Chapter

Fermentation of Cocoa Beans

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Abstract

Cocoa bean fermentation is a spontaneous process driven by an ordered microbial succession of a wide range of yeasts, lactic acid and acetic acid bacteria, some aerobic sporeforming bacteria and various species of filamentous fungi. The process of cocoa fermentation is a very important step for developing chocolate flavor precursors which are attributable to the metabolism of succession microbial. The microbial ecology of cocoa has been studied in much of the world. In Venezuela, studies have been carried out with Criollo, Forastero, and Trinitario cocoa, fermented under various conditions, the results obtained coinciding with the reported scientific information. Fermentation must be associated with the type of cocoa available, carried out knowing the final processing and derivative (paste, butter, powder). The results shown in this chapter correspond to investigations carried out with cocoa from three locations in Venezuela. The quantification, identification, isolation, functionality of the most representative microbiota involved in the fermentation of these grains was sought. This to give possible answers to the fermentation times and improvement of the commercial quality. Likewise, generate greater interest on the part of the producers in carrying out the fermentation.

Keywords: cocoa beans, fermentation, microbial ecology

1. Introduction

Cocoa beans (*Theobroma cacao* L) are the basis for the production of cocoa powder and butter, as well as chocolate. In order to obtain fine cocoa beans of aroma it is necessary to carry out a process of benefit (harvesting, shelling, fermentation, drying) in which the spontaneous fermentation of the grains has great influence, since in addition to an interesting occurrence Microbial succession favors the production of enzymatic, chemical, physicochemical reactions among others that still need to be studied.

The part used of the cacao tree *Theobroma cacao* L. are the beans and the edible is its cotyledons, which undergo important changes during the fermentation, drying and manufacturing process, giving rise to a flavor and aroma that is appreciated by chocolate consumers around the world. The raw cocoa has an extremely bitter and astringent taste, so it must be treated by a process in which the microorganisms, through fermentation, modify their components. The fermentation of the cocoa begins with the opening of the ear and the extraction of the cocoa beans. The grains that generally (depending on the maturity and time of opening) at the moment of being extracted are wrapped in a white mucilaginous pulp that comprises 40% of the wet basis weight of the fresh grain. Composed mainly of 82–87% water, 10–15% fermentable sugars (glucose, fructose and sucrose), 2–3% pentoses, 1–3% citric acid, 1–1.5% pectin, in addition to 1–2% of other hemicellulosic polysaccharides.

Proteins, amino acids, vitamins (mainly vitamin C) and minerals are also present, the mucilaginous pulp is an excellent substrate for microbial growth [1–3]. The process of fermentation of the cocoa is natural, since usually they are not intentionally added initiating microorganisms to the grains, which are sterile inside the ears and are contaminated with different microorganisms coming from all the surfaces with which they come into contact (tear, knives and the hands of the people who manipulate it).

The fermentation process of cocoa is characterized by a microbial succession in which according to various authors [3–9], the yeasts participate first, then the lactic acid bacteria (LAB) act and, finally, the acetic bacteria (AAB) intervene. Additionally, spore-forming bacilli of the genus Bacillus, and filamentous fungi. This process is essential both to modify the beans, eliminating the mucilage, and to prepare the grain that requires a battery of enzymes responsible for modifying its color, taste and smell.

They have been reported [3–6, 10–12] more than 100 microbial species that show different metabolic properties, identifying themselves new species due to the improvement in cultivation techniques together with the use of molecular biology tools. Two dominant bacterial species *Lactobacillus fermentum* and *Acetobacter pasteurianus*, together with four yeast species *Saccharomyces cerevisiae*, *Hanseniospora thailandica*, *H. opuntiae* and *Pichia kudriavzevii*, represent the central component of the bacterium-fungus association that lead to the fermentation of cocoa in many of the regions where it is produced [13].

The term cocobiota, was introduced [13] to refer to the microbial association of bacteria and fungi involved in the spontaneous fermentation of cocoa beans, which originate metabolites present in cocoa powder and dark chocolate, which can have beneficial effects on health. Five main groups of microorganisms participate in cocoa fermentation: filamentous fungi, yeasts, lactic acid bacteria, acetic acid bacteria, and various Bacillus species [5].

Venezuela has specimens of cacao (*Theobroma cacao* L) in almost all its territory; however, the study of the microbial characterization developed during fermentation has been very little studied compared to the reports found today worldwide. Therefore, the present investigation was based on quantifying, identifying, isolating, and knowing the functionality of the most representative microorganisms involved in the fermentation of cocoa beans grown in three locations, where the work systems were completely different.

