

Physicochemical and microbiological dynamics of the fermentation of the CCN51 cocoa material in three maturity stages

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Abstract - The physicochemical and microbiological dynamics during the fermentation phase of the CCN51 cocoa material was evaluated in three maturity stages (stage 2: 130-152 days after anthesis (DAA), stage 3: 153-174 DAA, and stage 4: 175-196 DAA). Through the microfermentation technique, the physicochemical variables (pH, titratable acidity, total soluble solids, and fermentation index) were monitored every 24 hours. The microbiological dynamics were evaluated by counting the colony forming units (CFU) in a plaque, quantifying three types of microorganisms (*Bacillus*, acetic acid bacteria and yeasts). In the three maturity stages assessed, a decrease in the pH of the beans was observed as the fermentation progressed; between 72 and 96 hours after fermentation, the pH of stage 4 beans was different from stages 2 and 3. Simultaneously, an increase in the pH of the pulp was observed without differences between the stages. Brix degrees did not show statistical differences between stages, however, the values of total soluble solids showed that the microorganisms present in stage 3 were more effective in metabolizing these in comparison with those of stages 2 and 4. Regarding the fermentation index, stage 4 reached the maximum value (1.35 ± 0.16) at 120 hours, meanwhile stages 2 and 3 reached it at the end of the fermentation (1.35 ± 0.01 and 1.55 ± 0.29 , respectively) at 168 hours. The microbiological dynamics in the three cocoa fruit maturity stages evidenced the absence of lactic acid bacteria and the presence of mesophilic aerobic bacilli. Yeasts were present throughout the fermentation in the three maturity stages, being more favored by stage 2, meanwhile, acetic acid bacteria were favored by stage 3. Outstanding microorganism species were characterized molecularly as *Pichia kudriavzevii*, *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus tequilensis*. Stage 3 showed the best characteristics to obtain good quality cocoa.

Index Terms: Maturity, microorganisms, quality, *Theobroma cacao*.

Dinâmica físico-química e microbiológica da fermentação de material de cacau CCN51 em três fases de maturidade

Resumo - A dinâmica físico-química e microbiológica durante a fase de fermentação do material de cacau CCN51 foi avaliada em três fases de maturidade (fase 2: 130-152 dias após a antese DDA, fase 3: 153-174 DDA e Fase 4: 175 -196 DDA). Através da técnica da microfermentação foi monitorizado a cada 24h as variáveis físico-químicas (pH, acidez titulável, total de sólidos solúveis e índice de fermentação); para a dinâmica microbiológica, por contagem de unidades formadoras de colônias na placa (UFC) foram quantificados três tipos de microorganismos (*bacillus*, bactérias ácido acéticas e leveduras). Nas três fases de maturidade, foi observado uma diminuição do pH no grão quanto progrediu a fermentação progrediu, entre 72 e 96 horas após a fermentação, o pH do grão na fase 4 foi diferente das fases 2 e fases 3. Paralelamente observou-se um incremento do pH da polpa sem diferenças entre as fases. Os graus Brix não apresentaram diferenças significativas entre fase, no entanto, os valores de sólidos solúveis mostraram que os microrganismos presentes na fase 3 foram mais eficazes no metabolismo do conteúdo de sólidos solúveis totais em relação à fase 2 e 4. No respeito a taxa de fermentação, a fase 4 atingiu o valor máximo de (1.35 ± 0.16) às 120 horas, enquanto as fases 2 e 3 atingiram-o no final da fermentação (1.35 ± 0.01 e 1.55 ± 0.29 , respectivamente) (168 h). A dinâmica microbiológica nas três fases de maturidade dos grãos de cacau, demonstrou a ausência de bactérias ácido lácticas e a presença de bacilos aeróbios mesófilos; as leveduras mostraram-se na totalidade da fermentação, nas três fases de maturidade, sendo favorecidas pela fase 2 enquanto as bactérias ácido acéticas foram favorecidas pela fase 3. As espécies de microrganismos em destaque foram molecularmente caracterizadas como *Pichia kudriavzevii*, *Bacillus subtilis*, *Bacillus Megaterium* e *Bacillus Tequilensis*. O estágio 3 apresentou as melhores características para obter um cacau de boa qualidade.

Termos para indexação: Maturidade, microrganismos, qualidade, *Theobroma cacao*.

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Introduction

Cocoa tree (*Theobroma cacao* L.) is a commercial crop of great economic importance in the world and is the source of the raw material for the manufacture of chocolate (THI et al., 2015, GUTIÉRREZ et al., 2011). Between years 2002 and 2010 the consumption of chocolate products increased by 10% worldwide, especially in the European countries, the United States, Brazil, Japan and Australia, reaching an amount of around 5.54 million tons (ECONOMICS COMMITTEE, 2014). Consumption preferences are given by its pleasant taste and nutritional benefits, as it is a source of antioxidants (BABAA et al., 2000) and an anticarcinogen (YAMAGISHI et al., 2001).

