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Inoculum of a Native Microbial Starter Cocktail to Optimize Fine-Aroma Cocoa (*Theobroma cacao*) Bean Fermentation

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Abstract: Fine-aroma cocoa (*Theobroma cacao*) is one of Ecuador's most iconic export products and comprises 63% of world production. Nevertheless, few advances have been made to improve fermentation processes that might benefit the development of chocolate's organoleptic characteristics. The study of starter cultures, which seek to improve organoleptic properties or decrease fermentation time, has been investigated in other countries. The aim of this study was to analyze the effect of a native microbial cocktail based on two yeasts (*Torulaspora delbrueckii* and *Hanseniaspora uvarum*), a lactic acid bacterium (LAB) (*Limosilactobacillus plantarum*), and an acetic acid bacterium (AAB) (*Acetobacter ghanensis*) inoculated at the beginning of the fermentative process while tracking physical and biochemical variables, microbial population dynamics, and bean fermentation time. The starter culture caused changes in sugar and acid content and increased polyphenols, which in turn generated temperature and pH changes in the dough. The dynamics of yeast, AAB, and mesophilic microorganisms remain higher than the controls throughout the process. A decrease in filamentous fungi that affect the flavor and quality of beans was observed due to the production of acetic acid or secondary metabolites from yeasts and LAB, and resulted in 24% greater fermentation than spontaneous fermentation in only 96 h.



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Keywords: cocoa bean fermentation; yeast; lactic acid bacteria; acetic acid bacteria; initiator microbial consortium

1. Introduction

Cocoa (*Theobroma cacao*) is one of the main traditional Ecuadorian export products. According to the National Institute of Statistics and Censuses (INEC), the cocoa sector (dedicated to cocoa) contributes approximately 5% of the economically active population (EAP) and 15% of the rural EAP, which makes it a mainstay in the economy of those who live in the Ecuadorian coast, the foothills of the Andes mountains, and the Ecuadorian Amazon. Ecuador is the world's third largest cocoa bean producer, contributing 7% of the total world production and is the world leader in the production of the variety of fine-aroma cocoa with a participation of 63%. The European Union and the United States are the main destinations for Ecuadorian cocoa [1]. To effectively serve these markets, it is essential that the harvest and post-harvest (fermentation and drying) be carried out in the best and most efficient way, generating a quality final product that can be marketed at a better price.

Two of the most important steps to developing the desired chocolate flavors in a cocoa bean are fermentation and drying. In these processes, the harvested beans can immediately undergo spontaneous fermentation using microbial action on the mucilaginous pulp to produce ethanol and acids and release heat. The fermentation of the cocoa bean generally

takes 5 to 10 days and is initiated by microorganisms native to the area inhabiting the surfaces of the pods and the soil [2,3].

In the first phase, several species of yeast appear and disappear, giving way to *Hanseniaspora guilliermondii*, which dominates for the first 24 h. Then, *Saccharomyces cerevis* and *Pichia membranaefaciens* dominate until the end of fermentation [4]. The yeasts transform the simple sugars of the mucilage into ethanol, degrading the pectin and lowering the acid. Acetic acid and alcohol cause the bean embryo to die on the second day, while the yeast consortium consumes oxygen, creating an anaerobic environment that favors the development of lactic acid bacteria. The yeast activity is inhibited by the alcohol concentration, which increases the pH level [3,5].

In the second phase, lactic acid bacteria (LAB) ferment residual carbohydrates. As conditions become more favorable, lactic acid bacteria dominate [6]. The bacteria of the *Lactobacillus* type (*L. collonides*, *Lb. fermentum*, *L. mali*, and *L. plantarum*) have mainly been isolated in stacked bean fermentations. Lactic acid bacteria convert a wide range of sugars and some organic acids (such as citric and malic acids) to lactic acid, acetic acid, ethanol, and carbon dioxide depending on the type of *Lactobacillus* [4,7]. Lactic acid bacteria contain “pectinolytic” enzymes, allowing them to hydrolyze pectins, which causes a decrease in the viscosity of the mucilage pulp or facilitates the entry of air. It is likely that acetic bacteria will flourish in this less acidic and aerobic environment due to the consumption of citric acid [7].

In the third phase of cocoa fermentation, acetic acid bacteria (AAB) transform ethanol produced by yeasts into acetic acid, which diffuses into the bean along with the high temperatures [4,5]. The most important acetic bacteria isolated from cocoa fermentation are *Gluconobacter oxydans*, *Acetobacter aceti*, *Acetobacter pasteurianus*, and at the end of fermentation *A. tropicalis* [3,5,7].

In the fourth and final phase, the *Bacillus* genus is detected. *Bacillus* sp. interact with each other and triggers many physical changes and chemical reactions that promote promising biochemical characteristics in beans [3]. Turning the stacked beans favors the presence of *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis* [6]. High temperatures favor the development of the *Bacillus* genus, known for producing numerous enzymes that could contribute to flavor with the production of organic acids and flavorings such as 2,3-butanediol [8,9]. An added benefit to this process is that filamentous fungi which generate mycotoxins (such as ochratoxin and citrinin) and can decrease the quality of the cocoa bean are lessened [2,3]. The extent of biochemical conversions depends on the rate, duration, and intensity of pH decrease, temperature increase, and sweat release. Flavor precursors are produced through the reduction of sugars, hydrophilic peptides, and hydrophobic amino acids, while cocoa beans undergo purplish whitening and/or brown coloration [8].

In cocoa bean fermentation, the bacterial species *Limosilactobacillus fermentum* (previously *Lactobacillus fermentum*) [10] and *Acetobacter pasteurianus* can be considered good candidates to make starter cultures; *Lb. fermentum* is especially useful owing to its heterofermentative metabolism, fructose growth capacity, citrate conversion, mannitol production, and acid, heat, and ethanol tolerance, characteristics that are especially desirable for starter strains in cocoa fermentation. *Bacillus* species have shown a positive effect on cocoa fermentation [4].

Quality standards for cocoa in Ecuador, including Servicio Ecuatoriano de Normalización (INEN), European, and American standards, must be met for cocoa exports to meet the demand for sustainable, uniform, and consistent supply, which can be achieved through standardized fermentation of beans [11]. To improve fermentation quality, starter cultures of yeasts, LAB, and AAB should be used [12], with careful selection of the appropriate LAB strain to avoid overproduction of lactic acid [4]. Thus, to reduce fermentation time and improve flavor and aroma, it is important to study the behavior of starter cultures made from autochthonous species of the area by analyzing their specific growth rate and target metabolites.

The aim of this study was to examine the use of a native microbial cocktail as a starter culture for fermenting Nacional variety cocoa beans. The study investigated physical and biochemical variables, microbial population dynamics of yeasts, LAB, AAB, filamentous fungi, and mesophilic microorganisms, polyphenol content, and fermentation time.

2. Materials and Methods

2.1. Inoculum Preparation

In a previous study, microbiological, biochemical, and molecular analyses identified the microbiota diversity from fine cocoa bean varieties Criollo (named Nacional) and Trinitario CCN-51 during commercial fermentation [13]. Pure isolates of *Torulaspora delbrueckii*, *Hanseniaspora uvarum* (yeasts), *Limosilactobacillus plantarum* (lactic acid bacterium—LAB), and *Acetobacter ghanensis* (acetic acid bacterium—AAB) were collected from the fermentation of cocoa beans from the COFINA company. These microorganisms were cultured in different liquid media, without agar. Yeasts were grown in NYD medium, composed of nutrient broth (8 g/L), yeast extract (5 g/L), and dextrose (10 g/L), adjusted to pH 6.6. LAB was grown in MRS medium, composed of bacteriological peptone (10 g/L), dextrose (20 g/L), dipotassium phosphate (2 g/L), magnesium sulfate (0.2 g/L), manganese sulfate (0.05 g/L), beef extract (8 g/L), sodium acetate (5 g/L), yeast extract (4 g/L), ammonium citrate (2 g/L), and 1 mL/L of tween-80, adjusted to pH 6.6. AAB was grown in YGC medium, composed of yeast extract (5 g/L), dextrose (20 g/L), and chloramphenicol (0.1 g/L), adjusted to pH 6.6. All media were sterilized by autoclaving at 121 °C and 15 psi for 20 min.

Strains were grown in 10 mL of liquid media at 28 °C with shaking at 100 rpm. After 12 h, these precultures were used to seed 900 mL into a laminar airflow cabinet. The mixture was incubated at 28 °C for 72 h with agitation at 100 rpm. Microbial enumeration was performed by using the serial dilutions and plating method [14,15]. The final concentrations were 1×10^8 CFU/mL.