In most producing countries, including Venezuela, cocoa fermentation is a process that is carried out in an artisanal way, using systems such as plastic baskets, wooden drawers, and staggered wooden boxes. These systems are generally covered with leaves of Musaceae or heliconia. Specifically, in Venezuela, some localities use a fermentation system in plastic containers for a time that is between 12 and 24 hours. This fermentation system in Venezuela has allowed women's inclusion in the workplace, thereby achieving gender equality that has caused so many problems worldwide. It is fulfilled with the fifth (5th) Goal to Transform Our World proposed by the United Nations for its 2015–2030 plan.

2. Cocoa fermentation

According to the fermenter used, cocoa beans' fermentation is done in quantities of 25 kg up to groups of approximately 200–250 kg—the type of cocoa influences the time and the technique used. Forastero and Trinitario cocoa require between 5 to 7 days of fermentation, while Criollo requires 2 to 3 days. After seven days of fermentation, some off-flavors may be formed by some fungal species. During

fermentation, temperature changes occur that favor both the evolution of microorganisms and a set of enzymes [14]. The changes result in the formation of cocoa aroma precursors (amino acids and reducing sugars). Precursors are enhanced in the stages of drying and roasting.

The fermentation process is characterized by a well-known microbial succession. The initial pH (3.6) of the pulp caused by the presence of citric acid, together with low oxygen levels, favors yeasts' colonization. The proliferation of these leads to the production of ethanol and the secretion of pectinolytic enzymes.

2.1 Yeast

The yeast population increases in number within the first 24 h of fermentation, after which it slowly decreases. Studies in cocoa fermentation from different areas of Venezuela show that the behavior of the yeasts (**Table 1**) occurs in a similar way when it has been fermented for three days for Criollo cocoa (from Sur del Lago, Zulia state) and five to seven days for Forastero cocoa (from Barlovento, Miranda state).

Table 1 shows the log CFU/g of the yeasts found in the fermented cocoa beans in the different sampled sites. The yeast population was present from the beginning of fermentation in all samples, with counts from 1.00 LogUFC/g for replica 2 (B^2) from the INIA Tapipa experimental station, Miranda state, to 5.31 LogUFC/g for replica 3 (C^3) of the INIA Chama experimental station, Zulia state. The yeasts' behavior was variable, finding maximum counts of 1.69 LogUFC/g for the first day of fermentation (C^1) up to 1.00 LogUFC/g (C^1 and C^2). Likewise, for the Miranda state's replicas, various behaviors were observed, which experienced increases or decreases according to the days of fermentation.

According [15], there is an increase in the number of yeasts by 10^3 CFU/g around the sixth to the seventh day of fermentation. This is possibly due to the growth of thermotolerant yeasts that use some of the acids, coinciding with an increase in oxygen content in the mass and survivors in the fermentation system's colder external zones. The experimental stations in Zulia showed initial values of 5.31 Log CFU/g and a significant decrease in the population at the end of the process. The variations found in the yeast population count for the studied sites could probably be due to deviations inherent in each locality's fermentation process. Genetic variations of the plantations, fermentation methods; plastic baskets (used in the

	Fermentation time (Days)					
Sample	**0**	**1**	**2**	**3**	**4**	**5**
A	3.48 ± 0.03^{c}	3.27 ± 0.23^{a}	1.67 ± 0.06^{a}	1.00 ± 0.01^{a}	4.17 ± 0.17^{a}	3.61 ± 0.08^{a}
B^1	4.42 ± 0.21^{b}	1.00 ± 0.01^{b}	1.80 ± 0.07^{a}	1.83 ± 0.07^{a}	4.55 ± 0.30^{a}	1.67 ± 0.15^{b}
B^2	1.00 ± 0.01^{e}	1.69 ± 0.04^{b}	1.73 ± 0.05^{a}	1.74 ± 0.06^{a}	3.21 ± 0.12^{b}	4.33 ± 0.10^{a}
C^1	2.49 ± 0.17^{d}	1.69 ± 0.07^{b}	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}		
C^2	2.76 ± 0.09^{d}	1.33 ± 0.05^{b}	1.00 ± 0.01^{a}	1.00 ± 0.01^{a}		
C^3	5.31 ± 0.06^{a}	1.00 ± 0.01^{b}	1.00 ± 0.01^{a}	1.35 ± 0.03^{a}		

^{a,b,c,d,e}Different letters for each row indicate significant differences ($p < 0,05$).
Mean ± standard deviation.
A: La Trinidad fermented cocoa, Miranda state B^1 and B^2: INIA Tapipa fermented cocoa, Miranda state C^1, C^2, C^3: INIA Chama fermented cocoa, Zulia state. 1,2,3: indicates the replica number.