The demand of the cocoa world market for good quality cocoas has been increasing; it has been shown that the complex composition of the cocoa bean flavor depends on several factors, including the genotype, the chemical composition of the soil, the age of the tree, and the postharvest processes carried out by producers. Among these, preconditioning, fermentation, drying and industrial processes as roasting, grinding, and product formulation are crucial (KONGOR et al., 2016).

In the fermentation process the action of the microorganisms takes place in the pulp surrounding the beans, which is rich in glucose, fructose, and sucrose (total content of 10-15%) and the initial pH is relatively low (pH: 3.3-4.0), mainly due to a high concentration of citric acid (1-3%) (ARDHANA and FLEER, 2003). The fermentation of cocoa is a spontaneous microbiological process; it is known that the microorganisms that initiate the fermentation are yeasts, thanks to the high content of sugars, low pH and the limited availability of oxygen in the pulp (THOMPSON et al., 2013).

The main activity of yeasts is the production of ethanol from carbohydrates, in addition to the assimilation of citric acid and the degradation of pectin (SCHWAN and WHEALS, 2004); this activity leads to the production of CO₂, which favors anaerobic conditions allowing the development of lactic acid bacteria (LAB) that helps in the decomposition of sugar and produces lactic acid, acetic acid and mannitol (PRABHAKARAN, 2010), and consumes citric acid. This stage lasts around 48 hours. Then, the practice of turning the mass periodically stimulates the growth of acetic acid bacteria (AAB). These bacteria oxidize the ethanol produced by the yeasts to acetic acid, which diffuses through the testa causing a breakdown of polyphenols and lipid membranes of the cell vacuoles, and the contents of the cells are mixed. Various enzymatic reactions are carried out and the polyphenols are oxidized. This reaction is partly responsible for the elimination of the bitter taste of the beans (VOIGT et al., 1994).

Since the transformation of ethanol into acetic acid is an exothermic reaction, heat is produced favoring

the development of bacteria of the *Bacillus* genus, which contributes to generating the taste and the production of pectinolytic enzymes; these species can also be present from the beginning of the fermentation (OUATTARA et al., 2008).

When fermentation was considered as a crucial step in the generation of flavors and aromas for chocolate, many countries worldwide have been concerned to follow the microbiological and physicochemical dynamics in the fermentations carried out in their territory, including countries as Ghana (CAMU et al., 2007), Côte d'Ivoire (GUEHI et al., 2008), China (GU et al., 2013), Indonesia (ARDHANA AND FLEER, 2003) (LISDIYANTI et al., 2003), Thailand (LISDIYANTI et al., 2003), the Philippines (LISDIYANTI et al., 2003), Trinidad and Tobago (ROMBOUTS, 1952), the Dominican Republic (GÁLVEZ et al., 2007), Brazil (PAPALEXANDRATOU et al., 2011) and Mexico (ROMERO et al., 2013). In Colombia, there are still no studies in this regard, which places the country in a disadvantageous position, not knowing the internal factors that affect the quality of the product that is generated. Furthermore, another important aspect that influences the fermentation is fruit maturity, since immature pods have yet not developed completely their juices or mucilage that favors the microbiological dynamics. On the contrary, if the pods are overripe, the almonds do not have sufficient amount of substrate for the microorganisms to act and to develop an adequate fermentation; all the previously mentioned generates defects in chocolate flavor and aroma (TENEDA, 2016).

Taking into account that the cocoa material CCN51 is the most representative in the cocoa production industry in Colombia, and that the different maturity stages that occur in the crops affect the quality of the bean, the aim of this study was to evaluate the physicochemical and microbiological dynamics of the fermentation phase of the CCN51 cocoa material in three maturity stages.

Material and methods

Biological material - The cocoa clone CCN51 was used as the evaluation material obtained from an established culture of 12 years of age, with a planting distance of 3 x 3 m, without shading, and with drip irrigation. The study defined three cocoa fruit maturity stages considering days after anthesis (DAA): stage 2 (130-152 DAA, with a red wine color and green and yellow pints), stage 3 (153-174 DAA, completely red color), and stage 4 (175-196, red color with black spots). These stages were selected according to the BBCH scale described by Niemenak et al. (2010), and by employing growth equations for the region, maturity fruit ages to assess, were established.

Study site - A cacao producing farm was selected in the department of Huila, Colombia, located in the municipality of Garzón, between coordinates 02° 12.941' N and 75° 36.064' W, with an average altitude of 876 meters above the sea level. The area is characterized by having a tropical climate with a dry *As* summer according to the classification of the Köppen-Geiger system, with an average annual temperature of 23.4 °C and average rainfall of 1,104 mm.

Sampling - Thirty (30) cocoa fruits or pods were collected from each maturity stage and were taken to the Agrifood Science Laboratory of the Nataima research center of AGROSAVIA (Km 9 via Espinal – Ibagué, department of Tolima).