2.2. Geographical Location of the Study

The study was conducted at the production and fermentation facilities of COFINA/República del Cacao, which is located in the province of Los Ríos, Vinces canton, Antonio Sotomayor parish, at an altitude of 17 m.a.s.l., 1°36'00" S; 79°42'29" W. This area has a humid tropical climate, annual rainfall of 1000 to 2000 mm, temperatures ranging from 24 to 30 °C, relative humidity of 98%, and is classified as tropical humid megathermal [16].

2.3. Inoculation and Beans Sampling during Fermentation

At the COFINA/República del Cacao facilities, Nacional cocoa beans were fermented in jute sacks (0.95 m long $\times$ 0.60 m wide) placed on wooden pallets, both for the treatment inoculated with *T. delbrueckii*, *H. uvarum* (yeasts), *L. plantarum* (LAB), and *A. ghanensis* (AAB) as well as for the spontaneous fermentation treatment. The inoculum of each species was poured directly (Figure 1A) and then manually homogenized. Every 24 h, the temperature of the fermenting mass was registered and 150 g samples were collected from hour zero (beginning of the fermentation process) to hour 96 (end of the fermentation process). A plastic tube notch, 2 inches in diameter and 1 m long, was utilized as an agricultural auger to extract random samples of fermenting bean biomass from jute sacks (Figure 1A), with two replicates per treatment. Each sample was transferred to a plastic bag, coded, and stored at −20 °C for chemical and biochemical analysis.

2.4. Temperature, pH, and Water Content

The fermentation temperature of the cocoa mass was monitored using an IFC 400 digital sensor (MadgeTech, Inc., Warner, NH, USA). The chemical parameters of pH were taken according to the methodologies described by [8], for which 15 g samples of cocoa beans were taken and 135 mL of distilled water was added, which was then vortexed for 20 s and the supernatant of the sample was measured. The water content (percent of humidity) was

determined based on international standards (drying at 103 °C for 16 h) for the inoculated and non-inoculated bean samples.

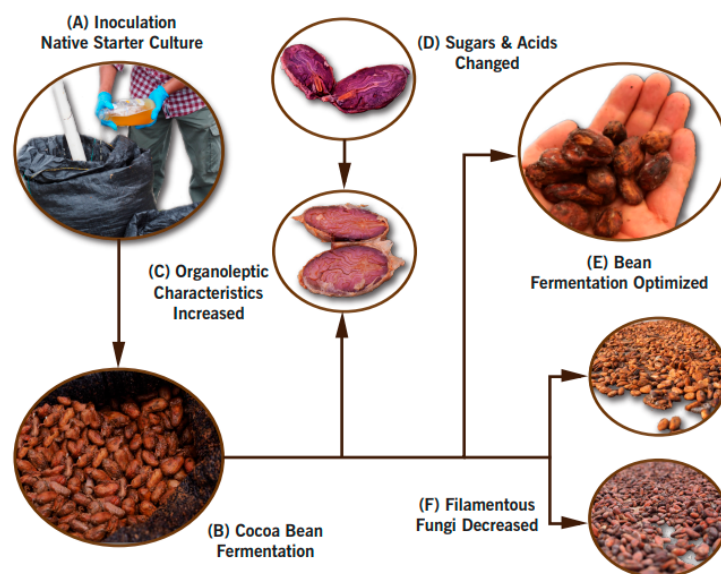


Figure 1. Research flow of a native microbial cocktail as a starter culture in fine-aroma cocoa (*Theobroma cacao*) bean fermentation. (A) Inoculation of two yeasts (*Torulaspora delbrueckii* and *Hanseniaspora uvarum*), an LAB (*Limosilactobacillus plantarum*), and an AAB (*Acetobacter ghanensis*). (B) The inoculum boosts yeast and AAB populations more than the control with spontaneous microbiota. The composition of sugars and organic acids, as well as temperature and pH levels, undergo significant changes during the fermentation process. (C) As a result, the sensory properties of cocoa beans are enhanced. In practice, good fermentation is indicated by brown beans with cracks, brittle cotyledons, and easy separation from the shell. (D) The starter culture increased polyphenol content, and both sugars and organic acids underwent some changes. (E) Bean fermentation efficiency improved by 24% compared to spontaneous fermentation in only 96 h. (F) Fungi that affect bean quality decreased due to acetic acid or secondary metabolites from yeasts and LAB.

2.5. Quantification of Population Dynamics

The population dynamics of microorganisms present during fermentation was determined by mixing 20 g of beans from the initial 150 g cocoa sample with 180 mL of sterile distilled water in sterile plastic bags (Ziploc). Each sample was homogenized at 8000 rpm for 30 s in a compact homogenizer and then diluted using 9 mL of autoclaved buffer (NaCl 0.05%) according to the methodology of [13,15]. Dilutions 3 and 4 were plated in triplicate for each media. Microbial enumeration for filamentous fungi and yeasts was performed on malt extract agar medium composed of maltose (12.75 g/L), dextrin (2.75 g/L), glycerol (2.35 g/L), peptone (0.78 g/L), and agar (15 g/L), adjusted to pH 4.7; and rose Bengal agar with chloramphenicol composed of bacteriological agar (15 g/L), bacteriological peptone (5 g/L), chloramphenicol (0.1 g/L), dextrose (10 g/L), magnesium sulfate (0.5 g/L), potassium phosphate (1 g/L), and rose Bengal (0.05 g/L), adjusted to pH 7.0. LAB enumeration was performed on MRS plus agar; AAB was performed on YGC plus agar; and for mesophilic microorganisms on NYD plus agar [17]. All growth media were incubated at 28 °C for 1 to 4 days. Yeasts, filamentous fungi, AAB, and mesophilic microorganism agar were incubated under aerobic conditions, while LAB plates were incubated under anaerobic conditions. After incubation, a colony count was performed.

2.6. Biochemical Characteristics

Samples of 150 g of cocoa beans of the Nacional variety were stored at −80 °C, lyophilized, and later homogenized for analysis by high-performance liquid chromatography (HPLC) (Agilent Technology 1260, Waldbronn, Baden-Württemberg state, Karlsruhe

district, Germany). Changes in the concentration of sugars (sucrose, fructose, and galactose) and organic acids (citric, acetic, lactic, malic, and oxalic acids) were detected by using the methodologies described by [8,9], with modifications.

2.6.1. Sugar Concentration

Bean pulp samples (500 mg) were mixed with MilliQ water (50 mL) and sonicated for 20 min. Then, 100 mL of the solution (adjusted to pH 13.5 using 0.3 M NaOH) was mixed with 0.5 M methanol (100 μ L) and incubated for 2 h at 70 °C. The pH was adjusted using 0.3 M HCl, and the resulting solution was evaporated to dryness and mixed with chloroform, followed by vigorous shaking. The chloroform layers were removed and the aqueous residues were eluted in an Eclipse Plus C18 HPLC device, inner diameter 4.6 mm, 250 mm length, 5 μ m, with a mobile phase of 0.1 M phosphate buffer and acetonitrile in a ratio of 83:17 (*v/v*,%) at a flow rate of 1 mL/min, 20 μ L injection volume, 35 °C, and detected by the UV equipment/VIS Detector at a wavelength of 245 nm [18].

2.6.2. Organic Acids Concentration

Organic acid extracts were prepared with 5 g of each lyophilized cocoa sample, dissolved in 60 mL of MilliQ water, and shaken for 10 min. The homogeneous mixture was centrifuged at 5000 rpm for 30 min at 5 °C, and the pellet was washed twice with 20 mL of MilliQ water. The supernatants were centrifuged again for 15 min and filtered through a 0.45 μ m Millex Millipore membrane (Saint Louis, MO, USA). The organic acid extracts were removed with an Eclipse Plus C18, inner diameter 4.6 mm, 250 mm length, 5 μ m HPLC device with a mobile phase of 50 mM KH₂PO₄ (monopotassium phosphate) (pH = 2.8 with phosphoric acid) at a flow rate of 0.7 mL/min, and an injection volume of 10 μ L at 20 °C, and detected by the 1260 Infinity II Multiple Wavelength Detector (Agilent 1260 II Array Diode Detector—G7117C) at 210 nm. The concentration of organic acids was determined by comparison with standard curves, constructed from stock solutions of commercial acids [18], and expressed in milligrams per gram of cocoa dry matter (mg/g DM).

2.7. Total Polyphenol Content

The total content of polyphenols (TPC) during fermentation was determined by spectrophotometry using the methodology described by [11], with modifications. The 100 μ L methanolic extract (from 2 g of the initial cocoa sample dissolved in 40 mL of methanol) was mixed with 50 μ L of Folin–Ciocalteu reagent, 750 μ L of distilled water, and 100 μ L of a Na₂CO₃ solution and reaction vessels were stored in the dark at room temperature. The absorbance was measured after one hour at a wavelength of 760 nm, based on a calibration curve of gallic acid as a reference. The TPC was expressed in milligrams of gallic acid per gram of cocoa dry matter (GA mg/g DM) [19].

