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Effect of Addition of a Specific Mixture of Yeast, Lactic and Acetic Bacteria in the Fermentation Process to Improve the Quality and Flavor of Cocoa Beans in Colombia

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Abstract

Cocoa fermentation process is fundamental to generate flavors and aromas that are characteristics of chocolate. In Colombia, this process is carried out by microbiota that spontaneously colonizes cocoa beans, therefore the quality of the fermentation is inconsistent. Taking into account that the fermentation of cocoa beans is carried out by a consortium of microorganisms, the aim of this research was to describe the effect of the addition of a specific mixture of yeasts, acetic acid bacteria, and lactic acid bacteria on the physicochemical and organoleptic characteristics of cocoa beans (clone CCN 51). Isolates of two yeasts (*Wickerhamomyces anomalus* and *Debaryomyces hansenii*), three acetic acid bacteria (AAB), (*Gluconobacter japonicus, Acetobacter tropicalis,* and *Acetobacter pasteurianus*) and three lactic acid bacteria (LAB) (*Pediococcus acidilactici, Lactobacillus brevis,* and *Lactobacillus plantarum*) obtained from previous cocoa fermentations selected for their pectinases and acid production capacities in a specific mixture were used. Using the micro-fermentation technique, the effect of a biological starter was evaluated under different viable microorganisms ratios (Yeasts: LAB: AAB as follows, 1: 1: 1, 1: 2: 2, 1: 2: 1, 1: 1: 2, 2: 1: 1, 2: 2: 1, 2: 1: 2, and 2: 2: 2). The concentration of each microorganism was standardized at $1x10^7$ cfu/mL, then the biomass of 4 mL for ratio 1 and 8 mL for ratio 2 of each suspension of microorganisms was added at time zero. Different doses of inoculum were 0%, 1%, 2%, 3%, 4%, and 5% v/w mL inoculum/g cocoa beans. A beneficial effect on the sensory quality of cocoa beans was evidenced by the addition of microorganisms; the best proportion of microorganisms was 2:1:2 (yeasts:LAB:AAB) and the best inoculum dose was 3% (v/w) showing lower acidity, astringency, and bitterness, and emphasizing the cocoa flavors, fruity, nutty, and panela malt.

Keywords: cocoa yeast, lactic acid bacteria, acetic acid bacteria, organoleptic properties, CCN 51 cocoa clone, starter cultures, fermentation

INTRODUCTION

Fermented cocoa beans are the primary raw material for preparation of chocolate, a product highly consumed worldwide due to its unique taste, and a source of antioxidants (Di Mattia *et al*., 2014; Schinella *et al*., 2010; Żyżelewicz *et al*., 2018), it also has anticancer potential (Martin *et al*., 2013; Maskarinec, 2009), and cardiovascular protection (Gómez *et al*., 2011). The fermentation of cocoa beans is a crucial step in the technological transformation of cocoa into chocolate because unfermented, bitter, and astringent cocoa beans lack the pleasant taste of chocolate. The fermentation of cocoa beans is, therefore, the first stage of the chocolate-making process, which comprises the natural microbial fermentation process of the mucilaginous pulp that surrounds the *Theobroma cacao* beans and lasts ca. 5-7 days (Schwan & Wheals, 2004). This triggers the biochemical transformation within the beans, which leads to the reduction of bitterness and astringency, and the development of taste precursors like free amino acids, peptides, and sugar (Afoakwa *et al*., 2011). Specific chocolate flavors as fruity, floral, spicy, herbal, nutty, caramel, or malty, are developed during fermentation, a process carried out by a series of microorganisms that lead to different types of biochemical reactions that induce the generation of specific characteristics in cocoa (Schwan & Wheals, 2004).

Currently, there is a great need for chocolate manufacturers with properly fermented cocoa products under safe conditions that maintains their organoleptic and nutritional qualities. In this sense, various studies have been carried out on the inoculation of microorganisms to the cocoa fermentation mass (Batista *et al.*, 2016; Crafack *et al*., 2014; Lefeber *et al*., 2012; Moreira *et al*., 2017; Sandhya *et al*., 2016; Visintin *et al*., 2017), demonstrating that these influence the chocolate taste, the speed and dynamics of the fermentation, and the degraded substrates and metabolites produced. Moreover, it has been proven that they can be applied in fermentations under natural and controlled environments (Schwan, 1998).

In Colombia, serious deficiencies (non-uniform criteria for cocoa post-harvest management, unappropriated processing facilities, lack of hygiene practices for cocoa management) have been identified in the fermentation processes of the cocoa beans in all of the cocoa-producing regions (Castellanos *et al*., 2007), which hinder competitiveness in an international market (Barragan & Rey, 2004). Currently, there is an unsatisfied demand for good quality cocoa, but it is necessary to transfer new technologies to Colombian cocoa producers to reach quality levels and be able to compete with producers worldwide and enter high-value markets. This will improve their socioeconomic conditions and quality of life.

Studies have suggested the use of a microbial starter culture as the best approach to improve the fermentation process (Yao *et al*., 2017). An effective cocoa fermentation develops when there is a correct microbial succession of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Mota-Gutierrez *et al*. (2018) affirm that microbial communities have been an essential factor in the evolution of aroma compounds. Consequently, the objective of this study was to select a diverse group of microorganisms obtained from previous fermentations of cocoa beans and evaluate their effect on the physicochemical and organoleptic characteristics of cocoa beans in Colombia.

MATERIALS AND METHODS

Biological materials

A total of 39 isolates (yeasts, LAB and AAB) of microorganisms obtained previously from fermenting cocoa beans from cocoa-producing farms and belonging to the work bank of the Corporación Colombiana de Investigación Agropecuaria - AGROSAVIA (Table 1), were evaluated in the current investigation.

Pectinolytic activity screening

A total of 16 yeast isolates were evaluated for their pectinases production in two solid culture media. 1) Pectin-medium: 1% citrus pectin, 0.5% casein peptone, 0.5% yeast extract, 0.1% $CaCO_3$, 0.1% KH_2PO_4, 2% agar, and 1,000 mg/L of chloramphenicol, at pH 4.0. 2) Cocoa pulp agar (CPA) medium: 50% cocoa pulp and 2% agar. A suspension of the microorganism was prepared considering the turbidity of McFarland No. 2 tube. Then, 10 µL were placed in five points of the Petri dish containing CPA medium and pectin medium. These were incubated for six days at 30°C. Afterward, the diameter of each colony formed was measured. Then, 7 mL of the iodine-potassium iodide solution (1.0 g of iodine, 5.0 g of potassium iodide and 330 mL of distilled water) was added to each Petri dish, and after 15 min, the dye was removed, and the formation of halos was quantified (Mahmoud *et al.*, 2008).