Table 1.
Log CFU/g of yeasts during the fermentation of cocoa beans from one location and five experimental stations.

fermentation of cocoa beans from the Miranda experimental stations) or wooden crates (used in the fermentation of cocoa beans from the Zulia experimental stations), failures in the control of the artisanal process, fermentation incomplete, excessive turning of the grains, changes in oxygen content, pH, accumulation of ethanol and other compounds can affect the fermentation system.

Studying the chemical and microbiological composition [16] during the fermentation process of cocoa beans from the Chuao area in Aragua state, found a yeast count for the first days of fermentation in the order of 3.77 to 5.38 Log CFU/g, while [17], in Venezuelan cocoa beans of the Carenero variety, grown in Merecure, Miranda State, reports yeast populations of 8.21 Log CFU/g, at week 1 of fermentation reaching a population density of 6.80 CFU/g at week 3 fermentation, decreasing its number to 3.00 Log CFU/g at the end of the fermentation process. The yeasts are predominant at 12 and 36 hours after the start of the fermentation of cocoa beans [3, 17].

In fermented cocoa beans in the Southeast region of Ivory Coast [17], found a rapid increase in the yeast population, going from 10^6 CFU/g to 10^7 CFU/g at 36 h, after 84 h, this decreased to 10^1 CFU/g. Studying [18] the diversity of yeasts involved in the fermentation of cocoa from six of the principal producing regions of Ivory Coast founded a yeast population of 10^4–10^5 CFU/g up to 10^7–10^8 CFU/g between 12 and 24 hours of fermentation. Growth kinetics were very similar for the six regions, with an increase in the yeast population during the first 24 hours of fermentation and a progressive decrease after 24 hours after starting the fermentation process.

Table 2 shows the macroscopic morphological characteristics of fourteen (14) yeast strains isolated at the sampled sites. 4 isolated from the cocoa beans fermented in $A^{1,2}$ from the beans fermented in B^1, 2 from B^2, 2 from C^1, C^2, and C^3. In most isolates, the morphology found agrees with the described by [19].

Strain	Shape	Margin	Elevation	Surface	Color	Brightness	Texture
A	Oval	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose
A	Oval	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose
A	Ovoid	Whole	Flat	Smooth	Cream	Brilliant	Butyrose
A	Elliptical	Whole	Flat	Smooth	Cream	Opaque	Butyrose
B^1	Ovala	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose
B^1	Elliptical	Whole	Flat	Smooth	Cream	Opaque	Butyrose
B^2	Oval	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose
B^2	Oval	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose
C^1	Spherical	Whole	Convex	Smooth	Pink	Brilliant	Butyrose
C^1	Round	Rhizoid	Umbilicada	Smooth	Cream	Opaque	Butyrose
C^2	Oval	Curly	Umbilicada	Rough	Crema	Opaque	Butyrose
C^2	Oval	Rhizoid	Umbilicada	Smooth	White	Opaque	Butyrose
C^3	Spherical	Whole	Convex	Smooth	Pink	Brilliant	Butyrose
C^3	Oval	Curly	Umbilicada	Rough	Cream	Opaque	Butyrose

A: La Trinidad fermented cocoa, Miranda state B^1 and B^2: INIA Tapipa fermented cocoa, Miranda state C^1, C^2, C^3: INIA Chama fermented cocoa, Zulia state. 1,2,3: indicates the replica number.

Table 2.
Morphological characteristics of yeasts isolated from fermented cocoa beans in one location and five experimental stations.

At a macroscopic level, we could observe six (6) different phenotypic of yeast in the isolated colonies' morphology. Most (8) colonies with oval shape, wavy margin, umbilicated, cream-colored, rough surface, opaque, and butyric texture were found in all the studied sites. Specifically, from sample A, there was (1) one ovoid colony, whole, flat, smooth cream-colored isolated and another colony (1) with an elliptical shape, a whole margin, flat, smooth, shiny, cream-colored, and butirose. For samples C^1 and C^3. Two (2) isolates with a spherical shape, entire margin, convex, smooth, shiny, and pinkish color. One (1) colony with a round shape, with rhizoid margin, umbilicated, smooth, opaque, cream-colored for sample C^2. Finally, one isolated yeast colony of sample C^3 oval, with rhizoid margin, smooth, opaque. Existence of at least six different yeast genera in the fermented cocoa beans [19]. The isolates identified would correspond to the genus *Saccharomyces*, *Debaryomyces*, *Hanseniospora*, *Rhodotorula*, *Pichia*, and *Candida*.

However, to confirm this, more biochemical tests must be performed and correlated with molecular tests. All these genera and many others have been found in cocoa beans' fermentation from different countries [4, 6, 10, 20–24]. The macroscopic characteristics found in the isolates are due to the fact that during cocoa fermentation an ecological succession occurs with micro-environmental changes such as the availability of nutrients, changes in pH, temperature, oxygen, among others. The yeasts are capable of changing their characteristics and behavior, according to the environmental conditions in the substrate that they are [25–27].