2.8. Fermentation Percentage

One hundred cocoa beans were sampled from different areas of the bag, both from the inoculated treatment and from the spontaneous fermentation, and were cut in half and quickly evaluated to avoid oxidative processes. Beans considered well-fermented had well-defined internal grooves and had a brown color (light or dark), without considering partially fermented, slaty, with white spots, moldy, over-fermented, or unfermented beans, according to the Ecuadorian Technical Regulations for cocoa beans NTE INEN 176 for Arriba Superior cocoa (CBAS) [20,21].

2.9. Data Analysis, Experimental Design, and Statistical Analysis

Data was collected and analyzed by the HPLC equipment software. The concentration of organic acids and sugars was determined by comparing them with standard curves constructed from standard solutions. Data were expressed in milligrams per gram of cocoa dry matter (mg/g DM).

Two factors were evaluated: (i) type of inoculation (inoculated with *T. delbrueckii*, *H. uvarum*; LAB: *L. plantarum*; AAB: *A. ghanensis*; and spontaneous fermentation) and (ii) fermentation time (0, 24, 48, 72, 96 h). The experiment used jute bags with 60 kg of cocoa beans, with two replicates for both inoculated and spontaneous fermentation (as control). It followed a completely randomized design (DCA) arranged in a divided plot (2×5) with four repetitions. The mathematical model is as follows:

$$Y_{ijk} = \mu + P_i + S_k(i) + T_j + [(PT)]_{ij} + e_{ijk}$$

where

Y_{ijk} = Random variable.

μ = Overall mean.

P_i = Effect of the i th type of inoculant.

$S_k(i)$ = Error for the type of inoculant.

T_j = Effect of the j -th hour of fermentation.

$[(PT)]_{ij}$ = Effect of the interaction Type of inoculant $\times$ Fermentation time.

e_{ijk} = Error for fermentation time.

The variables of temperature, pH of the fermented dough, population dynamics, and total content of polyphenols, sugars, and organic acids were characterized by descriptive statistics (mean and standard deviation). To compare the variables between treatments, an analysis of variance was performed, and means were compared by using the LSD Fisher test at 5% for treatments, times, and interactions. All analyses were performed using the INFOSTAT software for statistical analysis for Windows <https://www.infostat.com.ar/?lang=en> (accessed on 10 April 2023).

3. Results

3.1. Variation of Temperature and pH during Fermentation

The spontaneous fermentation and the treatment with a microbial cocktail starter showed an increase in temperature from hour 0. The spontaneous fermentation increased from 27.7 to 44.5 °C, and the starter culture increased from 24.7 °C to 45.4 °C, after 96 h of fermentation. Regarding the pH, it increased over time from 3.70 to 4.80 in the spontaneous fermentation and from 3.60 to 4.70 in the inoculated treatment (Figure 2).

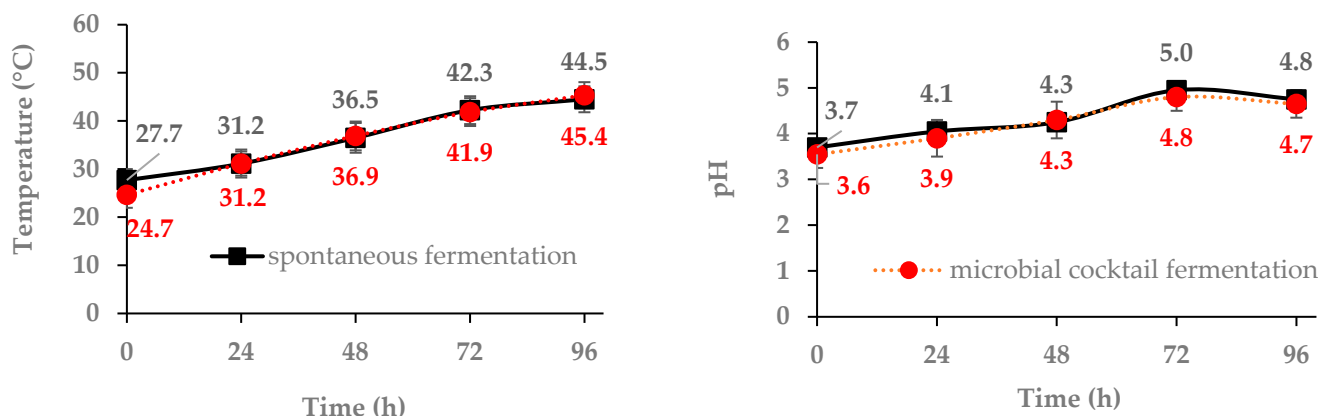


Figure 2. Changes of temperature and pH during fermentation of Nacional variety cocoa beans. Black lines show spontaneous fermentation (as control) and red lines microbial cocktail as a starter culture. Values represent the means of eight replicates and vertical bars indicate the standard deviation of the means.

3.2. Quantification of Population Dynamics during Fermentation

Both spontaneous fermentation and treatment with a starter culture had similar yeast populations at the beginning of the process, as shown in Figure 3. In the spontaneous fermentation (control) a decrease was observed from 0 h (7.9×10^7 CFU/mL) until 96 h

(4.6×10^4 CFU/mL); meanwhile, in the inoculated treatment, this decrease was moderated, with a reduction in the population from 7.9×10^7 CFU/mL to 5.8×10^6 CFU/mL being observed. It could possibly be because the cocktail was composed of two yeasts.

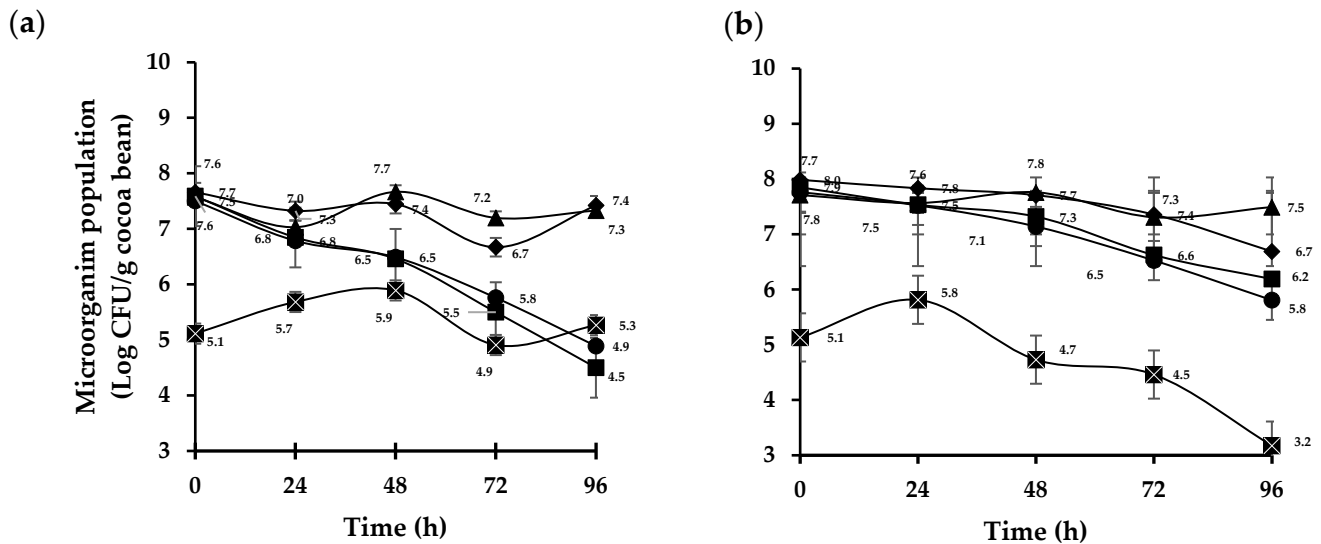


Figure 3. Changes in the microbial population of yeasts (●), lactic acid bacteria (◆), acetic acid bacteria (■), mesophilic microorganisms (▲), and filamentous fungi (⊠) during the bean fermentation of Nacional-variety cocoa by spontaneous fermentation (control) (a) and a microbial cocktail fermentation with a starter culture (b). Values represent the means of eight replicates and vertical bars indicate the standard deviation of the means.

In the treatment with the microbial cocktail, a greater LAB population was observed from 0 to 72 h (7.9×10^7 CFU/mL to 7.8×10^7 CFU/mL), which decreased to 4.9×10^6 CFU/mL at 96 h. Meanwhile, the opposite occurred in the spontaneous fermentation, with a decrease in the population from 0 to 72 h (7.8×10^7 CFU/mL to 6.87×10^6 CFU/mL), followed by an increase at 96 h to 7.6×10^7 CFU/mL (Figure 3).