Microfermentation of cocoa beans - The protocol established by Vidales (2014) was followed. The pods were washed with a 1% hypochlorite solution and were then opened to obtain the seeds. The seed mass was homogenized and placed in plastic cups of 900 g each forming a closed system for the anaerobic conditions that the first 96 hours require, and placing these in an oven at 35 °C. Thereafter, the oven temperature was increased to 45 °C and the closed system was broken, homogenizing the dough every 24 hours until 168 hours of fermentation was achieved (CAMPOVERDE, 2010, MENEZES et al., 2016).

For the physicochemical and microbiological analyzes, 23 g of the center sample of the cocoa mass was obtained aseptically every 24 hours, from hour zero (0) until achieving 168 hours of fermentation. Of these, 3 g were used for the physicochemical analysis and 20 g was employed for the microbiological analysis.

Physicochemical analysis - To measure pH, titratable acidity, total soluble solids, and fermentation index, the pulp and the tegument were separated from the beans with a scalpel to be processed independently (beans and pulp). Two grams (2 g) of each were weighed and macerated with 20 mL of distilled water, filtered, and in the filtrate was used to measure pH with a potentiometer (HANNA® Instruments). Then, the titratable acidity was determined with NaOH (0.1 N), and total soluble solids were measured in the pulp solution with a digital brand refractometer (ATAGO®).

To determine the fermentation index (FI), a sample of 0.5 g of dry cocoa beans (7% w/w) was obtained, macerated and then 50 mL of methanol: HCl solution (97: 3) was added. Afterward, the sample was homogenized and left to rest at 8 °C for 19 hours (NAZARUDDIN, et al., 2006). Subsequently, the absorbance ratio at 460 nm and 530 nm was determined by spectrophotometry in a Thermo Scientific Helios Zeta UV-VIS spectrophotometer according to equation 1.

$$FI = \text{absorbance value at 460 nm} / \text{absorbance value at 530 nm} \quad (\text{Equation 1})$$

The fermentation index is related to the color changes in the cocoa beans, i.e. it has an intense violet color at the beginning due to the polyphenols content that later turns to brown due to the condensation of anthocyanins that increases the absorbance value to 460 nm (NAZARUDDIN, et al., 2006). Values above one (1) indicate well-fermented beans, meanwhile, values below 1 indicate an insufficient fermentation (CAPORAZO et al., 2018, SUNOJ et al., 2016).

Statistical analysis - The data were analyzed by a simple ANOVA using a completely randomized design and Tukey's multiple comparison test at $\alpha = 0.05$ level.

Microbiological analysis - For the quantification of the microbial populations, 20 g of bean sample was placed in a sterile glass bottle, and then, 25 mL of sterile saline (NaCl) solution at 0.85% w/v was added. The mixture was shaken vigorously for 3 minutes and filtered through sterile gauze to remove the pulp from the microbiological suspension. From this suspension, six serial dilutions (10⁻¹ to 10⁻⁶) were made with saline solution (NaCl at 0.85% w/v). Then, 200 µL of each dilution were cultured in triplicate on the surface of plates of four solid culture media.

Culture media: Agar Merck malt extract (48 g/L) supplemented with chloramphenicol (1,000 mg/L) and oxytetracycline (100 mg/L) with a pH 3.5 (ARDHANA and FLEER, 2003; CROZIER et al., 2006) was employed for yeast counting. Agar-MRS supplemented with 10% tomato juice (ARDHANA and FLEER, 2003) and cycloheximide (200 mg/L) at a pH 5.5 was used for the quantification of LAB and *Bacillus* lactic bacteria. For the quantification of AAB, two media were used. A) Yeast extract (1%), peptone (4%), glucose (6%), calcium carbonate (1%), agar (2%) with 100 mg/L of cycloheximide and 150 mg/L of penicillin with a pH 5.0 (DU TOIT and PRETORIUS, 2002). B) Glucose (2%), yeast extract (0.8%), peptone (0.5%), ethanol (0.5%), calcium carbonate (0.3%) and agar (3%) with 150 mg/L of penicillin with a pH 3.5 (LISDIYANTI et al., 2003). All media were autoclaved at 121°C for 15 minutes.

Quantification: The plates sown were incubated at 30 °C for 72 hours (LEFEBER, et al., 2012). Then, the CFU count was performed in each medium.

Microorganism characterization, evaluation, and selection

Characterization: The colonies formed were analyzed macroscopically (shape, elevation, edge, surface, consistency, appearance, and color) using a Nikon SMZ745T stereoscope. Colonies with different characteristics were isolated in culture media by exhaustion. Once pure, these were stained with lactophenol blue. The bacterial isolates were characterized with Gram staining, catalase, and oxidase.

For the molecular characterization, the isolates obtained were selected according to the production of pectinases for yeast and production of acid for AAB. For the molecular characterization of mesophilic aerobic bacilli (MAB), those that presented macroscopic differences in the colony and microscopic differences in cells were selected from the group obtained.

Yeast selection: The yeast isolates were selected for their pectinolytic activity, for which two solid culture media were used, as follows. 1) cocoa-pulp agar (50% cocoa pulp, 2% agar; pH 3.8), and 2) pectin medium (citrus pectin, casein peptone, yeast extract, CaCO_3 , K_2HPO_4 , agar and chloramphenicol; pH 4.0).