LAB acidification capacity

A total of 16 LAB isolates were evaluated for their lactic acid production. The evaluation of the acidification capacity was carried out in a liquid medium. The initial pH of the medium was adjusted to 6.6 with sterile 0.5 N NaOH. The concentration of the inoculum was standardized to a McFarland No. 2 tube. The microorganisms were incubated under low oxygen conditions and without agitation for five days. Inoculums were plated in triplicates, including a control. After the fermentation, the cultures were centrifuged at 9.410 x g for 10 minutes, and the biomass production was established. The supernatant was used for the determination of lactic acid production. For the titration, 0.5 N NaOH was used. The culture medium used was elaborated as follows: peptone 1%, yeast extract 0.4%, glucose 2%, sodium acetate 0.5%, magnesium sulfate 0.02%, manganese sulfate 0.005%, sodium citrate 0.2%, ammonium sulfate 0.3%, dipotassium phosphate 0.2%, and 1 mL of Tween 80 per liter. The liquid medium was supplemented with 10% tomato pulp (Ardhana & Fleet, 2003), and 1% cocoa pulp (Freitas, 1998)

AAB acidification capacity

The acidification capacity of seven isolates of acetic acid bacteria was evaluated in a liquid medium. The initial pH of the media was adjusted to 6.6 with sterile 0.5 N NaOH. The concentration of the inoculum was standardized to a McFarland No. 2 tube. The microorganisms were left to ferment for 48 hours under agitation. Inoculums we plated in triplicates, including a control. After the fermentation, the cultures were centrifuged at 9.410 x g for 10 min, and the biomass production was established. The supernatant was used for the determination of acetic acid production with 0.5 N NaOH.

Table 1. Isolates used in this study obtained from various cocoa fermentations in different cocoa-producing regions of Colombia

No.	Code	Species	Origin and number of fermentation hours of the cocoa beans
6	RHALM12-1	*Pediococcus acidilactici*	Rivera Huila, 144 h
7	RHALM12-2	*Pediococcus acidilactici*	Rivera Huila, 144 h
12	SVCHALM2-3	*Pediococcus acidilactici*	San Vicente de Chucurí Santander, 24 h
13	SVCHALM2-5	*Pediococcus acidilactici*	San Vicente de Chucurí Santander, 24 h
24	SVCHALM5-6	*Lactobacillus brevis*	San Vicente de Chucurí Santander, 60 h
34	SVCHALM8-2	*Lactobacillus mesenteroides*	San Vicente de Chucurí Santander, 96 h
44	MANTALM9-1	*Pediococcus acidilactici*	San Vicente de Chucurí Santander, 108 h
46	MANTALM10-2	*Lactobacillus farraqinis*	Maceo Antioquia, 120 h
56	MANTALM13-2	*Lactobacillus plantarum*	Maceo Antioquia, 156 h
62	RHLM3-1	*Wickerhanomyces anomalus*	Rivera Huila, 36 h
63	RHLM3-2	*Wickerhanomyces anomalus*	Rivera Huila, 36 h
65	RHLM3-4	*Wickerhanomyces anomalus*	Rivera Huila, 36 h
67	RHLM13-1	*Debaryomyces hansenii*	Rivera Huila, 156 h
73	SVCHLM5-1	*Meyerozyma guillermondii*	San Vicente de Chucurí Santander, 30 h
75	SVCHLM5-2	*Debaryomyces hansenii*	San Vicente de Chucurí Santander, 60 h
78	SVCHLM5-5	*Debaryomyces hansenii*	San Vicente de Chucurí Santander, 60 h
87	MANTLM5-1	*Meyerozyma guillermondii*	Maceo Antioquia, 60 h
88	MANTLM7-2	*Meyerozyma guillermondii*	Maceo Antioquia, 84 h
90	MANTLM9-2	*Meyerozyma guillermondii*	San Vicente de Chucurí Santander, 108 h
92	MANTLM12-2	*Meyerozyma guillermondii*	Maceo Antioquia, 144 h
93	MANTLM13-1	*Meyerozyma guillermondii*	Maceo Antioquia, 156 h
94	MANTLM13-2	*Meyerozyma guillermondii*	Maceo Antioquia, 156 h
95	MANTLM14-1	*Meyerozyma guillermondii*	San Vicente de Chucurí Santander, 168 h
97	MANTLM14-3	*Meyerozyma guillermondii*	Maceo Antioquia, 168 h
99	MANTLM4-2	*Meyerozyma guillermondii*	Maceo Antioquia, 48 h
209	RHALM6-1	*Lactobacillus rhamnosus*	Algeciras Huila, 72 h
286	MANTAAM12-3	*Gluconobacter* sp.	Maceo Antioquia, 144 h
287	MANTAAM12-4	*Gluconobacter japonicus*	Maceo Antioquia, 144 h
307	AHAAM6-3	*Acetobacter tropicalis*	Algeciras Huila, 72 h
308	AHALM6-1	*Lactobacillus plantarum*	Algeciras Huila, 72 h
309	AHALM4-1	*Lactobacillus plantarum*	Algeciras Huila, 48 h
310	AHAAM6-1	*Acetobacter tropicalis*	Algeciras Huila, 72 h
311	AHAAM6-2	*Acetobacter pasteurianus/pomorum*	Algeciras Huila, 72 h
312	AHAAM6-4	*Acetobacter tropicalis*	Algeciras Huila, 72 h
313	AHAAM6-5	*Acetobacter malorum/tropicalis*	Algeciras Huila, 72 h
314	AHALM4-3	*Lactobacillus plantarum*	Algeciras Huila, 48 h
315	AHALM6-4	*Lactobacillus plantarum*	Algeciras Huila, 72 h
316	AHALM6-3	*Bacillus coagulans*	Algeciras Huila, 72 h
317	AHALM6-2	Unidentified	Algeciras Huila, 72 h

Notes: RH: Rivera (Huila), SVCH: San Vicente de Chucurí (Santander), MANT: Maceo (Antioquia), AH: Algeciras (Huila) L: Yeasts, AL: Lactic acid bacteria, AA: Acetic acid bacteria, M: Isolation moment (M1: 12 h after fermentation, M14: 168 h after fermentation).

Effect on physicochemical and organoleptic

Isolates

Different ratios and doses of eight selected isolates were evaluated on the physicochemical and organoleptic characteristics of cocoa beans of the clone CCN 51. The isolates employed were two yeasts (*Wickerhanomyces anomalus* isolate RHLM3-4, and *Debaryomyces hansenii* isolate RHM13-1), three LAB (*Pediococcus acidilactici* isolate MANTALM9-1, *Lactobacillus brevis* isolate SVCHALM5-6, and *Lactobacillus plantarum* isolate AHALM4-3), and three AAB (*Gluconobacter japonicus* isolate MANTAAM12-4, *Acetobacter tropicalis* isolate AHAAM6-1, and *Acetobacter pasteurianus* isolate AHAAM6-2).