In the case of AAB, both the control with spontaneous fermentation and the treatment with starter culture showed a decrease in their populations from 0 to 96 h, from 7.9×10^7 CFU/mL to 6.1×10^5 CFU/mL and from 7.1×10^7 CFU/mL to 1.5×10^6 CFU/mL, respectively (Figure 3).

Regarding filamentous fungi, the population increased from 5.7×10^5 CFU/mL to 5.8×10^5 CFU/mL within 48 h, decreased to 4.6×10^4 CFU/mL at 72 h, and increased again to 5.8×10^5 CFU/mL at 96 h in the spontaneous fermentation. However, in the inoculated treatment, the populations increased from 5.3×10^5 CFU/mL to 5.9×10^5 CFU/mL within 24 h and then decreased to 3.4×10^3 CFU/mL at 96 h (Figure 3).

During the fermentation process, both the control and inoculated treatments of mesophilic microorganisms presented populations ranging from 7.9×10^7 CFU/mL to 7.5×10^7 CFU/mL (Figure 3).

3.3. Variation in Concentrations of Sugars, Organic Acids, and Polyphenols

The analysis of the sugars and ethanol during the cocoa-shell-mucilage bean fermentation is shown in Figure 4. There was no significant difference in the interaction between treatment and hour for glucose content. However, in the spontaneous fermentation and the treatment with the microbial cocktail a higher glucose consumption was observed during the fermentation process. The glucose content in the control, starting with a concentration of 4.71 mg/g DM reached complete consumption at 48 h, while the treatment with the microbial cocktail began with 5.33 mg/g DM and decreased to zero after 72 h (Figure 4).

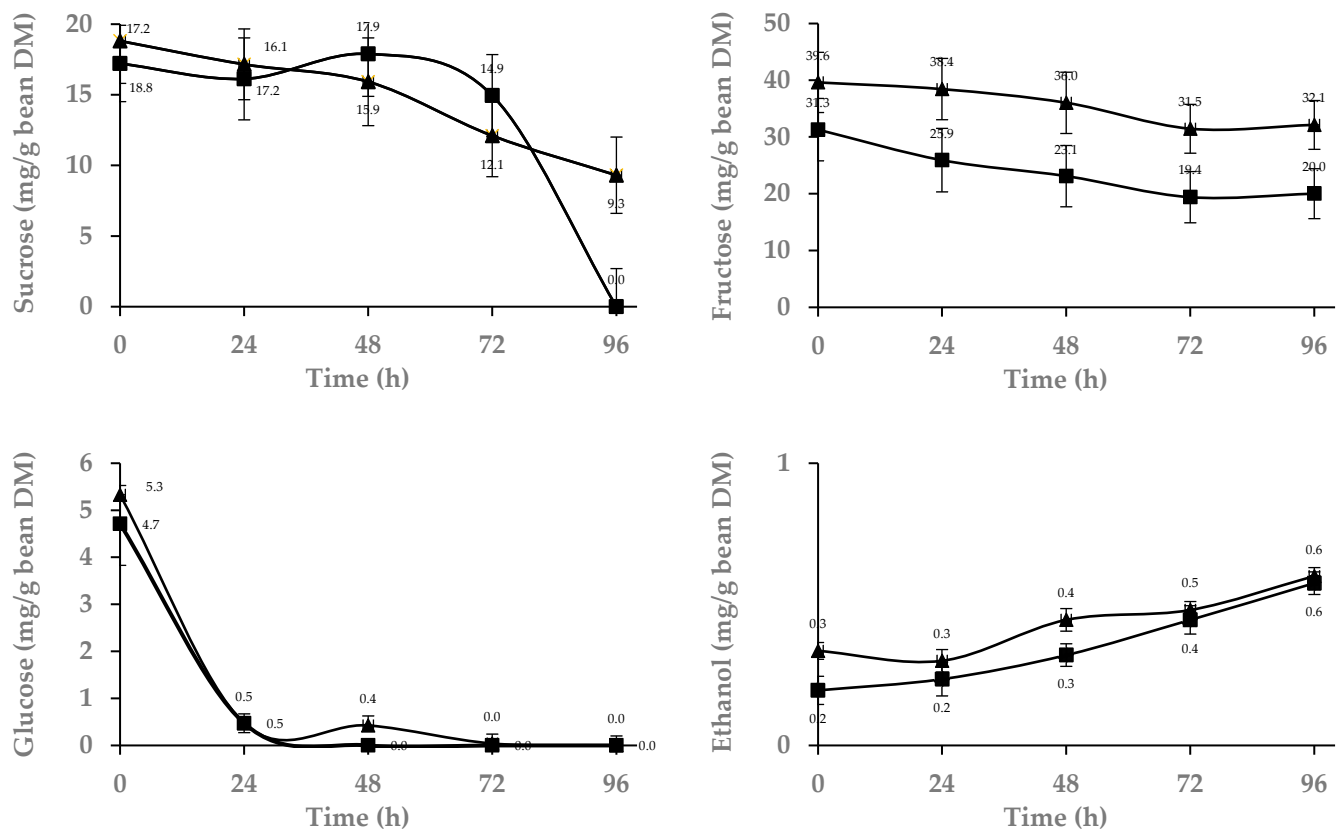


Figure 4. Changes in the sugars and ethanol content (mg/g bean DM) during the bean fermentation of the cocoa variety Nacional. Lines represent spontaneous fermentation (■) and the microbial cocktail as starter culture (▲). Values represent the means of eight replicates and vertical bars indicate the standard deviation of the means.

The fructose content for the control and the treatment with the starter culture decreased from 31.3 to 19.4 mg/g DM and from 39.6 to 31.5 mg/g DM, respectively, within 72 h; from that moment, in both cases, a slight increase was observed up to 20.0 mg/g DM in spontaneous fermentation and 32.1 mg/g DM in the treatment (Figure 4).

There was no significant difference in the interaction between treatment and hour for ethanol content, but significant effects were separately found for each. An increase in values was observed in both the control and inoculated treatments, from 0.2 and 0.3 mg/g DM and to 0.575 and 0.599 mg/g DM, respectively, after 96 h (Figure 4).

There were no significant interactions between treatment and hour for the lactic acid content. Spontaneous fermentation had the highest content at 72 and 96 h (15.3 and 15.4 mg/g DM, respectively), while the treatment with starter culture achieved the highest values of 12.93 and 12.53 mg/g DM at 48 and 96 h, respectively (Figure 5).

There were also no significant interactions between treatment and hour for the content of acetic acid, but it was observed that both the treatment and the control showed two peaks with greater production of this acid at 48 and 96 h, with values of 1.23 and 5.81 mg/g DM for the control and 2.35 and 3.30 mg/g DM for the inoculated treatment (Figure 5).

There were significant interactions between treatment and hour for citric, oxalic, and malic acids. Citric acid showed a low concentration in the inoculated treatment (11.72 mg/g DM), but a high concentration (26.67 mg/g DM) in the control, at 96 h. The oxalic acid content presented a higher concentration in the inoculated treatment at 96 h (11.71 mg/g DM) compared to the control, which, despite having its highest value at 72 h (14.02 mg/g DM), subsequently decreased at 96 h (7.67 mg/g DM). For malic acid, the inoculated treatment obtained a concentration of 4.62 mg/g DM at 96 h, while for the

control, as with oxalic acid, its highest concentration was found at 72 h (3.51 mg/g DM), which then decreased to 2.62 mg/g DM at 96 h (Figure 5).

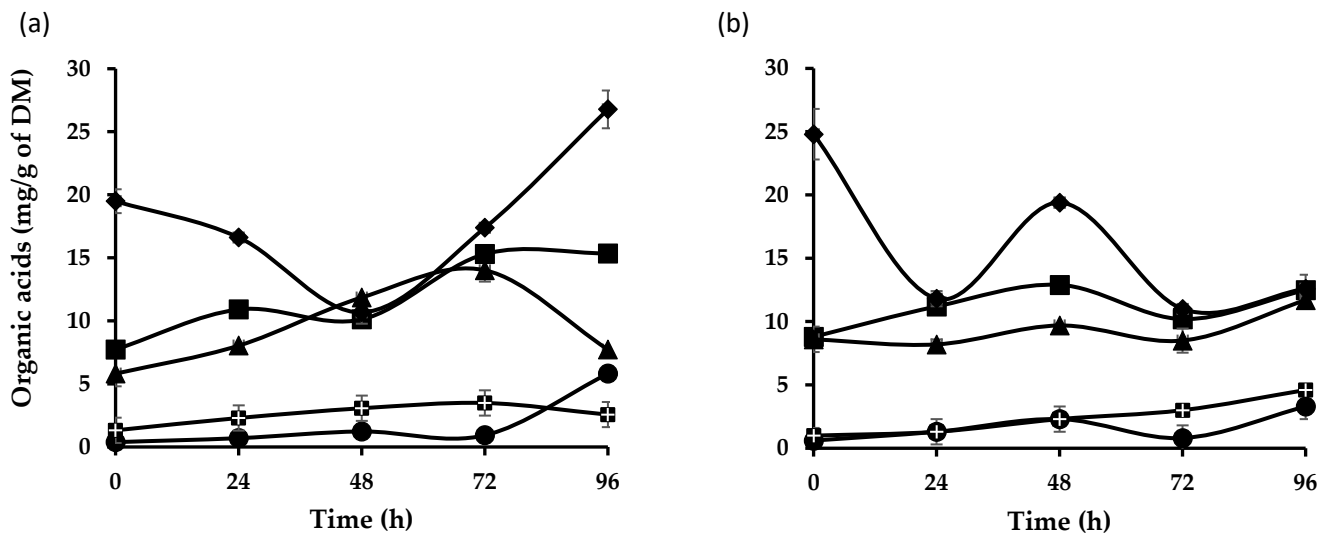


Figure 5. Changes of organic acid content for lactic (■), acetic (●), citric (◆), oxalic (▲), and malic (▣) acids during bean fermentation of the Nacional cocoa variety, comparing spontaneous fermentation (a) to microbial cocktail fermentation with a starter culture (b). Values represent the means of eight replicates and vertical bars indicate the standard deviation of the means.