Subsequently, a suspension of the microorganism to be evaluated was prepared taking into account the turbidity of the Macfarland tube No. 2, then in five equidistant points and in each culture medium, 10 μL of the cell suspension was placed. The cultured boxes were incubated for 6 days at 30 °C, and then, the diameter of each of the colonies was measured. In the pectin medium, 7 mL of iodine-potassium iodide solution (1.0 g of iodine, 5.0 g of potassium iodide, and 330 mL of distilled water) was added, leaving it to act for 15 min. Then, the solution was removed with distilled water and the halo formed was measured (PRIYA and SASHI, 2014). The most active isolates were sent for their molecular characterization.

Selection of AAB: Acetic acid bacteria were selected considering the production of acid in a liquid culture medium, for which a suspension of the microorganism to be evaluated was prepared, taking into account the turbidity of the Macfarland tube No. 2. Afterward, 1 mL of the suspension was added to a liquid medium with yeast extract (1%), peptone (4%), glucose (6%), calcium carbonate (1%), 100 mg/L of cycloheximide and 150 mg/L of penicillin at a pH 6.6. Then, these were incubated for five days under agitation. The biomass was separated by centrifugation at 12,000 rpm for 10 minutes, where 20 mL of the supernatant was used to titrate with 1 N sodium hydroxide solution (NaOH) expressing the results as percentage of titratable acidity, using equation 2:

$$\% \text{ of titratable acidity} = (B * N / W) * 100 \quad (\text{Equation 2})$$

Where:

B is the NaOH consumed in the titration (mL), N is the normality of NaOH 1 N, and W is the biomass weight.

DNA extraction: The broth cultured with each of the strains was transferred to 2 mL microtubes to proceed with their respective DNA extraction. The ZR-96 Fungal/Bacterial DNA kit (Zymo Research, USA, CA, Irvine) was used following the instructions of the manufacturer. At the end of the procedure, the concentration and purity of the DNA obtained by spectrophotometry was verified in the Nanodrop equipment and its subsequent visualization was carried out using a 1% agarose gel.

PCR starters and conditions: Amplification of the fragments was carried out with primers (NL1-F, LR6-R, ITS1-F, and ITS1-R). The PCR assays were carried out in 25 μL of reaction mixture containing 21 μL of Master mix, 1 μL of inverse primer, 1 μL of reverse primer and 2 μL of genomic DNA, under the following conditions. An initial denaturation step was performed at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 63 °C for 40 s, and extension at 72 °C for 90 s. Subsequently, an extension stage was carried out at 72 °C for 10 min. Finally, the amplified products were visualized by electrophoresis with 2 μL of PCR product amplified on a 1.5% agarose gel. The resulting gel was registered using a light transilluminator. The PCR products were sequenced by Corpogen (Bogotá) in alliance with Macrogen (Korea).

Results and discussion

Physicochemical dynamics during the microfermentation of the cocoa clone CCN51

pH: The pH of the beans showed a downwards behavior during the fermentation, starting with values of 6.22 ± 0.08 (stage 2), 6.31 ± 0.17 (stage 3) and 6.35 ± 0.11 (stage 4), and ending with 5.6 ± 0.31 (stage 2), 5.75 ± 0.18 (stage 3) and 5.54 ± 0.24 (stage 4), coinciding with the results reported by Ardhana and Fleer (2003). In the three stages evaluated, a desirable pH was reached in the fermentation, however, statistically significant differences were found ($P \leq 0.05$) at 72 and 96 hours of fermentation, where stage 2 recorded the highest pH values (Figs. 1a and 1b). These results indicate that possibly, the microorganisms did not generate the acids and appropriate conditions for their entrance to the bean, and therefore, a decrease in pH occurs, as what happened in stages 3 and 4. The final pH of the cocoa beans after the fermentation process occurs is very important since the type of product of the enzymatic proteolysis of the proteins (cocoa and chocolate aromas and taste precursors)

changes with the pH (KRATZER et al., 2009; YUSEP et al., 2002). This indicates that cocoa beans contain carboxypeptidase enzymes with optimal activity at a pH close to 5.8, releasing hydrophobic amino acids whose activity decreases considerably at pH values below 5.0.

Regarding the pH of the pulp, initial values registered were 3.52 ± 0.17 (stage 2), 3.45 ± 0.23 (stage 3), and 3.66 ± 0.14 (stage 4), with a gradual increase ending in values of 4.09 ± 0.13 (stage 2), 4.19 ± 0.08 (stage 3) and 4.25 ± 0.12 (stage 4). At 72, 120 and 144 hours of fermentation, the pH of the pulp showed statistically significant differences ($P \leq 0.05$), showing stage 2 the lowest rise in pH (Figure 1b). This confirms that in this stage, the microorganisms were not able to metabolize the acids present in the pulp, and therefore, the fermentation process was different from stages 3 and 4 (THOMPSON et al., 2013).

