in this table, it was observed that some microorganisms showed a significant contribution during the cocoa fermentation process, for example, *Saccharomyces cerevisiae, Candida krusei, Lactococcus lactis, Lactobacillus plantarum*, and *Acetobacter aceti*, so that they could be considered for the formulation of a defined inoculum to conduct the cocoa bean fermentation process in Mexico.

A study performed in the Dominican Republic by Lagunes- Gálvez *et al*. 2007 were isolated 43 autochthonous yeast strains from cocoa bean fermentation. The yeasts found in this study mainly belonged to five genera *Kloeckera, Candida, Hanseniaspora, Pichia*, and *Yarrowia*, as some of those found in present work. Besides, 44 LAB strains were isolated, of which the most abundant genus was *Lactobacillus*, with *L. plantarum* being the most abundant species, although *L. brevis, L. pentosus*, and *L. paracasei* were also found. In the case of AAB, only *Acetobacter lovianiensis* was reported in this study. Pereira *et al*. 2012 found that *S. cerevisiae, P. kluyveri, H. uvarum, L. plantarum, L. fermentum, A. malorum, A. cerevisiae, A. tropicalis*, and *A. ghanensis* were the predominant microorganisms during small-scale cocoa performed in Brazil.

On the other hand, from the yeast DNA extraction, PCR products with approximately 442 bp, including the ITS1 and ITS2 regions, have been obtained and sequenced. BLAST analysis of the sequence showed a coincidence of 98.42% with the sequence coded in the GeneBank database as KY105983.1 corresponding to the *Yarrowia lipolytica* ITS1-5.8s-ITS2 region. This yeast was previously reported as part of fermented cocoa bean microbiota (Lagunes-Galvez *et al*., 2007; Pereira *et al*., 2013; Da Veiga Moreira *et al*., 2013).

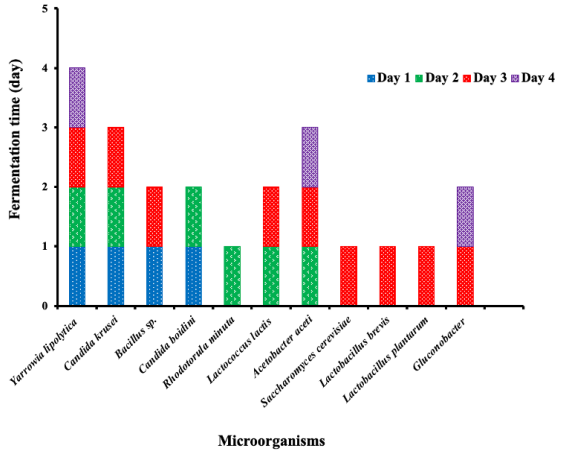

Fig. 1. Main microorganisms identified during spontaneous cocoa bean fermentation performed in Tabasco, Mexico.

The strain of *Yarrowia lipolytica* was present during the four days of the cocoa bean fermentation process performed in Tabasco. *Candida krusei* and *Acetobacter aceti* were detected during days 1, 2, and 4 and days 2, 3, and 4, respectively. In the case of LAB, the most prevalent strain during the process was *Lactococcus lactis* found on days 2 and 3 of fermentation. Other LAB strains (*L. plantarum* and *L. brevis*) were only present during the second day of fermentation, as shown in Figure 1.

3.2 Formulation of defined inoculum for cocoa bean fermentation

Microbial consortiums have been used to steer the cocoa bean fermentation process on a small scale (Pereira *et al*., 2012; Sandhya *et al*., 2016; Saunshi *et al*., 2019; Saunshia *et al*., 2018) and large scale (Lefeber *et al*., 2012; Menezes *et al*., 2016; Mota-Gutierrez *et al*., 2018). The most commonly used microorganisms as inocula for this process are *Saccharomyces cerevisiae*, *Pichia kluyveri*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Acetobacter pasterianus*, due to their role in this process, their ability to adapt to the cocoa pulp fermentation conditions, and other biotechnological properties (Batista *et al*., 2015; Crafack *et al*., 2013; Lefeber *et al*., 2012). In this study, the microorganisms selected for inoculum formulation for the cocoa fermentation process were *Yarrowia lipolytica*, *Lactococcus lactis*, and *Acetobacter aceti*, due to the predominance of these microorganisms during cocoa bean fermentation. These microorganisms have also