There was no significant difference in the interaction between treatment and time for polyphenol content. However, the spontaneous fermentation showed a peak at 48 h (17.16 GA mg/g DM). At the end of the 96-h fermentation process, the starter microbial cocktail treatment had a higher polyphenol content (16.92 GA mg/g DM) compared to the control (11.23 GA mg/g DM) (Figure 6).

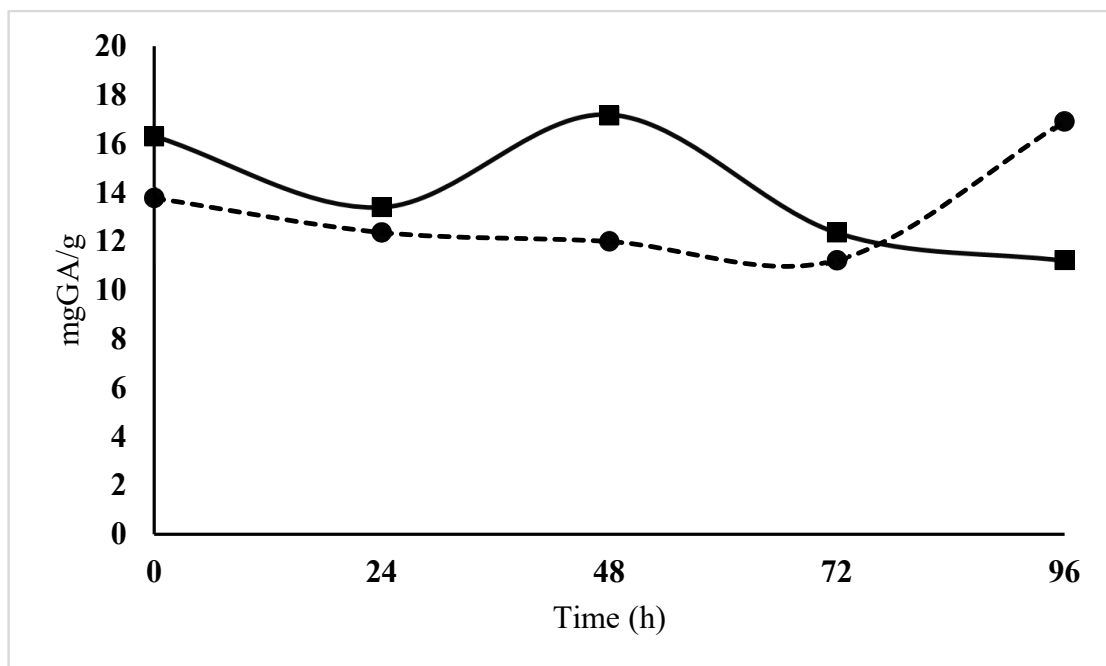


Figure 6. Changes in the content of polyphenols (gallic acid (GA) mg/g DM) during the fermentation of Nacional-variety cocoa beans due to the effect of a microbial cocktail as a starter culture (●) compared to spontaneous fermentation (■).

3.4. Fermentation Percentage

After 96 h of fermentation, the percentage of fermented beans in the treatment with spontaneous fermentation was 57.3%, while the treatment inoculated with the starter culture obtained a value of 77.0%, or 19.7% more fermented beans (Figure 7).

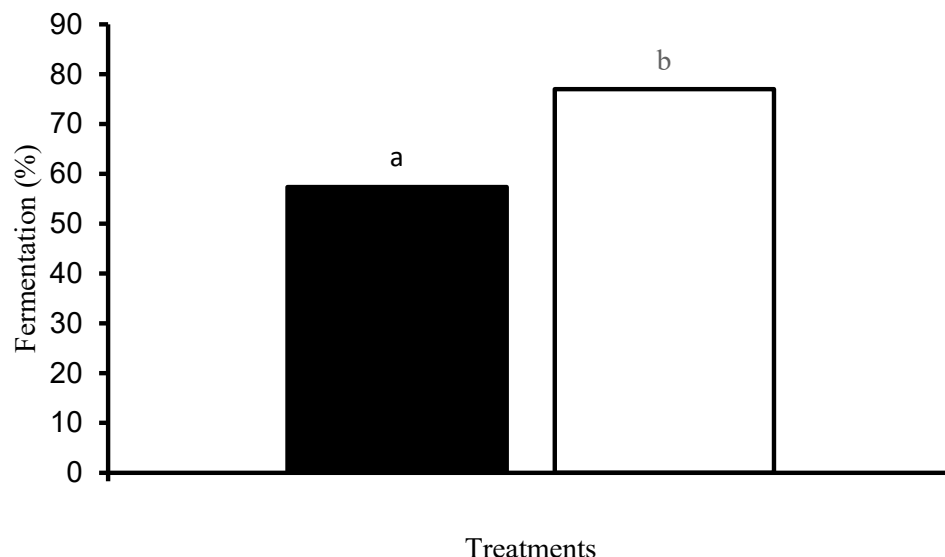


Figure 7. Fermentation percentage of Nacional-variety cocoa beans (a) by spontaneous fermentation and (b) by a microbial cocktail used as a starter culture.

When carrying out the cut test after the fermentation process, the treatment inoculated with the starter culture showed 24% more fermented beans (cotyledons with deep and brown striations and/or cotyledons with no striations, deep and slightly purple in color) (Figure 1C,D), 5% fewer purple beans, 15% fewer slaty beans (unfermented beans with a gray-black or greenish color inside), and no moldy beans (Figure 1E) compared with the spontaneously fermented cocoa beans (Table 1).

Table 1. Quality requirements for the Cocoa Beans Arriba Superior after processing (CBAS). Quality of Nacional-variety cocoa beans fermented spontaneously versus the addition of a microbial starter culture.

Requirements	Unit	Spontaneous Fermentation	Starter Culture	CBAS
Maximum humidity	%	60.64	58	7
100 beans weight	g	106.5	109.9	100–120
Fermented beans, minimal	%	57	77	53
Violet beans, maximum	%	43	20	25
Slaty beans, maximum	%	0	3	18
Moldy beans, maximum	%	0	0	4
Totals (analysis in 100 g)	%	100	100	100
Polyphenol content	GA mg/g DM	11.23	16.92	25–40

A starter culture containing mixed native microorganisms has been found to enhance the fermentation process, resulting in a final cocoa product with improved quality and flavor.

4. Discussion

4.1. Variation of Temperature and pH during Fermentation

For the purpose of obtaining the desired aromas, it is recommended that cocoa fermentation take place at a pH level of approximately 5.0 [22]. The pH values found in this study coincide with those previously reported [23], which found initial values of 3.7–3.9 that

progressively increased to 4.2–4.9 both in spontaneous fermentation and those inoculated with starter culture. The assimilation of citric acid by microorganisms causes the pH to increase [24]. The degree and time of acidification of the cotyledon during the fermentation process [25] together with changes in temperature, generated by a succession of various microorganisms (yeasts, LAB, AAB, and *Bacillus* sp.), create an optimal environment inside the cocoa bean for enzymes such as endoprotease, carboxypeptidase, invertase, and glycosidase to catalyze reactions that generate flavor precursor substances [26,27].