Percentage of titratable acidity: The results showed a progressive increase in the percentage of titratable acidity of the beans (Figure 1c) during the fermentation in the three stages, starting with values between 0.99 and 1.08%, and ending with values between 1.70 and 1.95%. There were significant statistical differences ($P \leq 0.05$) at 48 and 72 hours of fermentation. In general, stage 2 showed the lowest percentage of titratable acidity in the beans meanwhile stage 3 recorded the highest values. On the other hand, the percentage of titratable acidity of the pulp (Figure 1d) showed significant statistical differences at 0 and 24 hours of fermentation. Moreover, stage 2 started with $4.37 \pm 0.76\%$, meanwhile stages 3 and 4 recorded values of $6.19 \pm 0.84\%$ and $3.39 \pm 0.83\%$, respectively. For this variable, stage 3 presented the highest values between 120 and 168 hours of fermentation.

The endogenous acids of the beans (malic, tartaric, oxalic, phosphoric, and citric acids) are considered, probably, less important than those developed during the fermentation, being the diffusion of the lactic and acetic acids to the bean, the ones that dominate the acidity of the same (SCHWAN and WHEALS, 2004). It is known that the acidity of the pulp depends initially on the presence of citric acid (GÁLVEZ et al., 2007), but after the fermentation and due to the action of the microorganisms that intervene in it, acetic and lactic acids that are produced, are the ones that comprise the acidity (SCHWAN and WHEALS, 2004).

Total soluble solids: The total soluble solids of the pulp (Figure 1e) decreased considerably during fermentation, starting with values of 16.58 ± 3.31 (stage 2), 21.53 ± 0.47 (stage 3) and 17.15 ± 1.65 (stage 4), and ending with 6.42 ± 1.98 (stage 2), 5.11 ± 2.62 (stage 3) and 6.34 ± 1.96 (stage 4). This indicates that in stage 3 the microorganisms were more effective in metabolizing the content of total soluble solids compared to stages 2

and 4, by metabolizing 76% of the total soluble solids during fermentation. The statistical analysis showed significant differences ($P \leq 0.05$) at 24, 48 and 72 hours of fermentation, showing stage 2 the lowest values, being attributed to the scarcity of soluble solids to metabolize and generate acids for an adequate fermentation, which agrees with the analysis of pH and percentage of titratable acidity of the beans.

Fermentation index: Regarding the fermentation index (Figure 1f), the beans of stage 2 started with a value of 0.35 ± 0.02 , the ones of stage 3 showed a value of 0.408 ± 0.02 , and in stage 4 the value was 0.505 ± 0.18 ; at 120 h, stages 3 and 4 reached the maximum values of 1.70 ± 0.09 and 1.35 ± 0.16 , respectively, meanwhile stage 2 reached it at the end of the fermentation (168 h) with 1.35 ± 0.01 . The fermentation index as an unaddressed measure of the anthocyanin content has been considered a good indicator to establish the degree of fermentation of the cocoa beans, suggesting that a fermentation index value ≥ 1 indicates that the cocoa mass was fermented. However, other factors such as the pH of the beans must be taken into account in order to affirm this. This is the reason why, although in stages 3 and 4 a value ≥ 1 was reached at 120 h, the beans were not considered to have been fermented enough in this time period, as the pH of the beans was 6.38 and 6.31. As Yusep et al. (2002) suggested a pH close to 5.8 is necessary to generate special flavors and aromas in cocoa. Moreover, among the stages evaluated, no significant statistical difference was observed ($P > 0.05$) in this variable.

Microbiological dynamics during microfermentation of the cocoa clone CCN51: At zero (0) hours of fermentation, the presence of MAB was observed in the three maturity stages evaluated; the dynamics of this group was different according to the stage, and this is why in stage 2 these were observed at 24 and 72 hours ($2.3 \log \text{CFU/g}$); in stage 3, after starting with $3.6 \log \text{CFU/g}$, at 24 hours there was a decrease, and at 48 hours it increased to $8.3 \log \text{CFU/g}$, starting again a decrease. At 72 hours $1.3 \log \text{CFU/g}$ were found, and thereafter, these were not detected again. Regarding stage 4, bacilli were present throughout the fermentation process, starting with $4.3 \log \text{CFU/g}$ and presenting the highest presence at 48 hours with $7.6 \log \text{CFU/g}$, similar to stage 3; and after 96 hours, these stabilized, ranging from 4.1 to $5.8 \log \text{CFU/g}$ (Figure 2a). The role of bacilli during cocoa fermentation is not yet clear, however, it has been reported that they play an important role in the production of tetramethylpyrazine (ZAK et al., 1972), i.e. the main component of chocolate aroma (BONVEHÍ, 2005). Moreover, their capacity for the production of pectinolytic enzymes has also been demonstrated (OUATTARA et al., 2008) (YAO et al., 2017) since recently, it was

believed that the only responsible microorganisms for the production of this compound were the yeasts. Nonetheless, it has been stated that a high population can contribute to the acidity and perhaps to undesirable flavors in fermented cocoa beans (SCHWAN and WHEALS, 2004).