In relation to the physiological identification of the 6 groups of yeasts isolated from the fermented cocoa beans in the studied sites, **Table 3** shows the results obtained for the sugar fermentation. Glucose positively ferments for all genera with the exception of the strain identified as L2, which fermented it slowly and weakly, and the L4 strain gave a negative result for both glucose fermentation and the other seven sugars used in the test Likewise, the strain identified as L1 was the only one that showed positive fermentation for maltose, galactose and sucrose and weakly for raffinose, cellobiose was only slowly fermented by L3, while strains L5 and L6 only managed to ferment positively glucose. Lactose and xylose could not be fermented by any of the 6 groups of yeasts isolated and characterized in this study.

Investigating yeasts' diversity and role in the spontaneous fermentation of cocoa beans from Indonesia [22]. Isolated seven yeast strains that they identified based on similar phenotypic characteristics with eight reference strain *Saccharomyces cerevisiae*, *Saccharomyces bayanus, Candida tropicalis, Candida krusei, Candida lambica, Saccharomycopsis fibuligera, Saccharomycopsis fermentant*, and *Kloeckera* sp. These eight reference strains were selected based on the similarity in phenotypic characteristics with the seven isolates and that they are the yeasts most frequently

Strain	Glucose	D-Xilose	Maltose	Galactose	Lactose	Sacarose	Raffinose	Cellobiose
L1	+	—	+	+	—	+	W	—
L2	S	—	—	L	—	L	S	—
L3	+	—	—	—	—	S	—	+/−
L4	—	—	—	—	—	—	—	—
L5	+	—	—	—	—	—	—	—
L6	+	—	—	—	—	—	—	—

+: positive, L: positive delayed, S: slow positive, W: weak positive, −: negative.

Table 3.
Fermentation properties of eight (8) sugars from 6 yeasts isolated from cocoa beans fermented in one locations of Venezuela.

found in fermented cocoa. The carbon sources preferred by most yeasts are hexoses such as glucose and fructose, present in high concentrations in cocoa beans, and mannose since they can enter the glycolytic pathway directly. Before entering this metabolic pathway, the first and limiting step for glucose metabolism is its transport through the membrane. The consumption of this sugar is mediated by a large family of proteins associated with carbon sources [26].

The L1 strain was the only one that positively fermented sucrose. It has been shown that this sugar is converted to glucose and fructose in species such as *S. cerevisiae* for its use; this event takes place in the yeast cell membrane thanks to periplasmic invertase. The invertase gene expression (SUC2) is repressed when there are high concentrations of glucose and reactivated when the concentration of this sugar falls [23]. The cocoa bean pulp contains approximately 14% sugars (60% sucrose and 39% a mixture of glucose and fructose). Assimilation and fermentation with the efficiency shown are because the yeasts identified as L1 show a certain degree of specialization to ferment this substrate thanks to the proteins mentioned above and enzymes' presence.

Sugars such as maltose, galactose, cellobiose, and raffinose could also be used by these yeasts, although to a lesser extent than glucose and sucrose. Could tentatively identify This group of isolates identified as L1 as belonging to the species *S. cerevisiae*. This species of yeast isolated as one of the most dominant species in the fermentation of cocoa beans from several countries such as Belize, Brazil, Ivory Coast, Ecuador, Ghana, Indonesia, Mexico, Nigeria, Peru, Nicaragua, and the Dominican Republic [2, 6, 9, 10, 20–23, 27–33] due to that there is evidence that it is a fast-growing yeast and that it presents high tolerance to ethanol thanks to the presence of fatty acids contained in the cell and also because it presents a vigorous pectinase and endo and exo protease activity, which influences the quantity of free amino acids in the ferments contributing beneficially to the flavor of the cocoa beans [34, 35].

The isolates identified as L2 were the only ones that fermented glucose slowly after 14 days, as did galactose and sucrose slowly. These could be the *Debaryomyces hansenii* species. Yeasts "oilseeds" that accumulate lipids. It is a highly heterogeneous species, and therefore versatile, as evidenced by phenotypic differences between strains, such as variations in its ability to assimilate and ferment various carbon sources, the expression of different lipase and protease activities, and its diversity under conditions growth [36]. Although this species of yeast is not common in cocoa fermentation, it is used in starter cultures for the fermentation of cocoa beans [29, 37].