4.2. Quantification of Population Dynamics during Fermentation

The yeasts reached peak populations between 0 and 24 h for both inoculated and control treatments (Figure 3). These values exceeded previously reported numbers of 6.69 to 5.67 log CFU/g between 0 and 96 h [17]. Other authors point out that the highest population peak (1×10^7 – 1×10^8 CFU/g) of these yeasts occurs between the first 12 to 36 h, and then drops to around 1×10^3 CFU/g [23,27]. This great difference is due to the fact that, as the fermentation process progresses, the sources of nutrients for yeasts are depleted; in addition, high temperatures are generated, as well as the production of some acids that inhibit yeast multiplication [28,29]. Other authors [30] observed that, by inoculating starter populations of yeasts, LAB, and AAB, populations of 1×10^{10} CFU/g were obtained at 0 h which subsequently reduced by 50% at 24 h, and were 1×10^4 CFU/g at 96 h. In this study, those high numbers were not achieved and not all populations decreased to the low levels reported.

The trends in the LAB population of this study (Figure 3) were similar to those observed in another study [31], both for spontaneous fermentation and for the inoculated treatment. However, in [31], the treatment with LAB began with low densities of 1×10^6 CFU/g and after 50 h started to decrease progressively, while in the spontaneous fermentation, an increase was observed after 96 h. Other researchers [32] have shown that, when LAB are inoculated, an increase in the population is observed during the first 2 days (1×10^9 CFU/g) which then drops to 1×10^2 – 1×10^3 CFU/g after 72 h, while in spontaneous fermentation the population starts with 1×10^2 – 1×10^{-3} CFU/g and then increases to 1×10^7 CFU/g, decreasing from the fourth day. LAB plays a very important role since they ferment the sugars present in the pulp to lactic acid, acetic acid, and ethanol, as well as metabolize citric acid, producing lactic and acetic acids, among other functions [9,31].

For AAB, our results showed a similar trend to those obtained by [23], who used wooden boxes—another method commonly used for spontaneous fermentation—which are used for the daily turning of the beans during the fermentation process. Under these conditions, they obtained populations greater than 1×10^6 CFU/g during the entire process, observing a slight increase at 72 h. Perhaps the environmental conditions inside the mass become favorable for the development of microorganisms. When using a starter culture containing yeast, LAB, and AAB, inoculated at time 0, an increase was observed in the AAB populations up to 72 h, followed by a rapid decrease [32,33]. However, when inoculating with two yeast strains of *Torulaspora delbrueckii*, a decrease was observed in the AAB population from 0 to 96 h [34]. Despite the fact that in this study AAB was also inoculated, its population did not thrive. *Acetobacter ghanensis* does not have the best characteristics that allow it to dominate over other AAB populations, such as the dependence on glycerol and mannitol or lactate as energy sources, characteristics that *Acetobacter pasteurianus* does show [35]. However, *A. ghanensis* was chosen to be part of the starter cocktail because both occur frequently in spontaneous cocoa bean fermentation and it is involved in two aromatic compound degradation pathways [34]. The population decline may be due to insufficient aeration in the bag fermentation system, hindering microorganism growth [28].

Fungi such as *Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus ochraceus* produce mycotoxins that reduce cocoa bean quality and may harm consumers [36,37]. However, with the microbial cocktail inoculation, the population of filamentous fungi decreased until the end of fermentation (Data from Figures 1F and 3). Species such as *Lactobacillus* sp. and *Hanseniaspora uvarum*, as a result of their metabolism, produce acetic and lactic

acid and other substances that affect the growth of filamentous fungi that usually appear during the bean drying and storage stages [38]. Additionally, the starter cultures increase the organoleptic properties of the cocoa bean [38,39]. In practice, producers associate “good fermentation” with the brown color of the bean, the presence of cracks, and brittle cotyledons, with the bean separating easily from the shell as shown in Figure 1C,D.

No effect was observed on mesophilic microorganisms in either the starter microbial cocktail treatments or the spontaneous fermentation (Figure 3). Although the participation of mesophilic microorganisms in cocoa fermentation has not been studied in detail, these microorganisms produce a range of chemical compounds similar to yeasts, AAB, and LAB such as 2,3-butanediol, pyrazines, acetic acid, lactic acid, and short-chain fatty acids, which can contribute to acidity and perhaps off-flavors in fermented cocoa beans [29,30]. The concentration of mesophilic aerobes can indicate the presence of other microorganisms that may develop during cocoa bean fermentation [40]. This number may remain constant or slightly decrease during fermentation due to the dominance of other microbial species that can withstand increasingly adverse conditions [41,42]. According to the European Food Safety Authority, the presence of species of the genus *Bacillus* sp. in the different cocoa postharvest processes could be an indicator of poor hygiene conditions that should be avoided [43].

4.3. Variation in Concentrations of Sugars, Organic Acids, and Polyphenols

The consumption of glucose significantly dropped from 4.71 to 0.47 ± 0.16 mg/g DM within the first 24 h. High consumption of sucrose was observed when it was inoculated with the starter culture as well as with spontaneous fermentation, obtaining final values of 0 mg/g DM (Figure 4). The sugar concentration obtained was lower than the values usually found, which usually amount to 100 mg/g DM [40]. We did not detect sucrose, which contributed in part to the low total sugar content. This was indicative of an advanced ripening of the fruit [44]. Another substrate found was 5.9 ± 3.8 mg/g DM of citric acid. The decrease in sucrose is due to enzymatic processes (action of invertase enzymes) of hydrolysis, producing reducing sugars, mainly glucose and fructose, which are considered flavor and aroma precursors, involved during roasting in Maillard’s non-enzymatic browning reactions to generate some specific aromatic components [29]. In similar studies, where various microorganisms (AAB, LAB, and yeasts) were inoculated, the sucrose content was close to zero during the first 96 h of fermentation [45], while others did not obtain a higher consumption of this sugar when inoculating with *Saccharomyces cerevisiae* var. *chevalieri*, reporting initial values of 8 mg/g DM and final values of 3.5 mg/g DM [46]. In this investigation, the yeasts *H. uvarum* and *T. delbrueckii* were used because, in previous studies, they had a preference for consuming glucose [9], although they can also metabolize sucrose. In addition, *H. uvarum* is a species with a high potential to generate a greater amount of invertase enzyme. This would result in a more significant increase in the total solids at the end of cocoa bean fermentation [47].

Glucose is one of the main carbohydrate sources for yeasts. Yeasts consume 83% of glucose as their primary carbohydrate source within 120 h during spontaneous fermentations [9]. Similarly, inoculations with different microorganisms have been shown to cause high glucose consumption for up to 72 h, with values reaching close to zero [48]. This aligns with the findings of this study (Figure 4). On the other hand, an increase in glucose concentration resulting from sucrose hydrolysis was observed in yeast species that efficiently consume this sugar [8,49], but this increase was not observed in this study.

Glucose and fructose contribute to chocolate’s flavor by reacting with peptides and amino acids such as tyrosine, alanine, phenylalanine, and leucine, resulting in “sweet notes” [50]. Some strains of bacteria such as *L. plantarum*, used in this study, consume fructose and glucose, and like *Lb. fermentum* do produce a greater amount of lactic acid [51]. This may explain the low consumption of fructose in the inoculated treatment compared to spontaneous fermentation (Figure 4), since inoculating these species affected the balance of microorganisms and could cause a decrease in certain microorganisms such as *Lb.*

fermentum that possess genes involved in the uptake and consumption of fructose and/or sorbose [52,53].

The increase in ethanol concentration was very low compared to the 6, 12, and 40 mg/g DM of ethanol reported by [9,32,49], respectively. This is mainly due to the low glucose content that was present from the beginning of the process, this being the main source for the generation of ethanol, mainly by yeasts [17]. In addition, the increase in ethanol concentration could be produced by some strains of heterofermentative lactic acid bacteria when LAB species and no yeasts are inoculated [50].

For acetic acid, a higher content was found at 96 h (3.30 mg/g DM). This is due to the activity of microorganisms like AAB, which oxidize lactic acid and ethanol produced by LAB and yeasts. As a result, the cocoa experiences high acidity, and the embryo ultimately dies [5]. The content of acetic acid has an impact on the populations of filamentous fungi, leading to a decrease in their numbers [46]. The transformation of malic acid to lactic acid and CO₂ is conducted by some LAB such as *L. plantarum* [22]. Oxalic and malic acids, produced by yeasts and *L. plantarum*, enhance the flavor and aroma and have antimicrobial properties to preserve food quality [52].

In our study, treatment with the starter microbial cocktail resulted in a higher content of polyphenols (16.92 GA mg/g DM) compared to spontaneous fermentation (11.23 GA mg/g DM) (Figure 6). Our values were lower than those reported by [15], who found 44.29 GA mg/g DM on the first day of fermentation, decreasing to 36.78 GA mg/g DM on the last day for Nacional cacao. The low polyphenol content may be due to the harvest time and plant age; however, low polyphenol contents result in chocolates that are less astringent and bitter [25,26].

4.4. Fermentation Percentage

The percent of fermented cocoa beans agrees with other studies where inoculations of 0.5 g of yeasts (*Saccharomyces cerevisiae*) and 0.24 g of bacteria (*Acetobacter aceti*) for every 100 g of cocoa produced 72% of fermented cocoa beans [53], or those with inoculations of two yeasts, one AAB, and one ALB that obtained 81% fermentation at 120 h with a pH of 4.71 [54].