It should be noted that the fermentation of cocoa beans is a difficult process to control, especially when it is carried out in places exposed to the microbiota of each location, making it a substrate for an endless number of microorganisms that fulfill different functions and give as a result, good or bad flavor and aroma profiles (PAPALEXANDRATOU et al., 2011). The appearance of bacilli in fermentations of cocoa beans is less predictable than that of other microbial groups (MELO PEREIRA et al., 2012); Moreover, Thi et al. (2015) affirmed that LAB might not be necessary during a good cocoa fermentation, as they obtained this result when comparing the organoleptic quality with fermentation where only yeasts and AAB intervened.

Molecular characterization: The molecular characterization carried out identified *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus tequilensis* as the predominant MAB species in the fermentation of the CCN51 cocoa material (Figure 3).

Further, AAB are considered fundamental during the fermentation of cocoa, as they are responsible for oxidizing the ethanol generated by the yeasts to acetic acid, which together with ethanol, diffuses into the cocoa beans. This process heats the product of the exothermic bioconvection, causes the death of the embryo in the seed, and initiates biochemical changes, forming flavor and color precursor molecules of typical cocoa beans (CAMU et al., 2007). According to the characterization carried out (Gram staining, oxidase, and catalase tests), the colonies that grew on the AAB-specific agar media allowed the growth of these, however, it is considered important to carry out the molecular characterization to ensure this (Figure 4). Therefore, these are considered as possible AAB. Furthermore, these were found in stage 2 at 24 hours of fermentation, and later in stage 4, they were not found anymore. On the other hand, these were detected in stage 3 during the entire fermentation process, starting at zero hours with 4.3 log CFU/g, but at 72 hours they decreased; then at 120 hours, there was an increase (6.5 log CFU/g) finalizing with a value of 6.3 log CFU/g at 168 hours (Figure 2b).

Yeasts were present throughout the fermentation process in all maturity stages, confirming the importance of these in the fermentation of cocoa. According to Criollo et al. (2010), yeasts favor a greater migration of compounds from the outside of the almond that facilitates the enzymatic hydrolysis to the interior of the bean. In stage 2 at zero hours, the highest presence of yeasts was found with 6.3 log CFU/g, with a slight decrease at 24

hours, stabilizing their number at 48 hours until the end of fermentation with 4.7 log CFU/g. Stage 3 started with 4.14 log CFU/g, and at 24 hours, the highest presence of these microorganisms was observed (7.3 log CFU/g), descending to 48 hours, and stabilizing at 96 hours in ± 4 log CFU/g. In stage 4, it started with 2 log CFU/g, the lowest of the evaluated stages, presenting a gradual increase and finishing the fermentation with 4.3 log CFU/g (Figure 2c). The molecular characterization found that *Pichia kudriavzevii* was the dominant yeast species in the fermentation of cocoa clone CCN51 material (Figure 5).