Inoculum production

Each inoculum was seeded in a specific solid medium to produce the biomass of the microorganisms. Yeasts were cultured in a malt extract (5%) and agar (2%) media with a pH of 5.0. For LAB, a modified MRS medium was used with tomato pulp (10%) (Ardhana & Fleet, 2003), cocoa pulp (1%) (Freitas, 1998), and milk powder (2%), with a pH of 5.6. The AAB were sown in a medium composed of yeast extract (1%), peptone (0.4%), glucose (6%), calcium carbonate (1%), ethanol (0.5%), and agar (2%), with a pH of 5.7 (Ohmori, 1982; Hanmoungjai *et al*., 2007). All the media were left to incubate for 96 h at 30°C

Different ratios of microorganism

The effect of eight different microorganism ratios (1:1:1, 1:1:2, 1:2:2, 1:2:1, 2:1:1, 2:1:2, 2:2:1, and 2:2:2, yeast: LAB: AAB) were evaluated on the quality of the cocoa beans using the micro-fermentation technique. The isolates were cultured in solid media, and after 96 hours of fermentation, the biomass of the microorganisms was taken from the surface of the solid medium and resuspended in saline solution at 0.85%, where the concentration of each microorganism was standardized at $1x10^7$ cfu/mL by reading in the spectrophotometer at 600 nm for yeast and 540 for bacteria. Then, 4 mL for ratio 1 and 8 mL for ratio 2 of each suspension of microorganisms were obtained, and cells were separated by centrifugation at 9.410 x g and 4°C for 20 min (Heal Force, Neofuge 23 R). The supernatant was removed, and the biomass obtained was re-suspended in 9 mL of 0.85% saline solution and added to the experimental units. The mixtures of microorganisms were added at time zero, and the fermentation was monitored for 168 h. At this point, the natural drying process (i.e., solar exposure of the fermented beans) began and lasted approximately five days until obtaining an internal bean humidity of 7%. Seven experimental units per treatment were established. Each experimental unit consisted of 900 g of cocoa beans.

Doses of a microbes mixture

A starter culture (yeast: LAB: AAB) was added in different doses or treatments (0%, 1%, 2%, 3%, 4%, and 5% v/w) for the fermentation of the cocoa mass. The inoculum was prepared as above. Amounts of 4, 8, 12, 16 and 20 mL of a microbial suspension of each microorganism were taken, respectively, and the cells were separated by centrifugation at 9.410 x g and 4°C for 20 min (Heal Force, Neofuge 23 R); the biomass obtained was re-suspended in 4.5 mL of 0.85% saline solution and was added to the experimental units. In the control treatment (0%), no microorganisms were added, so this treatment was only comprised of 4.5 mL of 0.85% saline solution. The mixtures of microorganisms were added at time zero, and the fermentation was monitored for 168 h. Each experimental unit consisted of 400 g of cocoa beans. At this point, the natural drying process (solar exposure of the fermented beans) began and lasted approximately five days until obtaining an internal bean humidity of 7% as in the previous trial. Seven repetitions per treatment were established. Every 24 h, a replicate was extracted by treatment, and the physicochemical variables were measured. At the end of the fermentation (168 h), the sensory test was performed.

Micro-fermentation of cocoa beans

The cocoa pods of clone CCN 51 were harvested at their optimum maturity point and superficially sterilized by submersion in a solution of 1% sodium hypochlorite for three minutes, rinsed three times, and then sprayed with 70% alcohol. Finally, these were

rinsed again with sterile distilled water. Pods were opened in a laminar flow chamber, and the beans were separated from the placenta. Once the mass of beans was homogenous, for each experimental unit, 900 g and 400 g of cocoa beans were placed in plastic boxes with a capacity of 1000 and 500 mL respectively, and the amount of established inoculum was added. For the collection of the leachate, a box of 250 g was used integrated into a closed system to establish the anaerobic conditions that the micro-fermentation requires the first 48 h, and placing it in an oven at 35 °C. Subsequently, after 96 h, the temperature of the oven was raised to 45 °C, and the dough was homogenized every 24 h until completing 168 h.

Physicochemical and organoleptic characteristics

The measurement of pH, titratable acidity, fermentation index, cut test, and sensory analysis was carried out according to the following procedures.

Titratable acidity and pH: An amount of 2 g of beans were macerated with 20 mL of distilled water and filtered. Then, pH was measured with a potentiometer (Hanna® instruments), and titratable acidity was determined in 10 mL of the filtrate with NaOH (0.1 N).

Cutting test: Dry cocoa beans were cut longitudinally, observed in broad daylight and categorized into one of the following groups: complete fermentation (brown beans), partial fermentation (partially brown, partially violet beans), low fermentation (violet beans), and not fermented (flat and violet beans). The percentage of well-fermented beans was obtained according to:

$$\% \text{ WFB} = (\text{WFB}/\text{TBF}) \times 100$$

WFB = well fermented beans
TBE = total beans evaluated

Fermentation index: a sample of 0.5 g of cocoa beans representative of the cutting test was dried, macerated, and dissolved in 50 mL of a methanol: HCl solution (97:3), which was homogenized and left to rest at 8°C for 19 h. The fermentation index was measured by spectrophotometry at absorbance ratios of 460 nm and 530 nm (Nazaruddin *et al*., 2006)

Sensory analysis: Samples of 250 g of cocoa beans were roasted at 120°C for 35 min; when the process was finished, the shells were removed to obtain the cocoa nibs, which were grounded in a KitchenAid® food processor (St. Joseph, Michigan, U.S.A.) to obtain homogeneous samples of approximately 10 ìm in particle size. The liquor obtained was subjected to a sensory analysis test carried out by trained panelists. The panelist evaluated nine attributes that included basic (cocoa, acidity, astringency, and bitterness), and specific tastes (fruity, floral, green, nutty, and panela malt), as well as others (defects or acquired), which used a quantitative descriptive analysis with a scale of 0 to 10 points (0: absent, 1-2: low intensity, 3-5: medium intensity, 6-8: high intensity, and 9-10: very high intensity).

The liquors were melted in a water bath, preventing them from exceeding 40°C. The sample was homogenized. The monitoring of the variables previously described was carried out in 168 h of the fermentation. Five panelists evaluated two replicas of each sample. All samples were coded

The best mixture of microorganisms in the field

The 2:1:2 mixture of microorganisms (yeast: LAB: AAB) was evaluated in the field, for which *Wickerhamomyces anomalus*, *Debaryomyces hansenii*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Gluconobacter japonicus*, *Acetobacter tropicalis*, and *Acetobacter pasteurianus* were cultured on solid medium. After 96 h, the biomass was collected and standardized, as in

the section above. A volume of 120 mL of inoculum was added per experimental unit. Each experimental unit comprised of 12 kilos of cocoa beans placed in wooden boxes, and three replicates were established per treatment. The control treatment was established with cocoa beans without inoculation. A completely randomized design was used. The monitoring of the variables pH, titratable acidity, temperature, and fermentation were carried out every 24 h after the inoculation until 168 h. The sensory analysis was carried out in 6-days-old samples after the inoculation. The sensory panel consisted of five panelists; each sample was coded in triplicate.