been reported in other studies (Ardhana and Fleet, 2003; Camu *et al*., 2008b, 2007; Lagunes- Gálvez *et al*., 2007; Passos *et al*., 1984). However, only *Acetobacter aceti* has been used as part of a starter culture for cocoa fermentation (Sandhya *et al*., 2016; Saunshi *et al*., 2019; Saunshia *et al*., 2018). It is essential to highlight that this inoculum was added to the fermentation in two different ways, a mixed form, by inoculating the three microorganisms at the beginning of the fermentation, and a successive form by following the same microbial succession as the spontaneous cocoa bean fermentation.

Yarrowia lipolytica is a yeast that has been used as a starter culture for the fermentation of green coffee beans to modulate the concentration of volatile and no-volatile composition. The inoculation with this yeast modified the volatile and non-volatile components of the coffee beans, increasing the concentration of 2-phenyl ethanol and decreasing the concentration of acids, alkanes, and aldehydes (Lee *et al*., 2017a), resulting in the retention of 2-phenyl ethanol, 4-vinyl guaiacol, and 4-vinyl phenol when these coffee beans are lightly roasted (Lee *et al*., 2017b). Also, this yeast has been used as part of a starter culture with *Debaromyces hansenii* and *L. plantarum* to produce dry fermented sausages. The use of *Yarrowia lipolytica* showed intense lipolytic and proteolytic activity (Patrignani *et al*., 2007).

On the other hand, *Lactococcus lactis* has been used for the fermentation of green coffee beans. Wang *et al*. (2020) utilized a strain of *Lactococcus lactis* subsp. cremoris to modify the flavor of the green coffee through fermentation with and without glucose supplementation. Glucose-supplemented and non-supplemented fermented coffees had a higher production of volatile O- and N-heterocyclic compounds when they were roasted compared to non-fermented coffee. The fermentation supplemented with glucose produced a roasted coffee with a caramel aroma and preserved acidity and sweetness. Fermentation without supplementation resulted in a roasted coffee with a pronounced nutty aroma. Both fermentations reduced the undesirable smoke aroma present in the untreated coffee.

3.3 Microbial enumeration during small-scale cocoa bean fermentation protocols

There are a large number of microorganisms involved in cocoa fermentation, mainly yeasts, LAB, and AAB, which grow successively during this process, as shown in Figure 2 A. During the spontaneous cocoa fermentation, all the three microbial groups mentioned above are involved: yeast, LAB and AAB. Yeasts had a higher microbial growth from 12 h to 36 h of fermentation (7.75 log CFU g^{-1}). Simultaneously, a higher population growth of the LAB is observed from 24 hours of fermentation until its maximum growth is reached at 48 hours (7.56 log CFU g^{-1}). Whereas AAB have shown a higher rate of microbial growth until 48 h of fermentation, reaching their maximum at 84 h of fermentation (7.26 log CFU g^{-1}).

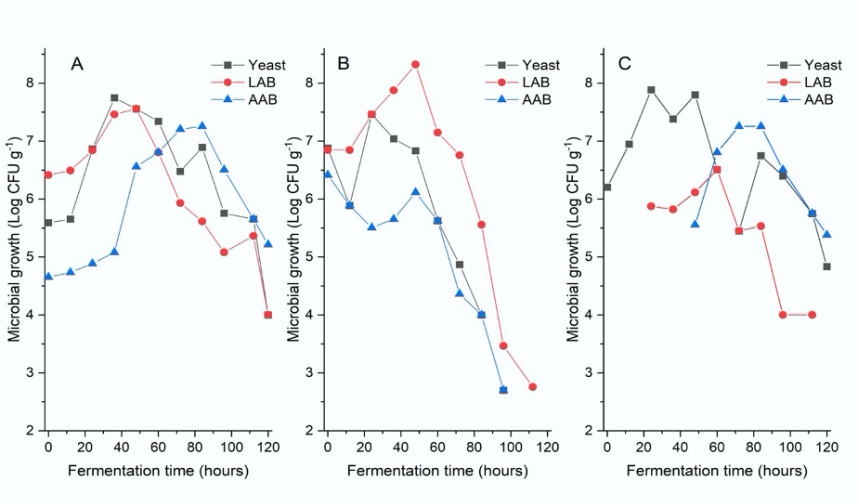

Fig. 2. Evolution of microorganisms during small-scale cocoa fermentation protocols. A) Spontaneous fermentation, B) Mixed inoculation form, and C) Succession inoculation form.