A fermentation time of only 4 days, instead of the traditional 6, is sufficient to produce quality cocoa with the potential for great flavor development [46]. The beans inoculated with our native starter culture meet the quality requirements for Cocoa Bean Arriba Superior (CBAS) (Table 1); this shortens the fermentation process and enables the beans to reach the drying stage sooner (Figure 1E).

5. Conclusions

Utilizing a native microbial cocktail as a starter culture drastically transforms the sugar and organic acid composition, temperature, and pH levels during cocoa bean fermentation.

The inclusion of this inoculum completely overhauls the dynamics of microorganisms, ultimately leading to significantly higher populations of yeasts and AAB in contrast to the control with spontaneous microbiota.

The use of a starter culture increased polyphenol content, increased fermentation efficiency by 24%, and reduced both sugars and acids, resulting in cocoa beans ready for drying in 96 h while also decreasing filamentous fungi that can negatively affect flavor and aroma.

New research aims to evaluate native microorganisms for improving cocoa products' organoleptic characteristics and specific flavors for targeted markets. The metabolic profiles of the microbial cocktail with mesophilic microorganisms such as *B. subtilis* must undergo laboratory-scale testing during cocoa fermentation to better understand their role in cocoa product quality.

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References

1. Agricultura e Industria Manufacturera, Cultivo de Cacao. Elaboración de Cacao, Chocolate. Ficha Sectorial, Cacao y Chocolate, Subgerencia de Análisis de Productos y Servicios; Corporación Financiera Nacional (CFN). 2022. Available online: <https://www.cfn.fin.ec/wp-content/uploads/downloads/biblioteca/2022/fichas-sectoriales-2-trimestre/Ficha-Sectorial-Cacao.pdf> (accessed on 26 May 2023).
2. Fowler, M.; Coutel, F. Cocoa beans: From tree to factory. In *Beckett's Industrial Chocolate Manufacture and Use*, 5th ed.; Beckett, S., Fowler, M., Ziegler, G., Eds.; John Wiley & Sons Ltd.: Hoboken, NJ, USA, 2017; pp. 9–49. [CrossRef]
3. Ganeswari, I.; Khairul Bariah, S.; Amizi, M.; Sim, K. Effects of different fermentation approaches on the microbiological and physicochemical changes during cocoa bean fermentation. *Int. Food Res. J.* **2015**, *1*, 70–76.
4. Ozturk, G.; Young, G.M. Food Evolution: The impact of society and science on the fermentation of cocoa beans. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 431–455. [CrossRef] [PubMed]
5. Nielsen, D. The microbiology of Ghanaian cocoa fermentations. *Int. J. Food Microbiol.* **2007**, *114*, 168–186. [CrossRef]
6. Nigam, P.; Singh, A. Cocoa and Coffee Fermentations. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C., Lou Tortorello, M., Eds.; Academic Press: London, UK; Burlington, NJ, USA; San Diego, CA, USA, 2014; pp. 485–492.
7. Camu, N.; De Winter, T.; Verbrugghe, K.; Cleenwerck, I.; Vandamme, P.; Takrama, J.S.; Vancanneyt, M.; De Vuyst, L. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Appl. Environ. Microbiol.* **2007**, *73*, 1809–1824. [CrossRef] [PubMed]
8. De Vuyst, L.; Weckx, S. The cocoa bean fermentation process: From ecosystem analysis to starter culture development. *J. Appl. Microbiol.* **2016**, *121*, 5–17. [CrossRef]
9. Ardhana, M.; Fleet, G. The microbial ecology of cocoa bean fermentations in Indonesia. *Int. J. Food Microbiol.* **2003**, *86*, 87–99. [CrossRef]
10. Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C.M.; Harris, H.M.; Mattarelli, P.; O'toole, P.W.; Pot, B.; Vandamme, P.; Walter, J.; et al. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 2782–2858. [CrossRef]
11. Zambrano, G. Evaluación de la Influencia del Proceso de Beneficio del Cacao (*Theobroma cacao*) CCN-51 de Altura en su Calidad Final, Mediante el Análisis Físico, Físico-Químico y Sensorial. Bachelor's Thesis, Universidad Central del Ecuador, Quito, Ecuador, 2018; pp. 29–32. Available online: <http://www.dspace.uce.edu.ec/bitstream/25000/16624/1/T-UCE-0008-CQU-044.pdf> (accessed on 9 May 2023).
12. Figueroa, C.; Mota, J.F.; Hernández, Z.; González, O.; Cocolin, L.; Suárez, M. The challenges and perspectives of the selection of starter cultures for fermented cocoa beans. *Int. J. Food Microbiol.* **2019**, *301*, 41–50. [CrossRef]
13. Yáñez-Mendizábal, V.; Falconi, C.E.; Palacios, H. Identification of microbial population in two fine cocoa varieties during the commercial fermentation process. *Food Microbiol. Saf.* **2023**, accepted.
14. Falconi, C.E.; Yáñez-Mendizábal, V.; Claudio, D.R. Native *Bacillus subtilis* strains efficiently control lupin anthracnose both under greenhouse and in field conditions. *Int. J. Adv. Sci. Eng. Inf. Technol.* **2022**, *12*, 2519–2526. [CrossRef]
15. Yáñez-Mendizábal, V.; Falconi, C.E.; Kanaley, K. Production optimization of antifungal lipopeptides by *Bacillus subtilis* CtpxS2-1 using low-cost optimized medium. *Biol. Control* **2023**, *185*, 105306. [CrossRef]
16. Dotan, S.; Amaya, J.; Fredy, N.; Palacios, B. Plan de Desarrollo y Ordenamiento Territorial 2015–2020. Gobierno Autónomo Descentralizado del Cantón Vices. Vices, Ecuador. 2015. Available online: http://app.sni.gob.ec/sni-link/sni/PORTAL_SNI/data_sigad_plus/sigadplusdiagnostico/1260001030001_Diagnostico%20Territorial%20del%20Cant%C3%B3n%20Vices%202015_2020_04-04-2016_15-59-59.pdf (accessed on 29 May 2023).
17. Ho, V.T.; Zhao, J.; Graham, F. Yeasts are essential for cocoa bean fermentation. *Int. J. Food Microbiol.* **2014**, *174*, 72–87. [CrossRef] [PubMed]
18. Violeta, N.; Trandafir, I.; Ionica, M. HPLC Organic Acid Analysis in Different Citrus Juices under Reversed Phase Conditions. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2010**, *38*, 44–48. [CrossRef]
19. Carrillo, L.; Londoño, J.; Gil, A. Comparison of polyphenol, methylxanthines and antioxidant activity in *Theobroma cacao* beans from different cocoa-growing areas in Colombia. *Food Res. Int.* **2014**, *60*, 273–280. [CrossRef]