Cellobiose was fermented only by the L3 strain. Depending on the species, different strategies have been observed among yeasts to use cellobiose. One of them consists of the expression of extracellular β-glucosidases on the cell surface; cellobiose is hydrolyzed extracellularly. After this, glucose is transported and metabolized inside the cell. The second strategy consists of the phosphorolytic pathway; cellobiose is transported into the cell by cellodextrins. An intracellular phosphorylase cuts the disaccharide with an inorganic phosphate, producing a glucose molecule and an α-glucose-1-phosphate that can be metabolized quickly. The hydrolytic pathway is the third strategy. Sugar is transported into the cell by cellodextrins to later be hydrolyzed by a β-glucosidase into two glucose molecules that can be easily metabolized by the cell [23].

According to [19], these isolates could be identified as *Hanseniospora opuntiae*. This species is considered one of the most frequent in cocoa fermentation processes [9, 38–41]. For example, it has been described as an elemental microbiota species in Malaysia's cocoa fermentation processes [42]. In general, this is a persistent species throughout the fermentation process, regardless of the cocoa's origin and the

method used to ferment the beans. A study with cocoa from Ecuador, both in boxes and in fermentation platforms, exhibited the presence of *Hanseniaspora* sp. (100% identity with *H. opuntiae, H. guilliermondii* and *H. uvarum*) and *P. kudriavzevii* [43].

The isolates grouped as L4 did not show fermentation for any of the eight sugars used; however, they could grow in the tubes. According to [19], several species of yeasts grow aerobically on sugars that fail to ferment. From the pattern found for this isolate, L4 could be classified as *Rhodotorula* spp. This species has not been reported in previous studies [23] on cocoa fermentation, identified *R. dairensis* in fermented cocoa beans in two locations in Ecuador.

L5 and L6 showed a very similar fermentation pattern for sugars used, which allows us to presume that they could be the same species, *Pichia kudriavzevii* or *Candida tropicalis*. The characterization by molecular biology will enable us to distinguish between which of the two species belong. Both species are recurrent in the cocoa fermentation processes [22, 39, 43–45]. The species *P. kudriavzevii* is also part of the central microbiota of cocoa ferments from Malaysia. In addition to his thermotolerance, its presence is favored by tolerance to highly selective environmental conditions present in the fermentation of cocoa pulp, such as the presence of chemical stressors, such as ethanol, acetic acid, and lactic acid [42, 46].

None of the isolated and identified species could ferment xylose; this is because the yeasts involved in cocoa ferments cannot operate the proton-symport system, subject to catabolic repression and the facilitated diffusion system for the transport and use of this sugar. Neither species could ferment lactose; this sugar [47] can be assimilated or fermented by yeasts through the transport via induction of a lactose-permease and its subsequent hydrolyzation to glucose and galactose by an intracellular β-glucosidase.

2.2 Lactic Acid Bacteria (LAB)

The values of the Log CFU/g of the LAB are shown in **Table 4**. A low population was obtained from 1.00 CFU/g to 2.75 CFU/g at the beginning of the fermentation process. LAB increased after two days and decreased in the last days of the cocoa beans' fermentation process. LAB population for the cocoa samples from the Zulia region (C^1-C^3) was 2.00 Log CFU/g, a logarithmic cycle above the representatives from the Miranda region (A and B^1). B2 was in the same order of C^1-C^3.

The LAB growth dynamics found for the regions of Miranda and Zulia could be due to several factors. The frequency of turning or removal of the mass of grains in fermentation, a process that It involves aeration or ventilation of the cocoa mass, generating exposure to oxygen and an increase in its concentration in the cocoa mass; in this investigation, this process was carried out every 24 hours. This fact could cause a significant reduction in the number of the LAB population during the last days of the fermentation process. In general, LAB are facultative anaerobic microorganisms. Their optimal growth conditions are in oxygen-free atmospheres, but they can tolerate low concentrations; therefore, oxygen is one of the various factors that generate microbial "stress" in this group. Similar results have been reported for different fermentation processes of cocoa beans from different regions of the world [21, 48].

In cocoa beans' fermentation, LABs exhibit the fastest growth rate during the 16–48 hours of fermentation and are present in large numbers, but not necessarily in biomass than yeasts for a short period [15].

The preliminary identification of LAB (**Table 5**) was selected colonies with bacillary or rounded shape, creamy white or beige color, positive Gram stain, catalase/oxidase negative. According to the results, there were four (4) different BAL phenotypes within the isolated strains. Presented Most of them (12) as long bacilli