It was noted that at the end of fermentation, these three microbial groups were still present; however, after 112 hours of fermentation, it is evident that the population rate of these groups decreased significantly.

Pereira *et al*. (2012) observed that during spontaneous 500 g cocoa fermentation, yeast, and LAB´s growth rates simultaneously reached their maximum at 12 h of fermentation (8 log CFU g^{-1}). The microbial growth of LAB remains until the end of fermentation, whereas the yeast population is undetectable after 72 hours of fermentation. In the AAB case, they can be detected from the 12th hour of fermentation, reaching a cell population of 6.36 log CFU g^{-1} and remaining in fermentation until 96 hours. The results found by Pereira *et al*. (2012) were different from the ones found in this work, in which an evident microbial succession can be observed, and the three microorganisms remain until the end of fermentation.

In the case of the mixed inoculation form fermentation protocol (Figure 2B), the natural microbial succession during this process was not observed, since the inoculation simultaneously with all microorganisms promoted the growth of the LAB over the yeast.

This effect can be observed from the beginning of fermentation until 60 hours. Simultaneously, it is observed that the growth of the AAB is not very favorable, and even their population decreases from initial time up to 24 hours, when a slight increase of the population is observed and reaches their maximum growth rate at about 48 hours. This limited growth of acetic acid bacteria was due to prevailing conditions during cocoa bean fermentation that was not ideal for their growth, such as the concentration of oxygen present, the pH, the amount of ethanol (Camu *et al*., 2007). Therefore, the pH value and temperature required during fermentation may not be reached, and consequently, not all the biochemical transformations needed for the fermented cocoa bean to have good quality are performed. Regarding the succession inoculated fermentation (Figure 2C), it was observed that yeast populations and acetic acid bacteria present a similar behavior to those observed during spontaneous fermentation. Nevertheless, the LAB population in this fermentation protocol is lower than that found in spontaneous fermentation.

3.4 Temperature and pH during small-scale cocoa bean fermentation protocols

All fermentation protocols were initiated at a temperature of 30 °C. Subsequently, the temperature was increased to a maximum of 37 °C for spontaneous and mixed inoculation form cocoa fermentation and about 42 °C for succession inoculation form fermentation. In the case of cocoa that was not subjected to fermentation (washed), the temperature is between 30 and 33 °C (Figure 3A). Therefore, only the fermentation carried out with succession inoculation reached a temperature higher than 40 °C.

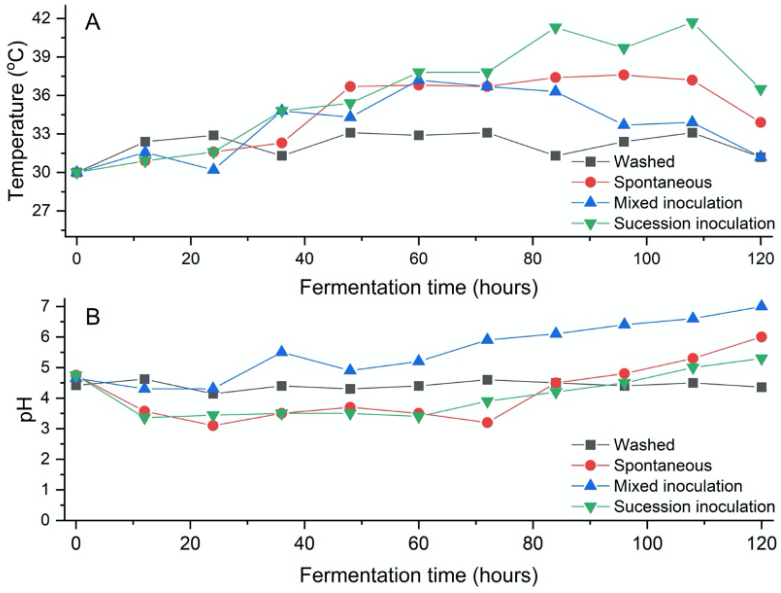

Fig. 3. Evolution of Temperature (A) and pH (B) during small-scale cocoa fermentation protocols.