20. INEN. NORMA TÉCNICA ECUATORIANA NTE INEN 176, Cocoa Beans Requirements, Sexta Revisión. Quito, Ecuador. 2021. Available online: https://corpalmesa.com/wp-content/uploads/2021/07/CORPALMESAnte_inen_.pdf (accessed on 4 September 2023).
21. Coexca. Instructivo Control Calidad/Coexca. Colombia Exporta Cacao Fino y de Aroma. Colombia. 2017, pp. 19–26. Available online: https://www.swisscontact.org/_Resources/Persistent/5/6/1/6/5616ce94e66df97f365ec26cdf9ad999fef0bc18/InstructivoControlCalidad.pdf (accessed on 5 September 2023).
22. Mayo, B.; Flórez, A. Lactic Acid Bacteria: *Lactobacillus plantarum*. In *Encyclopedia of Dairy Sciences*, 3rd ed.; McSweeney, P.L.H., McNamara, J.P., Eds.; Academic Press: Cambridge, MA, USA, 2022; pp. 206–217. [\[CrossRef\]](#)
23. Muñoz, A.; Gómez, S. Análisis Comparativo de la Diversidad Microbiana y la Producción de Compuestos Bioquímicos de Cacao (*Theobroma cacao* L.) Variedades Nacional y Trinitario ccn-51 durante la Fermentación. Bachelor's Thesis, Universidad de las Américas, Quito, Ecuador, 2020; pp. 27–32. Available online: <https://dspace.udla.edu.ec/handle/33000/12186> (accessed on 14 May 2023).
24. Sharmistha, S.; Tanmay, S.; Runu, C.; Maksim, R.; Mohammad, A.S.; Muthu, T.; Kannan, R.R.R. Dark chocolate: An overview of its biological activity, processing, and fortification approaches. *Curr. Res. Food Sci.* **2022**, *5*, 1916–1943. [\[CrossRef\]](#)
25. Colonges, K.; Seguine, E.; Saltos, A.; Davrieux, F.; Minier, J.; Jimenez, J.C.; Lahon, M.C.; Calderon, D.; Subia, C.; Sotomayor, I.; et al. Diversity and determinants of bitterness, astringency, and fat content in cultivated Nacional and native Amazonian cocoa accessions from Ecuador. *Plant Genome* **2022**, *15*, e20218. [\[CrossRef\]](#)
26. Janek, K.; Niewianda, A.; Wöstemeyer, J.; Voigt, J. The cleavage specificity of the aspartic protease of cocoa beans involved in the generation of the cocoa-specific aroma precursors. *Food Chem.* **2016**, *211*, 320–328. [\[CrossRef\]](#)
27. Cempaka, L.; Aliwarga, L.; Purwo, S. Dynamics of cocoa bean pulp degradation during cocoa bean fermentation: Effects of yeast starter culture addition. *J. Math. Fundam. Sci.* **2014**, *46*, 14–25. [\[CrossRef\]](#)
28. Voigt, J. Chocolate and Cocoa Aroma. In *Chocolate in Health and Nutrition*, Primera ed.; Watson, R., Preedy, V., Zibadi, S., Eds.; Humana Press: New York, NY, USA, 2013; pp. 89–93. [\[CrossRef\]](#)
29. Santander, M.; Rodríguez, J.; Vaillant, F.; Escobar, S. An overview of the physical and biochemical transformation of cocoa seeds to beans and to chocolate: Flavor formation. *Crit. Rev. Food Sci. Nutr.* **2019**, *60*, 1593–1613. [\[CrossRef\]](#)
30. Sârbu, I.; Csutak, O. The microbiology of cocoa fermentation. *Caffeinated Cocoa Based Beverages* **2019**, *8*, 423–446. [\[CrossRef\]](#)
31. Sandhya, M.V.S.; Yallappa, B.S.; Varadaraj, M.C.; Puranaik, J.; Jaganmohan, R.; Janardhan, P.; Murthy Pushpa, S. Inoculum of the starter consortia and interactive metabolic process in enhancing quality of cocoa bean (*Theobroma cacao*) fermentation. *LWT—Food Sci. Technol.* **2016**, *65*, 731–738. [\[CrossRef\]](#)
32. Saunshi, Y.P.; Sagar Sandhya, M.V.; Rastogi, N.K.; Murthy, P.S. Starter consortia for on-farm cocoa fermentation and their quality attributes. *Prep. Biochem. Biotechnol.* **2020**, *50*, 272–280. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Schwan, R.F. Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Appl. Environ. Microbiol.* **1998**, *44*, 1477–1483. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Visintin, S.; Ramos, L.; Batista, N.; Dolci, P.; Schwan, F.; Cocolin, L. Impact of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* starter cultures on cocoa beans fermentation. *Int. J. Food Microbiol.* **2017**, *257*, 31–40. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Illegheems, K.; Pelicaen, R.; De Vuyst, L.; Weckx, S. Assessment of the contribution of cocoa-derived strains of *Acetobacter ghanensis* and *Acetobacter senegalensis* to the cocoa bean fermentation process through a genomic approach. *Food Microbiol.* **2016**, *58*, 68–78. [\[CrossRef\]](#)
36. Ramos, N.; Castro, A.; Juárez, J.; de la Cruz, O.; Rodríguez, N.; Blancas, J.; Navarro, A. Evaluación de ocratoxina a en *Theobroma cacao* L. “cacao blanco” durante el proceso de cosecha, fermentado, secado y almacenado. *Rev. Soc. Química Del Perú* **2016**, *82*, 431–439. [\[CrossRef\]](#)
37. Ruggirello, M.; Nucera, D.; Cannoni, M.; Peraino, A.; Rosso, F.; Fontana, M.; Cocolin, L.; Dolci, P. Antifungal activity of yeasts and lactic acid bacteria isolated from cocoa bean fermentations. *Food Res. Int.* **2019**, *115*, 519–525. [\[CrossRef\]](#)
38. Romanensa, E.; Freimüller, S.; Volland, A.; Stevens, M.; Krähenmann, U.; Isele, D.; Fischer, B.; Meile, L.; Schwenninger, S. Screening of lactic acid bacteria and yeast strains to select adapted antifungal co-cultures for cocoa bean fermentation. *Int. J. Food Microbiol.* **2019**, *290*, 262–272. [\[CrossRef\]](#)
39. Schwan, R.; Wheals, A. The Microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 205–221. [\[CrossRef\]](#)
40. Gálvez, S.L.; Loiseau, G.; Paredes, J.L.; Barel, M.; Guiraud, J.P. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. *Int. J. Food Microbiol.* **2007**, *114*, 124–130. [\[CrossRef\]](#)
41. García-Gonzalez, E.; Mendez-Orejuela, J.H.; Sierra-Banguera, J.S.; Chamorro-Moreno, D.I.; Ordoñez-Narváez, G.A.; Ochoa-Muñoz, A.F.; Montalvo-Rodríguez, C. Ecology and population dynamics of yeast starter cultures in cocoa beans fermentation. *BioTechnology* **2022**, *103*, 343–353. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Batista, N.N.; Lacerda, C.; Dias, D.; Carla, A.; Marques, A.; Freitas, R. Dynamic behavior of *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT—Food Sci. Technol.* **2015**, *63*, 221–227. [\[CrossRef\]](#)
43. Bastos, V.S.; Santos, M.F.S.; Gomes, L.P.; Leite, A.M.O.; Paschoalin, V.M.F.; Del Aguila, E.M. Analysis of the cocobiota and metabolites of *Moniliophthora perniciosa*-resistant (*Theobroma cacao* L.) beans during spontaneous fermentation in Southern Brazil. *J. Sci. Food Agric.* **2018**, *98*, 4963–4970. [\[CrossRef\]](#) [\[PubMed\]](#)

44. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann, S. Aroma properties of cocoa fruit pulp from different origins. *Molecules* **2021**, *26*, 7618. [[CrossRef](#)]
45. Papalexandratou, Z.; Dennis, S.; Nielsen, D.S. It's Gettin' Hot in here: Breeding robust yeast starter cultures for cocoa fermentation. *Trends Microbiol.* **2016**, *24*, 168–170. [[CrossRef](#)]
46. Cempaka, L.; Rahmawati, E.A.; David, W.A. Sensory profiles of chocolate drinks made from commercial fermented cocoa powder and unfermented cocoa beans. *Curr. Res. Nutr. Food Sci.* **2021**, *9*, 988–999. [[CrossRef](#)]
47. Ooi, T.; Ting, A.; Siow, L. Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study. *LWT—Food Sci. Technol.* **2020**, *122*, 108977. [[CrossRef](#)]
48. Afoakwa, E.O.; Quao, J.; Takrama, J.; Budu, A.S.; Saalia, F.K. Chemical composition and physical quality characteristics of Ghanaian cocoa beans as affected by pulp pre-conditioning and fermentation. *J. Food Sci. Technol.* **2013**, *50*, 1097–1105. [[CrossRef](#)]
49. Viesser, J.; Pereira, G.; de Carvalho, D.; Vandenberghe, L.; Azevedo, V.; Brenig, B.; de Carvalho, D.; Rogez, H.; Góes-Neto, A.; Soccol, C. Exploring the contribution of fructophilic lactic acid bacteria to cocoa beans fermentation: Isolation, selection and evaluation. *Food Res. Int.* **2020**, *136*, 109478. [[CrossRef](#)]
50. Kresnowati, M.T.A.P.; Suryani, L.; Affifah, M. Improvement of cocoa beans fermentation by LAB starter addition. *J. Med. Bioeng.* **2013**, *2*, 274–278. [[CrossRef](#)]
51. Puerari, C.; Teixeira Magalhães, K.; Freitas Schwan, R. New cocoa pulp-based kefir beverages: Microbiological, chemical composition and sensory analysis. *Food Res. Int.* **2012**, *48*, 634–640. [[CrossRef](#)]
52. López, M.; Criollo, J.; Hernández, M.; Lozano, M. Physicochemical and microbiological dynamics of the fermentation of the CCN51 cocoa material in three maturity stages. *Rev. Bras. Frutic.* **2019**, *41*, 1–13. [[CrossRef](#)]
53. El Salous, A.; Angulo, A.; Solís, L. Acceleration of cocoa fermentation through the action of bacteria (*Acetobacter aceti*) and yeast (*Saccharomyces cerevisiae*). *Espiraes Rev. Multidiscip. Investig. Científica* **2019**, *3*, 1–20. [[CrossRef](#)]
54. Fonseca, D.; López, D.; Ortiz, S.; Nuñez, C.; Lozano, D. 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. *Pelita Perkeb. (Coffee Cocoa Res. J.)* **2020**, *36*, 154–172. [[CrossRef](#)]

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