	Fermentation time (Days)					
Sample	**0**	**1**	**2**	**3**	**4**	**5**
A	1.00 ± 0.01^{c}	2.79 ± 0.13^{ab}	3.27 ± 0.08^{ab}	2.92 ± 0.06^{a}	2.44 ± 0.16^{a}	1.33 ± 0.05^{a}
B^{1}	1.53 ± 0.09^{bc}	2.45 ± 0.23^{b}	2.48 ± 0.19^{cd}	2.49 ± 0.21^{ab}	1.99 ± 0.08^{a}	1.73 ± 0.06^{a}
B^{2}	1.87 ± 0.08^{abc}	1.63 ± 0.12^{c}	2.36 ± 0.14^{cd}	2.49 ± 0.17^{ab}	1.58 ± 0.13^{a}	1.33 ± 0.05^{a}
C^{1}	2.27 ± 0.15^{ab}	3.22 ± 0.21^{a}	1.68 ± 0.04^{b}	3.75 ± 0.12^{a}		
C^{2}	2.75 ± 0.08^{a}	1.73 ± 0.15^{c}	2.19 ± 0.087^{d}	2.50 ± 0.04^{ab}		
C^{3}	2.45 ± 0.17^{ab}	2.75 ± 0.03^{ab}	2.88 ± 0.06^{bc}	2.32 ± 0.09^{ab}		

^{a,b,c,d,e}Different letters for each row indicate significant differences ($p < 0,05$). Mean ± standard deviation.
A: La Trinidad fermented cocoa, Miranda state B^{1} and B^{2}: INIA Tapipa fermented cocoa, Miranda state C^{1}, C^{2}, C^{3}: INIA Chama fermented cocoa, Zulia state. 1,2,3: indicates the replica number.

Table 4.
Log CFU/g of LAB during the fermentation of cocoa beans.

	Morphology/Gram positive	Catalase	Oxidase	Glucose Gas	15°C	47°C
BAL1-A	Short bacillus	—	—	+	+	—
BAL2-A	Short bacillus	—	—	+	+	—
BAL3-A	Long bacillus	—	—	+	+	—
BAL4-A	Long bacillus	—	—	+	+	+
BAL1-B[1]	Short bacillus	—	—	+	+	+
BAL2-B[1]	Coccobacilli	—	—	—	—	—
BAL3-B[1]	Long bacillus	—	—	+	+	—
BAL4-B[1]	Long bacillus	—	—	+	+	—
BAL5-B[1]	Short bacillus	—	—	+	+	—
BAL1-B[2]	Long bacillus	—	—	+	+	—
BAL2-B[2]	Cocci	—	—	—	+	—
BAL3-B[2]	Long bacillus	—	—	+	+	—
BAL4-B[2]	Bacilluscocco	—	—	+	+	—
BAL5-B[2]	Cocci	—	—	—	+	—
BAL1-C[1]	Long bacillus	—	—	+	+	—
BAL2-C[1]	Long bacillus	—	—	+	+	—
BAL3-C[1]	Short bacillus	—	—	+	+	—
BAL1-C[2]	Long bacillus	—	—	+	+	—
BAL2-C[2]	Long bacillus	—	—	+	+	—
BAL3-C[2]	Short bacillus	—	—	+	+	+
BAL1-C[3]	Short bacillus	—	—	+	+	—
BAL2-C[3]	Long bacillus	—	—	+	+	—
BAL3-C[3]	Short bacillus	—	—	+	+	+
BAL4-C[3]	Long bacillus	—	—	+	+	—

+: Growth, —: no growth.

Table 5.
Morphological and biochemical characteristics of BAL isolated from fermented cocoa beans.

(BAL1), eight (8) isolated were short bacilli (BAL2), two (2) in the shape of coccobacilli (BAL3), and in the form of cocci, only three (3) isolated (BAL4). Similar results have been reported for different fermentation processes of cocoa beans from different regions of the world [7, 18, 21, 27, 30, 49].

According to these results, we could classify 22 isolates as Lactobacilli, belonging to the genus Lactobacillus and three (3) as Lactococci, genus Lactococcus. Twenty-one (21) of the isolates showed growth at 15° C, while only four (4) could grow at 15 and 47° C. Most of the twenty-two isolates (22) produced gas from glucose fermentation. Accordingly, the former could be heterofermenting LAB and the latter homofermenting LAB.

2.3 Acetic Acid Bacteria (AAB)

The quantification of AAB (**Table 6**) shows that populations between 2.32–3.62 Log CFU/g and 1.33–5.31 Log CFU/g were obtained in the first days of fermentation for samples from the Miranda and Zulia experimental stations, respectively.