RESULTS AND DISCUSSION

Pectinolytic activity in yeasts

Sixteen yeast isolates were evaluated for their production of pectinases in CPA and pectin media. All the isolates showed growth in these two media, demonstrating the production of pectinases (Table 2). *Wickerhanomyces anomalus* (isolate RHLM3-4) and *Debaryomyces hansenii* (isolates SVCHLM5-2 and RHLM13-1) showed high growth in CPA medium, while two isolates of the species *Meyerozyma guillermondii* (MANTLM14-1 and MANTLM12-2) showed the highest growths in pectin medium.

In our study, isolates RHLM3-4 (*W. anomalus*) and SVCHLM5-2 (*D. hansenii*) also showed high growth in the cocoa pulp agar medium, which indicates that these microorganisms are adapted to the exceptionally high acidity conditions of the cocoa pulp (i.e., pH 3.5) (Romero & Zambrano, 2012) with high concentrations of sugar (i.e., 10-15%) (Nielsen *et al.*, 2007). Further, these microorganisms are endowed with enzymes that act on the cocoa pulp, including polygalacturonase (PG), pectinesterase (PE), and lyases (pectin lyase (PL) and pectate lyase (PAL)) (Martos *et al*., 2013). *W. anomalus*, for example, has been reported in the formation of aromatic compounds in cocoa beans during the fermentation process carried out in Ivory Coast in Africa (Koné *et al*., 2016). Moreover, it has been previously isolated from the cocoa beans fermentation process in Brazil (Papalexandratou & De Vuyst, 2011).

Table 2. Screening of the pectinolytic activity of yeasts. Evaluation of yeasts isolates in CPA and pectin media

Code*		Species	CPA medium (mm)	Pectin medium (mm)
65	RHLM3-4	*Wickerhanomyces anomalus*	27.32±3.0 a	20.76±0.5 bc
75	SVCHLM5-2	*Debaryomyces hansenii*	20.62±1.5 ab	22.75±0.7 abc
67	RHLM13-1	*Debaryomyces hansenii*	20.28±1.1 abc	22.63±1.7 abc
97	MANTLM14-3	*Meyerozyma guillermondii*	16.97±0.6 abc	34.21±2.2 ab
99	MANTLM4-2	*Meyerozyma guillermondii*	16.87±0.2 abc	25.58±1.3 abc
73	SVCHLM5-1	*Meyerozyma guillermondii*	16.74±0.8 abc	22.17±1.2 abc
93	MANTLM13-1	*Meyerozyma guillermondii*	16.72±0.5 abc	36.80±2.9 ab
87	MANTLM5-1	*Meyerozyma guillermondii*	16.68±0.4 abc	28.51±0.8 ab
92	MANTLM12-2	*Meyerozyma guillermondii*	16.53±0.9 abc	38.06±3.4 a
63	RHLM3-2	*Wickerhanomyces anomalus*	16.53±0.4 abc	20.48±1.1 bc
78	SVCHLM5-5	*Debaryomyces hansenii*	16.42±0.9 abc	23.94±0.8 abc
94	MANTLM13-2	*Meyerozyma guillermondii*	16.26±0.2 abc	25.13±0.6 abc
88	MANTLM7-2	*Meyerozyma guillermondii*	16.24±0.4 abc	25.98±0.8 abc
62	RHLM3-1	*Wickerhanomyces anomalus*	15.59±0.9 abc	1.07±1.1 c
90	MANTLM9-2	*Meyerozyma guillermondii*	15.02±0.3 bc	24.95±1.1 abc
95	MANTLM14-1	*Meyerozyma guillermondii*	13.85±1.9 c	41.63±6.5 a

Notes: * Isolates obtained from cocoa fermentations in three regions of Colombia. Treatments with the same letter do not differ statistically according to Tukey's multiple range Honestly Significant Difference test (HSD 0.5%). Diameter in mm. Average values of 5 colonies ± Standard error.

Nonetheless, halos of the pectinolytic activity were not detected in any of the strains studied; however, all the isolates grow in pectin and CPA media, demonstrating the use of a carbon resource. Further, the high concentration of sugars in the CPA medium may have repressed the secretion of pectinolytic enzymes. This is like what was found by Schwan & Rose (1994), who reported a decrease in the pectinolytic activity of the strain *Kluyveromyces marxianus* isolated from the cocoa fermentation when the glucose concentration was higher than 100 g/L. Da Silva *et al*. (2005) did not detect pectinolytic activity by *D. hansenii* (FT20) in the pectin medium. Nevertheless, they found secretion of pectinases in media containing polygalac-turonic acid and glucose and/or galactose. Martos *et al*. (2013) reported that the yeast *W. anomalus* does not synthesize pectinases in pectin medium; the synthesis only occurs when pectin or galacturonic acid is used with glucose.

Lactic acid production in LAB

There were statistical differences in lactic acid production, where LAB as *Lactobacillus plantarum* (isolate MANTALM13-2, 56) and *Pediococcus acidilactici* (isolate MANTALM9-1, 44) recorded higher lactic acid production above 2% in liquid medium (Figure 1). Lactic acid bacteria (LAB) carry out a crucial role in the cocoa fermentation process since they metabolize sugars (glucose and fructose) and citric acid from the pulp to produce mainly lactic and acetic acids (Ho *et al*., 2015). These acids are essential because they penetrate the beans and activate the formation of aroma precursors (Janek *et al*., 2016). In this study, *L. plantarum, P. acidilactici*, and *L. brevis* were selected for inoculation. *Lactobacillus plantarum* produced 22.20 g/L of lactic acid, which is higher compared to the values reported by Peev *et al*. (2017). This species is homolactic and acid-tolerant (Lefeber *et al*., 2010), and therefore, it is well adapted to the cocoa ecosystem capable of growing at a pH of 3.5, and at high concentrations of ethanol (12%) and acetic acid (5%) (Visintin *et al*., 2016). *Pediococcus acidilactici* has antimicrobial activities against *Listeria monocytogenes, Bacillus cereus, Bacillus subtilis,* and *Escherichia coli* (Cizeikiene *et al*., 2013). Likewise, *Lactobacillus brevis* has been reported to suppress the growth of *Penicillium claviforme, Aspergillus awamori, Aspergillus niger,* and *Gibberella* (Romanens *et al*., 2018; Tropcheva *et al*., 2014), and to control *E. coli* and *Salmonella* spp. (Syukur *et al*., 2013). Moreover, just like *P. acidilactici* and *L. plantarum*, and thanks to its production of fatty acids and tolerance to pH changes, it is used industrially. Besides, studies have shown that *L. plantarum* attenuates immune-mediated colitis, and it can be a therapeutic agent for intestine diseases (Schultz *et al*., 2002).