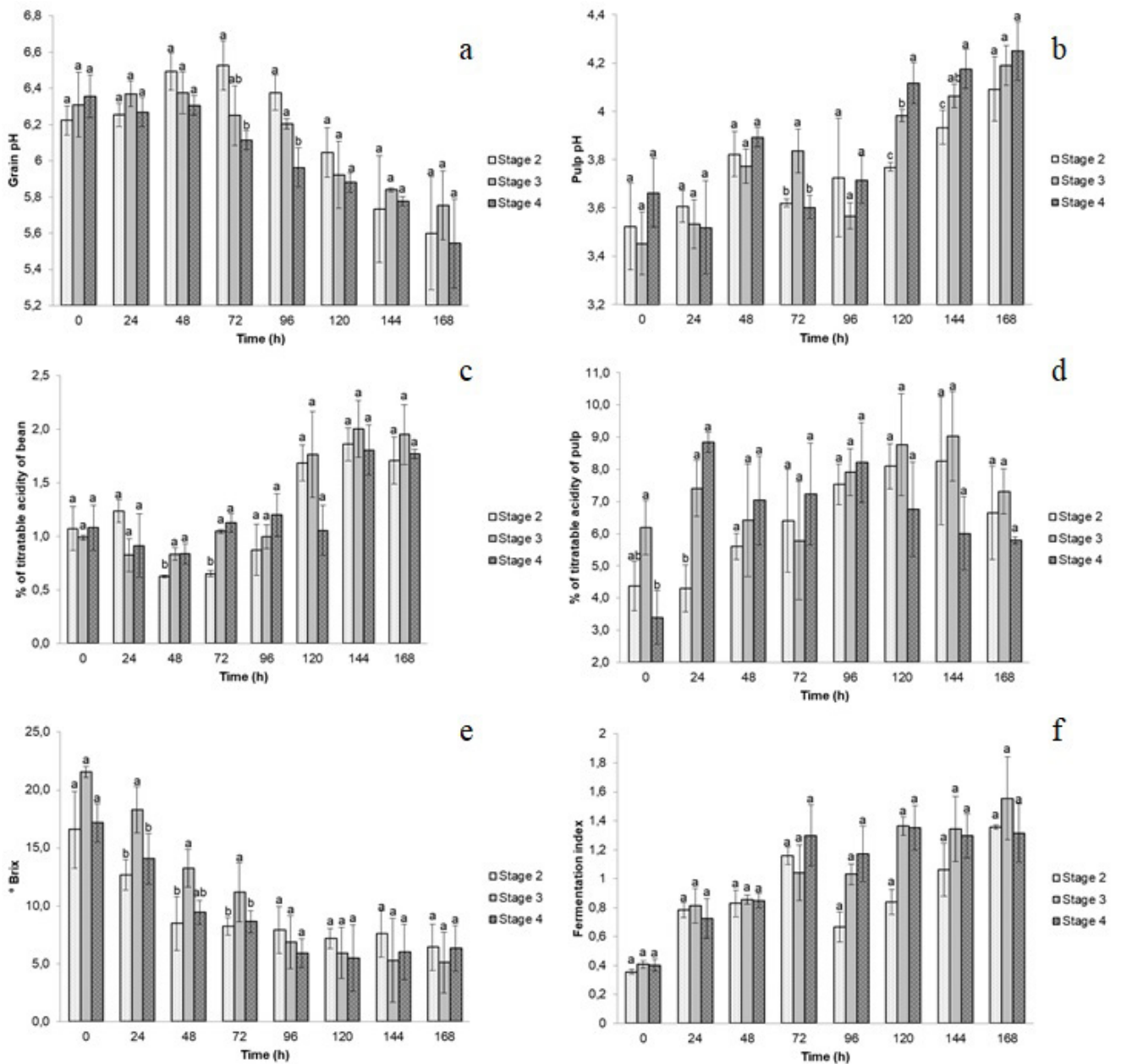


Figure 1- Dynamics of bean pH (a), pulp pH (b), % of titratable acidity of the beans (c), % of titratable acidity of the pulp (d), total soluble solids of the pulp (e), and fermentation index (f) in three maturity stages during the microfermentation of cocoa beans of clone CCN51. The bars indicate the standard deviation (n = 3). Deflection bars are not displayed if the size of the bar is smaller than the size of the symbol.

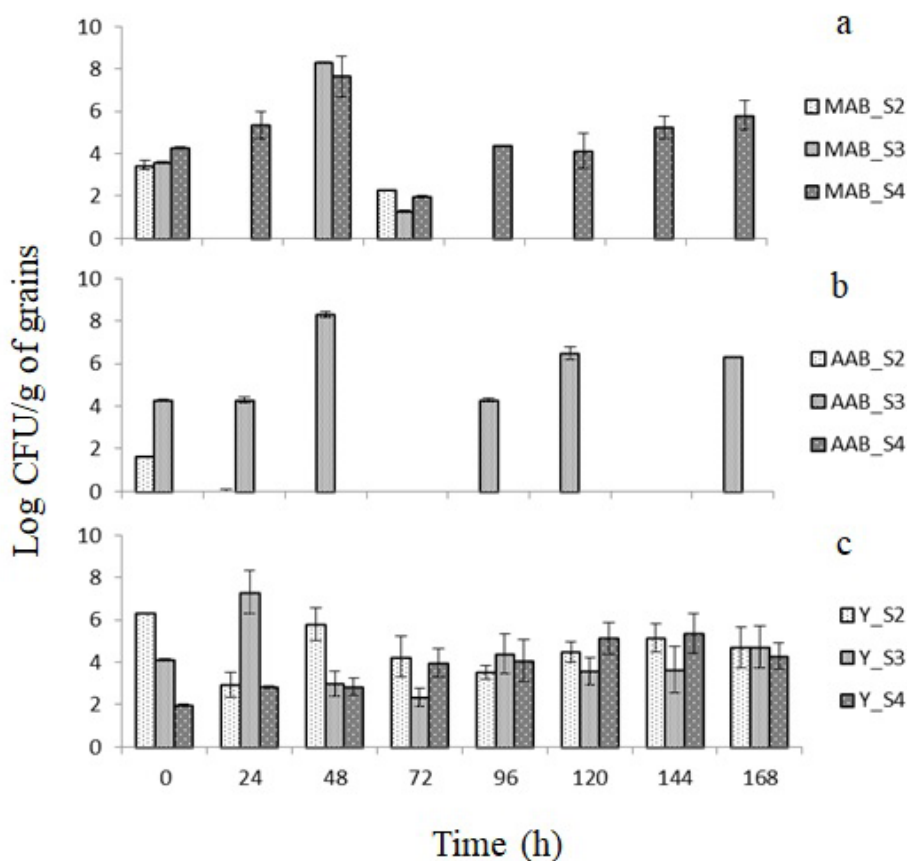


Figure 2- Microbiological dynamics of MAB (a), AAB (b) and yeasts (c) at three maturity stages during the microfermentation of the cocoa beans of clone CCN51. The bars indicate the standard deviation ($n = 3$). Deflection bars are not displayed if the size of the bar is smaller than the size of the symbol.