This temperature increase during the cocoa bean fermentation process is caused by the oxidation of the alcohol produced by the yeast into acetic acid by the acetic acid bacteria. The oxidation of alcohol is an exothermic process that causes the temperature of the fermenting mass to be increased, reaching temperatures higher than 45 °C (De Vuyst and Weckx, 2016; Gutiérrez, 2017). However, such temperatures above 45 °C were reported for large-scale fermentation at least 100 kg of cocoa as shown during fermentations performed by Hernández-Hernández *et al*. (2016), Lagunes-Gálvez *et al*. (2007), and Papalexandratou *et al*. (2019). In the case of small-scale cocoa fermentations, it has been reported that the maximum temperature reached is around or less than 45 °C. The temperature found during the mixed inoculation protocol that is lower than that found in succession inoculation protocol. A lower AAB growth rate can explain this effect during mixed inoculation protocol (Figure 2B) than in succession inoculation protocol (Figure 2C). The conditions of the fermentation mass can produce this reduction in the number of microbial cells upon inoculation (low concentration of available oxygen, low concentration of alcohol and lactic acid), that were not adequate for the growth of AAB. Concerning the spontaneous and the succession inoculated fermentation form protocols, it was observed that between 60 to 100 h, when the temperature of the fermented mass reached their maximum values, there was similar growth of AAB in both fermentations. However, there were significant differences in AAB growth rates between 48 to 60 h (Figure 2A and 2C), which could explain the differences found on the temperature of the fermented mass for these two fermentation protocols.

The comparison of the temperatures achieved during cocoa small-scale fermentation protocols performed in this study showed that the maximum temperature obtained in succession-inoculated fermentation is similar to that reported by Sandhya *et al*. (2016). They found a maximum temperature of 41 °C during spontaneous fermentation of cocoa beans, and 40 - 2 °C for ten kg-cocoa fermentations inoculated with a starter culture composed by *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Acetobacter aceti* at different inoculation levels (10-60%). In another study, Pereira *et al*. (2012) observed a maximum temperature of 47 °C during spontaneous 500 g cocoa fermentation carried out in plastic containers. The increase in temperature during fermentation has a significant effect on the loss of germination power of the cocoa bean. If the cocoa bean germinates, it will become a defect for marketing, and also constitutes an entry point for contaminants in the bean such as filamentous fungi. Besides, the heat generated during the process affects the enzymatic activity of the bean. The temperature is considered an essential parameter for the control of the fermentation process since it is an image of the microbial reactions that take place (Pontillon, 1998; Barel, 2013).

The initial pH value of the fermentation mass was around 4.70 for all fermentation protocols, except washed cocoa, as can be observed in Figure 3B. The pH of the washed cocoa showed variations between 4.14 to 4.62. The fermentation with mixed inoculation was the one that showed the most significant pH variation from 4.70 to 7. It is essential to highlight that, after 84 hours, in this fermentation protocol, the pH increased, leading to the growth of filamentous fungi in the fermenting mass. The occurrence of fungi species during cocoa fermentation is not desirable because they are frequently associated with spoilage, production of off-flavors, and accumulation of mycotoxins (Copetti *et al*., 2011, 2010; Gilmour and Lindblom, 2008; Schwan and Wheals, 2004).

On the other hand, the spontaneous fermentation and the succession inoculation showed a similar pattern, decreasing the initial pH from 4.70 to about 3.0 during the first 24 hours, after that the pH value was slightly increased to 3.75 at 48 h and then increased to a pH value of 6 for spontaneous and 5.3 for succession inoculation fermentations. The increase in pH values during cocoa fermentation can be caused by the utilization of the sugars by the yeasts and the use of citric acid by lactic acid bacteria (Lagunes-Gálvez *et al*., 2007). It reported that despite the production of acetic acid by the AAB, the fermenting mass´s pH could increase to a value of 5 or even 6 (Schwan and Wheals, 2004), similar to those found in this work.