Acetic acid production in AAB

The acetic acid bacteria (AAB) isolates did not show statistical differences in acetic acid production, registering values between 0.29 and 0.33% (Figure 2). The lower acetic acid production allows AAB to complement with LAB since the acid excess leads to an unpleasant flavor (Cardona, 2016; Figueroa *et al*., 2019); besides, lactic acid inhibits the degradation caused by acetic acid in the epicatechin and catechin (Eyamo *et al*., 2016). We included AAB because they play an essential role in cocoa fermentation as they contribute to the death of the seed embryo by oxidizing alcohol to acetic acid and liberate endogenous enzymes implied in ûavor precursors that are crucial to obtain high-quality cocoa (Teyssier & Hamdouche, 2015). The isolates used in this study, i.e., *Gluconobacter japonicus, Acetobacter*

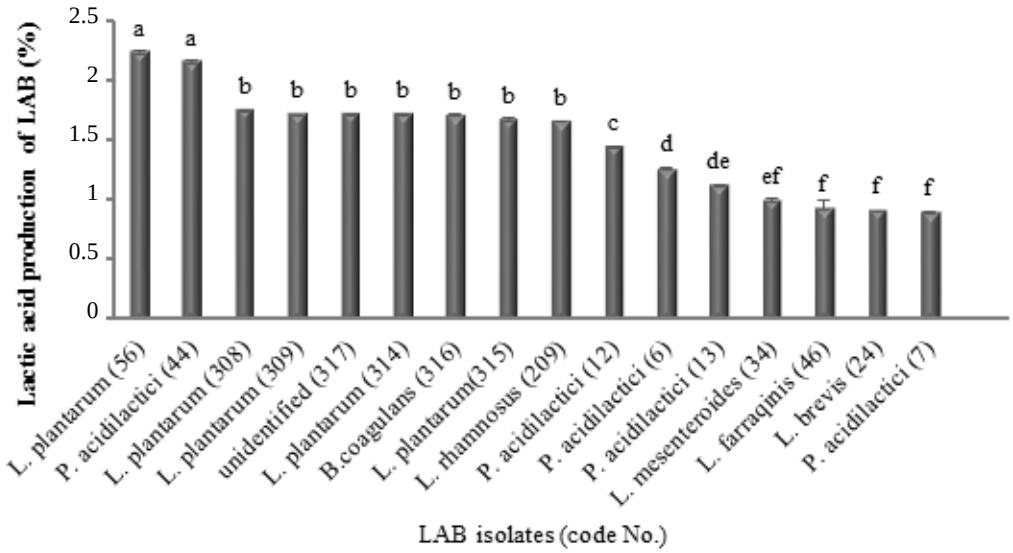

Figure 1. Evaluation of the lactic acid production by lactic acid bacteria (LAB) obtained from the fermentation of cocoa beans in Colombia. Treatments with the same letter do not show statistical differences. Tukey's HSD 0.5 protected by Fisher. Each value is the average of 3 replications. (Mean ± SE)

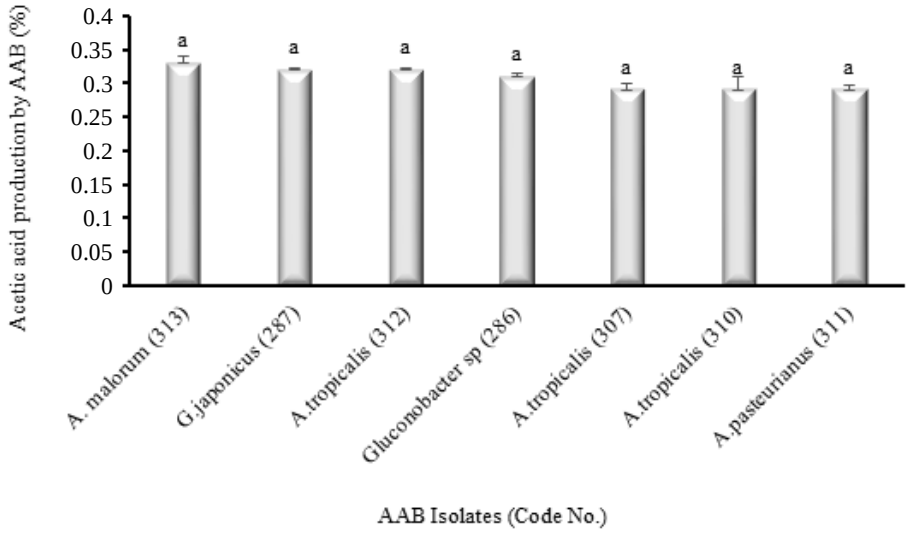

Figure 2. Evaluation of acetic acid production by acetic acid bacteria (AAB) obtained from the fermentation of cocoa beans in three regions of Colombia. Treatments with the same letter do not show statistical differences. Tukey's HSD 0.5 protected by Fisher. Each value is the average of 3 replications. (Mean ± SE)

tropicalis, and *Acetobacter pasteurianus*, tend to appear around the 48th hour of the fermentation and convert ethanol into acetic acid, and lactic acid into acetoin (Figueroa *et al*., 2019). This process increases temperature that, in turn, induces cellular hydrolysis, and activation of the endogen proteases, essential in cocoa flavor formation (Janeck *et al*., 2016; Vuyst & Weck, 2016). It must be noted that these species are used worldwide in the production of foods and beverages, and are currently used in the food and pharmaceutical industries (Raspor & Goranoviè, 2008; Sengun & Karabiyikli, 2011),

Different ratios yeasts, LAB and AAB and the physicochemical and sensory characteristics

The analysis of physicochemical variables of the cocoa beans treated with different mixtures of microorganisms (*Wickerhamomyces anomalus*, *Debaryomyces hansenii*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Gluconobacter japonicus*, *Acetobacter tropicalis*, and *Acetobacter pasteurianus*), indicated that the addition of these microorganisms improves the percentage of well-fermented seeds. Furthermore, the viable ratios 2:2:1 and 2:2:2 stood out with more than 90% of well-fermented beans on day 5 of the fermentation. Likewise, the fermentation index was higher in treatments with the addition of microorganisms, exceeding the value of 1 on the third day of fermentation. Similarly, the acidity of the almond is reached more quickly with the addition of microorganisms (Table 3). However, the sensory analysis indicated that two mixtures (2:1:2, 2:2:1) showed basic (cocoa) and specific flavors such as nutty and fruity, with low values of astringency and bitterness compared to the control (Figure 3).

It is worth mentioning that the increase in acetic acid concentration and the concomitant decrease in pH of the beans (from 6.3-7.0 to 4.0-5.5) destroys the internal membranes of the grain cotyledon compartments, in particular, reserve material and pigment cells during the fermentation process, causing the endogenous substrates and the enzymes to migrate and mix. Thus, important flavor precursors are produced, such as reducing sugars, hydrophilic peptides, and hydrophobic amino acids, as well as the bleaching of the purple color and/or the occurrence of the brown coloration of the cocoa beans.