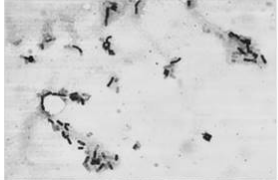
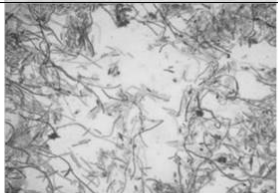
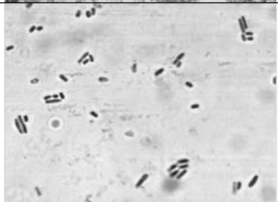
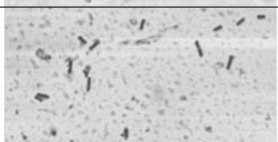
| Stage | Fermentation time | Gram | Catalase | Identification | Morphology |
|-------|-------------------|------|----------|-----------------------------|---|
| 3 | 24 | + | + | <i>Bacillus subtilis</i> |  |
| 3 | 24 | + | + | <i>Bacillus Megaterium</i> |  |
| 3 | 48 | + | + | <i>Bacillus Tequilensis</i> |  |
| 4 | 120 | + | + | <i>Bacillus subtilis</i> |  |

Figure 3- Characteristics of selected MABs and their molecular identification, Garzón, department of Huila (Colombia)

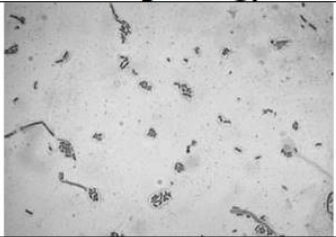

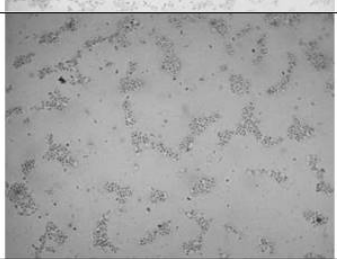
| 1* | 2 | 3 | 4 | 5 | 6 | 7 | Morphology |
|----|----|---|---|---|------------------|---------------------|---|
| 2 | 24 | - | + | - | 130.55 ±19.03 | Unidentified |  |
| 3 | 48 | - | + | - | 10.55 ±0.54 | Unidentified |  |
| 3 | 96 | - | + | - | 16.99 ±4.08 | Unidentified |  |

Figure 4- Characteristics of selected AAB and their molecular identification, Garzón, department of Huila (Colombia). * 1: Stage; 2: Fermentation time; 3: Gram; 4: Catalase; 5: Oxidase; 6: Production of acid; 7: Identification.




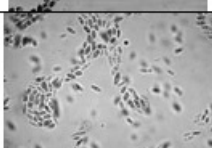
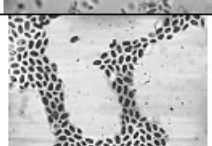
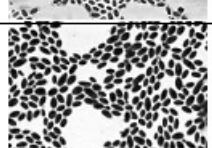


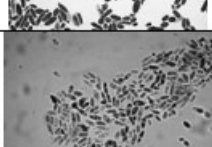
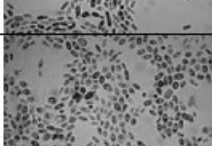
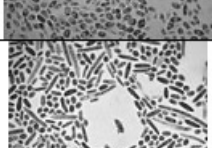
| Stage | Fermentation time | Halo size (mm) | | Morphology |
|-------|-------------------|----------------|------------|---|
| | | Polar | Equatorial | |
| 4 | 120 | 12.84 | 6.62 |  |
| 3 | 120 | 12.56 | 7.80 |  |
| 3 | 144 | 12.44 | 8.43 |  |
| 3 | 96 | 11.77 | 6.70 |  |
| 3 | 96 | 11.33 | 6.29 |  |
| 4 | 96 | 13.84 | 7.45 |  |
| 3 | 72 | 13.46 | 8.45 |  |
| 3 | 120 | 12.96 | 8.77 |  |
| 4 | 96 | 13.1 | 7.89 |  |
| 4 | 96 | 12.58 | 8.07 |  |
| 3 | 72 | 11.97 | 6.80 |  |

Figure 5. Characteristics of the selected yeast *Pichia kudriavzevii* and molecularly identified, Garzón, department of Huila (Colombia)

Conclusions

According to the results regarding pH of the beans, total soluble solids of the pulp, titratable acidity of the beans and pulp, and the fermentation index, the maturity stage three (153-174 DAA) for the cocoa material CCN51 was the most favorable for that the fermentation of the cocoa beans, responding to the theoretical criteria and obtaining a good quality cocoa.

The microbiological dynamics in the three maturity stages of the cocoa pods evidenced the absence of LAB and the presence of MAB. Maturity stage three (153-174 DAA) favored the development of AAB, observing their presence during all the periods monitored. In addition, it was possible to verify that even under controlled conditions, typical microorganisms of the cocoa fermentation are able to colonize and grow, developing their metabolic activity in favor of the fermentation of the beans.

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