	Fermentation time (Days)					
Sample	**0**	**1**	**2**	**3**	**4**	**5**
A	2.32 ± 0.14^{c}	2.25 ± 0.22^{d}	3.37 ± 0.20^{b}	3.41 ± 0.12^{b}	4.75 ± 0.12^{a}	5.27 ± 0.20^{a}
B^{1}	3.62 ± 0.24^{b}	1.73 ± 0.06^{d}	1.00 ± 0.01^{d}	1.76 ± 0.09^{c}	1.86 ± 0.074^{b}	5.44 ± 0.16^{a}
B^{2}	2.52 ± 0.16^{c}	6.18 ± 0.18^{a}	6.36 ± 0.07^{a}	6.04 ± 0.06^{a}	1.33 ± 0.05^{b}	1.00 ± 0.01^{b}
C^{1}	2.18 ± 0.18^{c}	3.43 ± 0.16^{c}	2.68 ± 0.21^{c}	3.60 ± 0.01^{b}		
C^{2}	1.33 ± 0.06^{d}	4.39 ± 0.09^{b}	3.19 ± 0.19^{b}	3.31 ± 0.14^{b}		
C^{3}	5.31 ± 0.20^{a}	4.26 ± 0.22^{bc}	3.56 ± 0.08^{b}	2.42 ± 0.11^{b}		

^{a,b,c,d,e}Different letters for each row indicate significant differences ($p < 0,05$). Mean ± standard deviation.
A: La Trinidad fermented cocoa, Miranda state B^{1} and B^{2}: INIA Tapipa fermented cocoa, Miranda state C^{1}, C^{2}, C^{3}: INIA Chama fermented cocoa, Zulia state. 1,2,3: indicates the replica number.

Table 6.
Log CFU/g of AAB during the fermentation of cocoa beans.

AAB carry out the transformation of the ethanol produced by the yeasts into acetic acid. The conversion of ethanol into acetic acid is an exothermic reaction. Ethanol and acetic acid diffuse into the cocoa beans, which finally generates the embryo's impossibility to develop a new cocoa plant.

The macroscopic, physiological, and biochemical morphological characteristics of twenty (20) BAA isolated from the fermented cocoa beans are shown in **Table 7**. Five (5) strains were isolated from A, two (2) from B1, four (4) from B2, three (3) from C1, two (2) from C2, and four (4) from C3. 100% of the isolates were catalase positive, oxidase negative, and capable of oxidizing acetate, a characteristic behavior of Acetobacter species. Except for A. peroxydans [50].

Two (2) morphotypes were found. Bacilli (BAA1), colonies with a point shape, 5 mm in diameter, convex, creamy beige, and coccobacilli (BAA2) elongated colonies, 2 mm, connected, shiny, beige; both Gram-negative, catalase-positive, and oxidase negative. From the above, there are four (4) subtypes. Eight (8) isolates cannot grow in 10% ethanol and 30% D-glucose; identified as BAc1, they could be the species *A. syzygii*, *A. lovaniensis*, *A. pomorum*, *A. aceti*, or *A. tropicalis*. Seven (7) isolates did not grow in 10% ethanol, but in 30% D-glucose classified as BAc2, presumably *A. ghanensis* or *A. cibinongensis*. Two (2) isolates grown in 10% ethanol (BAc3), *A. oeni*, or *A. pasteurianus*. Three (3) that grow in 10% ethanol and 30% D-glucose called BAc4, possibly they are the species *A. nitrogenifigens* [50] (**Table 7**).

	Morphology/ Gram negative	**Catalase**	**Oxidase**	**10% Ethanol Growth**	**30% D-glucose Growth**	**Acetate oxidation**
BAA1-A	Bacillus	+	—	—	—	+
BAA2-A	Bacillus	+	—	—	+	+
BAA3-A	Coccobacilli	+	—	—	—	+
BAA4-A	Bacillus	+	—	+	—	+
BAA5-A	Bacillus	+	—	—	—	+
BAA1-B[1]	Bacillus	+	—	+	+	+
BAA2-B[1]	Bacillus	+	—	—	+	+
BAA1-B[2]	Bacillus	+	—	—	+	+
BAA2-B[2]	Bacillus	+	—	+	+	+
BAA3-B[2]	Bacillus	+	—	—	+	+
BAA4-B[2]	Bacillus	+	—	—	—	+
BAA1-C[1]	Bacillus	+	—	—	+	+
BAA2-C[1]	Bacillus	+	—	—	—	+
BAA3-C[1]	Bacillus	+	—	—	—	+
BAA1-C[2]	Coccobacilli	+	—	—	—	+
BAA2-C[2]	Bacillus	+	—	—	—	+
BAA1-C[3]	Bacillus	+	—	—	+	+
BAA2-C[3]	Bacillus	+	—	—	+	+
BAA3-C[3]	Bacillus	+	—	+	+	+
BAA4-C[3]	Bacillus	+	—	+	—	+

+: Growth, —: no growth.

Table 7.
Morphological and biochemical characteristics of BAL isolated from fermented cocoa beans.

3. Evaluation of fermented cocoa

Once the cocoa is fermented, typing analysis is done to classify it. The cut test (**Figure 1**) is to identify cocoa beans that have been well-fermented, lightly fermented, and non-fermented. However, it will allow you to observe the grains that present problems, including; over-fermented, slaty, sprouts, affected by insects.