Table 3. Physicochemical variables of cocoa beans obtained from cocoa fermentations three, five and seven days after inoculation with different ratios of microorganisms (treatments)

Treatment (yeasts: LAB:AAB)	Three days			Five days			Seven days		
	F %	FI	pH	F %	FI	pH	F %	FI	pH
Control	36.00	0.85	6.36	68.18	1.5	6.28	91.30	1.2	5.5
1:1:1	30.00	1.24	5.64	77.27	1.9	4.59	87.50	1.9	5.6
1:2:1	54.55	1.38	5.18	68.18	1.9	4.91	86.36	1.4	5.2
1:1:2	54.17	1.43	6.04	81.82	1.8	4.60	90.91	1.6	4.54
1:2:2	45.83	0.99	4.91	83.33	1.6	5.43	95.45	1.6	4.63
2:1:1	40.91	1.29	4.71	81.82	1.7	4.68	92.86	1.5	5.05
2:1:2	36.36	0.94	5.92	72.73	1.5	5.26	91.67	2	5.07
2:2:1	54.55	1.29	5.75	90.91	1.7	5.39	100.00	1.5	5.56

Notes: F = Fermentation
FI= Fermentation index

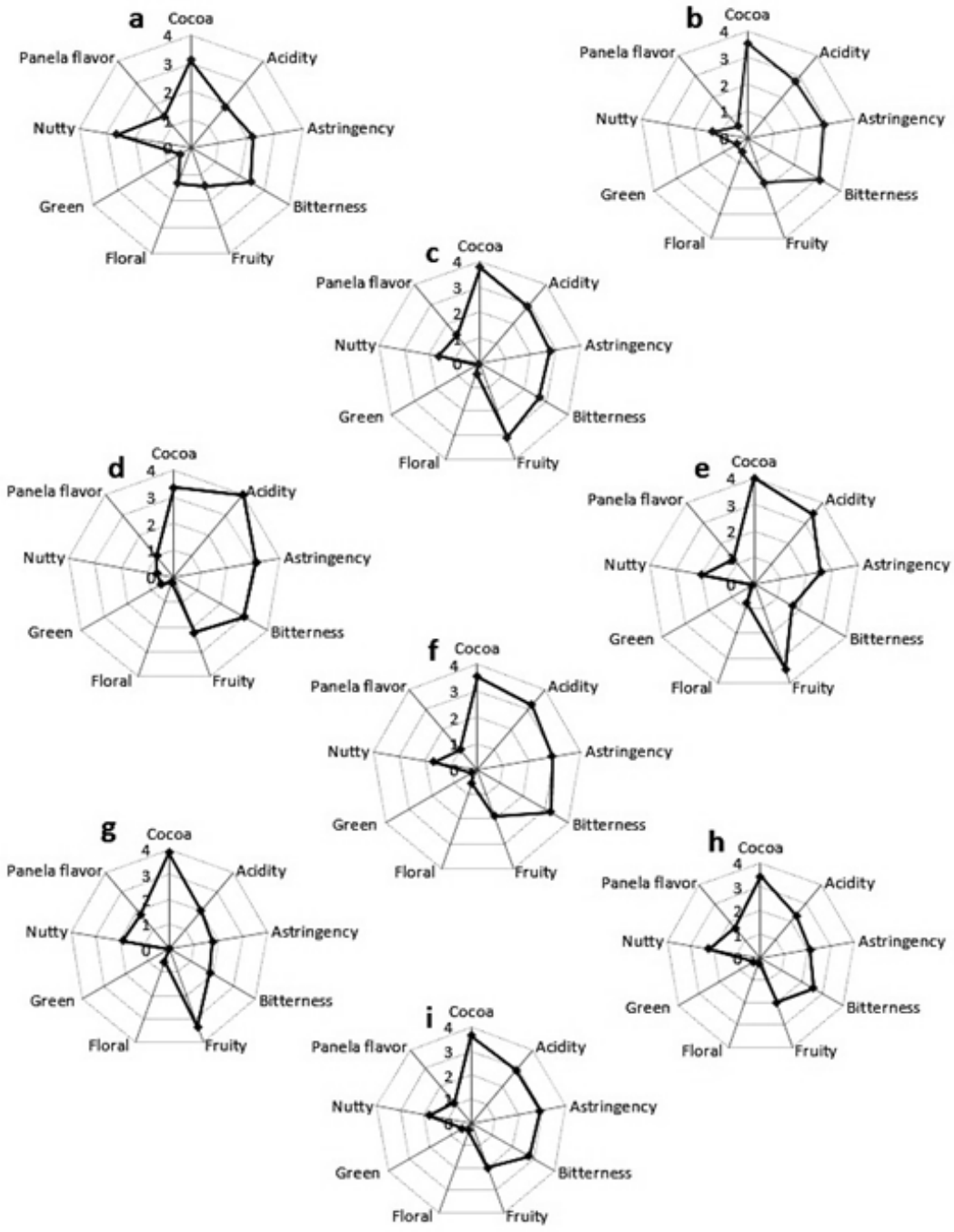

Figure 3. Profile of flavors obtained by fermented cocoa beans with addition of microorganisms (yeasts: LAB: AAB), as follows: a) control (no addition of microorganisms), b) 1:1:1, c) 1:2:1, d) 1:1:2, e) 1:2:2., f) 2:1:1, g) 2:1:2, h) 2:2:1, and i) 2:2:2. Each value is the average of the assessment of five panelists and each sample was coded in duplicate

Doses of microbe mixture on the physicochemical and organoleptic characteristics

The pH values showed statistical differences with the use of different doses of the mixed inoculum. All the pH values of the beans treated with the doses of the microbial inoculum were lower than the control (0%). Also, with the results obtained from the percentage of total acidity of the beans, the action of the microorganisms was observed to affect this variable compared to the control (0%), i.e., the increase in acidity during the fermentation was faster in the treatments with the addition of microorganisms.

Titratable acidity on day 1 of the fermentation process showed no statistically significant differences ($p < 0.05$), while on days 3, 5, and 7, differences were registered. On day 3, the 2% treatment was statistically different from the control and the 5% treatment. On day 7, the control treatment showed the lowest value compared to treatments with the addition of microorganisms. Moreover, for the fermentation index on days 1 and 3, no statistically significant differences were observed ($p < 0.05$), contrary to days 5 and 7, where statistical differences were recorded. The percentage of well-fermented beans shows that there is a statistically significant difference ($p > 0.05$) between the doses of the mixed inoculum on 24, 72, and 120 h of the fermentation processes (Table 4).

This proportions and doses of a specific mixture of microorganisms influenced the pH of the cocoa bean mass during the fermentation process, and this is considered important, since strong acidification of the cotyledon (pH 4.0-4.5) causes non-specific proteolysis of all proteins present in the cotyledon of the grain, producing cocoa with low flavor potential. Meanwhile, moderate acidification (pH 5.0-5.5) causes specific proteolysis of the storage proteins that produce cocoa with a higher flavor potential (Crafack *et al*., 2014).