In the study conducted by Sandhya *et al*. (2016), it was found that the pH variation in spontaneous fermentations of 10 kg of Forastero cocoa was between 3 to 5.4 and between 4.5 and 5.5 in the case of fermentations inoculated with a starter culture at 10%. Furthermore, it was reported that the pH of the fermentation inoculated with 30% of the same starter culture has a final pH value of 7.2, and the fermented cocoa bean has a low fermentation index, similar to the reported in the present study with mixed inoculation fermentation.

Table 3. Cut test evaluation of cocoa beans fermented by different fermentation protocols small-scale cocoa fermentation protocols.

Sample	Brown (%)	Purple (%)	Partly purple/brown (%)	Overfermented (%)
Spontaneous fermentation (SF)	79	0	0	0
Washed cocoa (WC)	0	10	45.50	0
Mixed inoculation form fermentation (MIF)	19	1	37.5	0
Succession inoculation form fermentation (SIF)	75	0	0	4

3.5 Quality assessment of fermented cocoa beans

The cut test is one of the quality assessment procedures for the fermented cocoa beans, and it indicates the process efficiency and suitability of the cocoa beans to be commercialized and processing (Camu *et al*., 2008a; Schwan, 1998). This method is validated by the NMF-F-352-S-1980 standard (Dirección General de Normas, 1980) to assess the cocoa and coffee bean fermentation efficiency. This method is based on the differences in the hydrolysis and oxidation of the anthocyanins, indicative of a proper cocoa bean fermentation. Therefore, the differences in color found inside the cocoa bean are useful for cocoa fermentation endpoint and could predict the aromatic potency of cocoa-related products (Sandhya *et al*., 2016). After performing the cut test of all cocoa small-scale fermentation protocols samples, it was found that only the samples of spontaneous fermentation and the succession inoculation form showed a high percentage of fermented cocoa beans, with 79% and 75% respectively (Table 3). These two fermentation protocols did not have a significant statistical difference in this cocoa quality test.

Regarding the mixed inoculation protocol, the quality of the fermented cocoa beans samples was low, with only 19% of fermented beans and 37.5% of partially fermented beans. This phenomenon could be explained by inadequate conditions in temperature, internal pH, and the concentration of acetic acid required to undergo the biochemical transformations inside the beans, such as hydrolysis and oxidation of anthocyanins at the acceptable levels. That can be confirmed with Figure 2, which showed a lower growth rate of acetic acid bacteria and a decreased production of acetic acid in this fermentation process. Also, a lower temperature and a higher pH value were achieved in this fermentation protocol compared to spontaneous fermentation and succession form inoculation (Figure 3A and 3B). The washed cocoa protocol samples had no brown cocoa beans since, in this case, the cocoa samples were not subjected to a fermentation process.

Conclusions

This research is the first study performed in which the use of defined inoculum is used to steer the fermentation of Mexican cocoa to produce homogeneously fermented cocoa beans. The inoculum used in this study was composed of strains previously isolated from a spontaneous cocoa fermentation. The autochthonous strains were identified as *Yarrowia lipolytica*, *Lactococcus lactis*, and *Acetobacter aceti*. During small-scale cocoa fermentations, it was observed that the variation in pH and temperature, as well as the percentage of fermented beans obtained by succession inoculation fermentation protocol, were similar to those obtained during spontaneous fermentation. Therefore, this fermentation protocol is suggested to direct the fermentation process on a large- scale. However, it is necessary to conduct more research about the production of metabolites,

aroma precursor compounds during fermentation, and perform sensory tests to determine the suitability of the use of this inoculum to obtain fermented cocoa beans with characteristics demanded by the national and international markets. Besides, the use of a defined inoculum to conduct the fermentation of the cocoa bean reduces the presence of toxigenic fungi during this process. This inoculation would guarantee that the fermented bean obtained has a homogenous quality and innocuousness.

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