Figure 1 schematizes the portion of a cutting test (carried out manually with the help of a knife) of cocoa beans. This test seeks to know the percentage of fermentation that specific cocoa has—the determination to be made in at least five (5) replicas of 100 grains each.

Cocoa is classified commercially according to the percentage of fermentation. In Venezuela, the established classification [51, 52] in Extra Fine (EF: 95%), Fine First (F1: 80%), Fine Second (F2: 20%). It is known as ordinary cocoa beans with fermentation percentages lower than F2.

It is an international consensus that cocoa beans with brown coloration and well-defined streaks on their cotyledons are fully fermented. Grains with violet/brown colorations are associated with slightly fermented grains. Grains purplish, compact, or very little change in the violet color (typical of Trinitarios and Forasteros) may be unfermented. **Figures 2–4** show cocoa beans with these characteristics.

In **Figure 2**, cocoa beans with violet coloration are shown, indicating that they are not fermented. In the dissection of these beans, it is observed that there are no well-defined cracks or channels, which are formed due to the microbiological succession and enzymatic reactions that occur during the fermentation process. The bean shown in the upper left part has a dark, blackish coloration, which indicates that it is a slate grain, not desirable in transforming from cocoa to chocolate. The dissection of the lower-left grain shows a violet coloration and compaction in it, indicative of non-fermented grains; it also presents white areas that could indicate the presence of fungi or attack by insects.

Figure 1.
Cutting test of cocoa beans from various cocoa-producing areas of Venezuela, representing fermented, slightly fermented, unfermented, and damaged beans.

Figure 2.
Unfermented cocoa beans (upper and lower left) and well-fermented cocoa beans (upper and lower right).

Figure 3.
Slate cocoa bean with insect attack.

The upper-right grain has a brown color and a notable presence of cracks or channels in its cotyledons that indicate that it has completed the fermentation process. Another indication of a well-fermented grain is observed in the lower-right grain, in which, although cracks and channels are not perfectly evident, it presents the separation or opening of its cotyledons, which allows classifying them as well or completely fermented.

Slate cocoa beans (**Figure 3**) generally appear due to a lack of fermentation or incomplete fermentation (which is interrupted by the producer for various reasons). Slate grains are associated with increased bitterness and astringency. Attributes that later affect the cocoa paste (liquor) and, therefore, the production of chocolate.

Figure 4.
Slightly or partially fermented cocoa beans.

This bean also has a compact appearance (not fermented). The bean shown a cavity in the center indicates that insects have attacked it. Generally, this occurs because there are poor storage conditions (relative humidity and temperature). Proliferating species such as *Ephestia elutella*, which in addition to causing damage to the beans, can affect the production of chocolate.

Figure 4 shows cocoa beans that have passed the fermentation process; however, it has not been complete for them. Obtaining slightly or partially fermented grains occurs because the fermentation mass is excessive; there are no controlled processes for removing the fermentation mass or due to the location of the grains in the drawers or fermentation systems, in addition to the amount of mucilage that each grain has. In this image (**Figure 4**), the dissection of the upper-right grain is an excellent example of partially fermented. The periphery of the grain is purple, and the center of the grain is brown, with the appearance of channels.

Forasteros-type grains, as they present a natural intense violet color, can often pass as unfermented or partially fermented. Therefore, it is necessary to know the evaluation criteria very well to avoid incurring classification errors.

Finally, it is essential to note that the purpose of the cutting test is to establish a commercial classification and does not establish a precise and exact indicator of what the aromatic or flavor quality of the cocoa beans will be.

Another parameter to consider in fermented cocoa is the percentage of acidity because many times high fermentation percentages (greater than 90%) generate a higher content of acetic acid (predominant acid in cocoa due to the action of acetic acid bacteria). The high acidity makes cocoa paste/liquor require long working times in chocolate conching machines. In the case of Venezuela, the relationship between cocoa producer-chocolatier-artisan's increases day by day. With this, criteria can be established regarding the final fermentation percentages that are desired for a specific type of cocoa.

4. Conclusion

Fermentation is a crucial stage in the post-harvest processing of cocoa. The succession of yeasts, lactic acid bacteria, acetic acid bacteria, and others more involved,

together with the system's conditions and the enzymes, allows the formation of aroma precursors (sugars and amino acids). Finally, the precursors are magnified in the roasting, resulting in a diversity of aromas in the final derived products.

It is necessary to deepen each type of cocoa fermentation to know and standardize the percentage or degree of fermentation that each bean requires. It is important to note that 100% fermented grains often provide an acetic acid index that ultimately translates into longer working time in chocolate conching machines.

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Conflict of interest

The authors whose names are listed in this paper certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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