The results of the sensory analysis of the liquors prepared from fermented beans inoculated with different doses of microorganisms showed on day 1 of the fermentation process basic tastes as astringency and bitterness, and specific ones as green. On day 3, all liqueurs began to have subtle cocoa flavors, although acidity, astringency, bitterness, and green tastes were still present. Finally, on day 7, the control (0%) and the dose of 2, 3, and 5% obtained very good ratings by the panelists, highlighting the tastes of cocoa, fruity, nutty, and panela malt. However, the dose of 3%, in addition to sharing these characteristics, obtained low acidity, astringency and bitterness values, aspects that are important when cataloging cocoa quality (Figure 4).

When the different doses of the mixed inoculum were evaluated, the percentage of well-fermented seeds showed that there is a statistically significant difference ($p \leq 0.05$) between the doses of the mixed inoculum on days 1, 2, and 5 of the fermentation. The analysis shows an evolution of the percentage

Table 4. Evolution of the physicochemical variables during cocoa beans fermentation by adding different doses of mixed microbiological inoculum

Treatment	3 days			5 days			7 days		
	F, %	F I	pH	F, %	F I	pH	F, %	F I	pH
0 %	26.8±0.0 c	0.90±0.19 a	6.2±0.1 a	63.3±0.9 ab	1.08±0.01 c	5.6±0.1 a	79.0±7.0 b	1.97±0.03 a	5.7±0.3 a
1 %	42.0±2.0 ab	0.76±0.08 a	5.6±0.3 b	72.7±5.8 a	1.32±0.08 bc	5.1±0.1 a	95.3±1.2 a	1.86±0.05 ab	5.1±0.3 b
2 %	37.9±4.6 bc	0.69±0.04 a	5.7±0.1 ab	66.0±2.4 ab	1.58±0.02 b	5.1±0.1 a	93.3±2.3 a	1.53±0.11 bc	5.0±0.1 b
3 %	50.0±2.0 ab	0.68±0.01 a	5.9±0.1 ab	65.0±1.0 ab	2.00±0.10 a	5.5±0.2 a	94.7±4.2 a	1.69±0.14 ab	5.0±0.3 b
4 %	51.3±11.0 ab	0.77±0.01 a	5.7±0.3 b	51.0±8.2 c	1.44±0.05 bc	5.4±0.3 a	84.7±6.4 ab	1.70±0.27 ab	5.0±0.2 b
5 %	54.0±3.5 a	0.91±0.14 a	5.9±0.1 ab	54.3±6.3 bc	1.34±0.32 bc	5.3±0.2 a	85.7±2.4 ab	1.38±0.07 c	5.4±0.2 a

Notes: Means with the same letter are not significantly different ($p > 0.05$). Average values of three replicates ± Standard error.
F = Fermentation; FI = Fermentation index

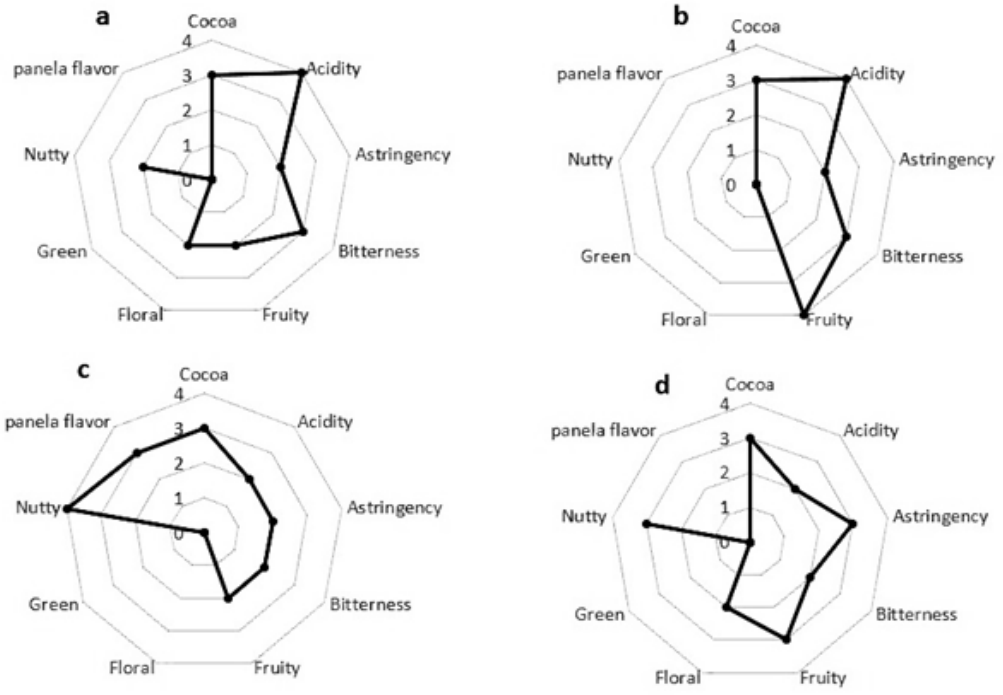

Figure 4. Sensory profiles found during cocoa bean fermentation by the effect of adding different doses of mixed microbiological inoculum. Dose on day 7. a) control (no addition of microorganisms), b) 2%, c) 3%, and d) 5%. Basic (cocoa, acidity, astringency and bitterness) and specific tastes (fruity, floral, green, nutty, and panela malt). Each value is the average of the assessment of five panelists and each sample was coded in duplicate

of well-fermented seeds as the fermentation days pass. Alterations in the color of the cocoa beans predict the flavor potential and the final day of the fermentation (Camu *et al*., 2008). The Colombian technical norm NTC 1252 "cocoa bean" specifies that 65 well-fermented beans per 100 beans sampled are the minimum value to belong to the "Prize" category. According to the above, the beans coming from the doses of 1%, 2%, and 3% would reach this category on day 5 of the fermentation; this may be a response to the diffusion of lactic and acetic acids into the seeds (Schwan & Wheals, 2004).

The evaluation of the mixture of microorganisms in the field indicated an effect of this mixture in the dynamics of the grain acidity, as well as an increase in the temperature that is reflected in the number of well-fermented beans. This indicates that the addition of microorganisms improves the fermentation of the cocoa beans, managing to reduce the astringency and the bitterness of the cocoa beans after six days of fermentation compared to the control.

Best mixture of microorganisms in field

The evaluation of the fermentation in the field showed that the treatment with microorganisms (2: 1: 2 Yeasts: LAB: AAB) exhibited a rapid increase in acidity, from 72 hours after fermentation and with statistically different values compared to the control; likewise, the stabilization of the pH of the beans was observed earlier in the treatment with microorganisms at 120 hours (Figure 5). Furthermore, the temperature increased much faster in this same treatment and was higher than the control between 24 and 120

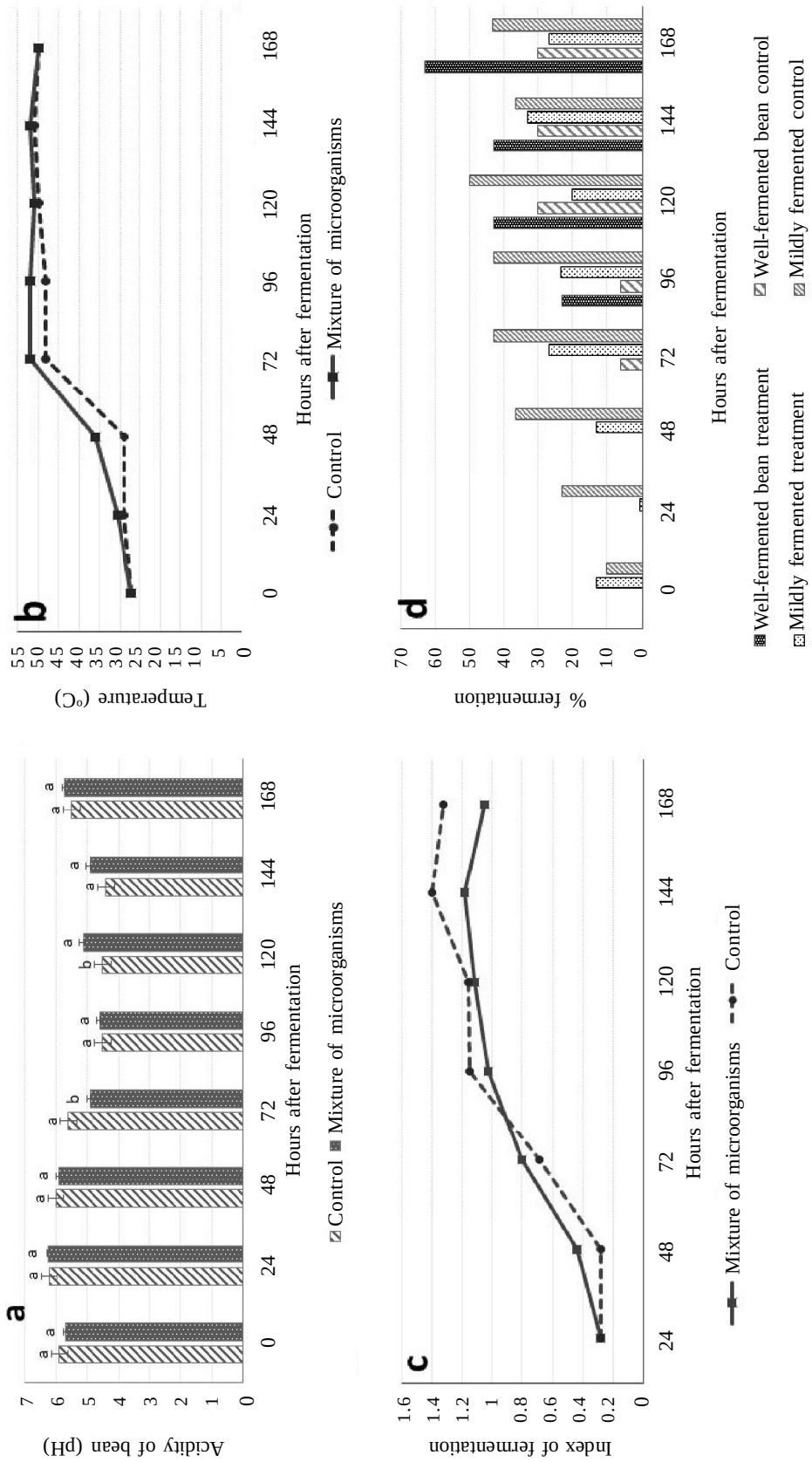

Figure 5. Follow-up variables of the cocoa fermentation in the field, including; a) Acidity, b) Temperature, c) Fermentation index, and d) Cutting test (percentage of fermented beans). Each value is the average of 3 replications

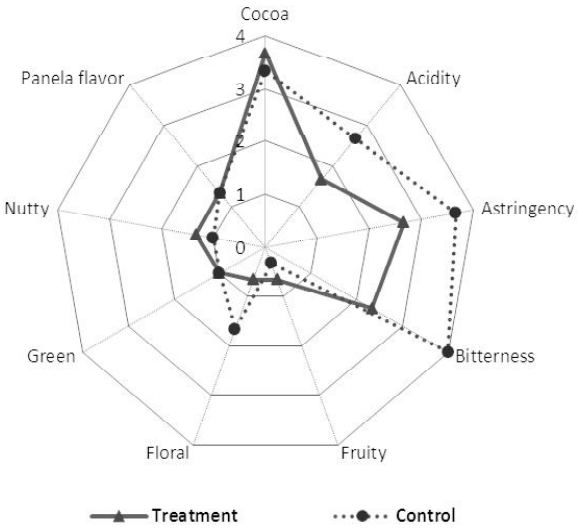

Figure 6. Flavor profile of fermented cocoa beans six days after fermentation in the field. Treatment: mixed microbiological inoculum (2: 1: 2 Yeasts: LAB: AAB). Control: without inoculum. Basic (cocoa, acidity, astringency, and bitterness) and specific (fruity, floral, green, nutty and panela malt) tastes. Each value is the average of the assessment of five panelists and each sample was coded in triplicate

hours, reaching 52 °C at 72 hours of fermentation (Figure 5). The fermentation index increased gradually in the two treatments; however, the control recorded higher values after 96 hours. On the other hand, the cutting test indicated that well-fermented beans were registered in the treatment with microorganisms at 96 hours of fermentation, reaching 63.3% of well-fermented cocoa beans at 168 hours compared to the control treatment that only registered 30% of well-fermented beans; furthermore, the treatment achieved 90% of seeds with desirable characteristics while the control only reached 73.3% of these type of beans (Figure 5). The sensory analysis was carried out at day 6 of the fermentation in the field, indicating that the treatment with microorganisms decreased the acidity, astringency, and bitterness of cocoa beans (Figure 6). Fermentation is the most important stage in the formation of the aroma and flavor of cocoa beans (Afoakwa *et al*., 2008; Lima *et al*., 2011). The use of starter cultures is a biotechnological alternative that allows controlling efficiently and standardizing the cocoa fermentation process and improves the quality of the product (Ozturk & Young, 2017; Soumahoro *et al*., 2015). The evaluation of the mixture of microorganisms in the field indicated that the addition of microorganisms improves the fermentation of the cocoa beans after six days of fermentation compared to the control.

CONCLUSIONS

Some proportions and mixture of microorganisms improved the physicochemical and organoleptic characteristics of the cocoa beans, highlighting special flavors such as nutty and fruity. After the analysis of the physicochemical and organoleptic variables, we can conclude that although on day 5, most physicochemical variables indicated that the fermentation could be stopped in some doses of inoculum used in this study, the sensory analysis showed that a complete fermentation of 6 to 7 days is desirable.

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