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Research article

Evaluation of stress tolerance and design of alternative culture media for the production of fermentation starter cultures in cacao

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ABSTRACT

Ecuador is one of the world's leading producers of cacao beans, and Nacional x Trinitario cacao represents one of the most distinctive varieties due to its flavor and aroma characteristics. This study aimed to evaluate the effect of the starter culture isolated from microbial diversity during the spontaneous fermentation of Nacional x Trinitario cacao. A total of 249 microbial isolates were obtained from spontaneous culture, with *Lactiplantibacillus* (45 %), *Saccharomyces* (17 %), and *Acetobacter* (2 %) being the most relevant genera for fermentation. Tolerance tests were conducted to select microorganisms for the starter culture. *Lactiplantibacillus plantarum* exhibited the highest tolerance at pH 5 and 6 % ethanol and tolerated concentrations up to 15 % for glucose and fructose. *Acetobacter pasteurianus* grew at pH 2 and 6 % ethanol, tolerating high sugar concentrations of up to 15 % for glucose and 30 % for fructose, with growth observed in concentrations up to 5 % for lactic and acetic acid. Subsequently, a laboratory-scale fermentation was conducted with the formulated starter culture (SC) comprising *S. cerevisiae*, *L. plantarum*, and *A. pasteurianus*, which exhibited high tolerance to various stress conditions. The fermentation increased alcoholic compounds, including citrusy, fruity aromas, and floral notes such as 2-heptanol and phenylethyl alcohol, respectively 1.6-fold and 5.6-fold compared to the control. Moreover, the abundance of ketones 2-heptanone and 2-nonanone increased significantly, providing sweet green herbs and fruity woody aromas. Cacao fermented with this SC significantly enhanced the favorable aroma-producing metabolites characteristic of Fine-aroma cacao. These findings underscore the potential of tailored fermentation strategies to improve cacao product quality and sensory attributes, emphasizing the importance of ongoing research in optimizing fermentation processes for the cacao industry.

1. Introduction

Cacao (*Theobroma cacao*) is an economically significant crop and a key component of Ecuador's agroindustry. This raw material is used to produce 100 % fine-aroma chocolate bars, employing cacao beans that are challenging to grow in other parts of the world due

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to the country's geographical and climatic characteristics [1]. There are four morphogenetic groups of cacaos: Forastero, Criollo, Nacional, and Trinitario, a hybrid between Forastero and Criollo [2]. Nacional is a niche cultivar grown in Ecuador, and these cultivars are the result of several generations of crosses between the ancestral Nacional and Trinitario [3]. It is genetically similar to Criollo [4], it is also known as "Fino de Aroma" or "Arriba," and is highly demanded in the "production of the finest chocolates [5,6].

Cacao beans and pulp composition suit bacterial growth [7]. The fermentation of cacao beans is carried out through a succession of activities involving yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [8–10]; where the main purpose is the elimination of primary pulp surrounding the kernels by endogenous yeast-mediated pectinolysis to improve the subsequent drying of the non-germinating kernels, as well as the death of the seed embryo, which occurs through the curing steps [11]. The acid, produced during fermentation, penetrates the husk and triggers biochemical reactions in the bean to form chocolate flavor precursors and reduce the astringent and bitter taste [12,13].

Cacao bean fermentation is considered the postharvest treatment stage that influences the quality of chocolate the most [14]. In South America, cacao beans are fermented mainly in large wooden boxes that can hold up to 1.5 tons. These boxes have slatted bases or holes in the sides and base, which allow the generated fluids to drain and provide air access [13]. Box fermentation systems facilitate turning, as bean movement is favored by gravity [15]. The duration of fermentation varies considerably from one country to another and even from one farmer to another; it also depends on the cacao variety and usually lasts between 5 and 10 days. In Ecuador, the optimal fermentation time for the maximum development of aromatic compounds in Nacional cacao is 4–5 days [15,16]. Subsequently, the drying process is carried out in covered solar dryers (greenhouse type), where the fermented cacao beans are spread on tables placed 1 m above ground level [17].

One of the main disadvantages of spontaneous fermentation is that the quality of cacao flavor and aroma does not remain constant across batches. This variability underscores the importance of developing robust starter cultures capable of withstanding the harsh conditions prevalent during the fermentation process. For example, the residual fructose content, together with the reduced water activity, can stimulate the proliferation of spoilage bacteria and filamentous fungi during the last days of fermentation, which produce undesirable microbial compounds such as C3–C5 free fatty acids and extracellular proteases and lipases. Also, the late growth of toxigenic fungi becomes a significant public health risk due to the production of mycotoxins [18]. Enterobacteriaceae may also be responsible for glucose gluconic acid production, which acts on the growth of glucose-dependent yeasts and LAB [11]. These factors contribute to expressing more intense astringency and bitterness in the sensory profile. Without proper fermentation of the cacao beans, the development of aromatic compounds that arise in this stage is limited [16].

Several studies support the benefits of applying starter cultures in cacao fermentation to reduce fermentation time [10,19]; and enhance phenolic [20] and antioxidant compounds [21]. Successful enhancement of aromatic compounds in fermentations with starter cultures has been achieved in Trinitario [22] and Forastero [23] cacao varieties in Costa Rica and Ivory Coast. Meanwhile, only Brazil has stood out in research on Forastero cacao in South America [18,24]. However, no studies have been done on applying starter cultures to enhance aroma and flavor compounds in Nacional cacao have been done in Ecuador. Furthermore, the design of alternative culture media for cultivating these starter cultures is crucial for their successful application from an economic and environmental perspective. Alternative culture media can reduce costs and environmental impact by utilizing sustainable resources, providing an economically viable and environmentally friendly approach to enhancing cacao fermentation processes. This research aimed to assess the impact of a starter culture composed of species isolated from a spontaneous fermentation process, considering the stability of microorganisms under different stress conditions and design suitable, economically, and environmentally sustainable culture media to enhance the formation of aromatic compounds associated with the quality of Nacional cacao.

2. Materials and methods

2.1. Isolation of microorganisms by culture-dependent methods

Samples were aseptically collected at 0, 24, 48, 72, and 96 h from the spontaneous fermentation of Nacional x Trinitario cacao conducted in wooden boxes, sourced from a cacao processing center located in the city of Milagro (Guayas, Ecuador). This spontaneous fermentation was carried out in the summer season.

1 g of cacao beans with adherent pulp were homogenized in 9 mL of peptone water (TM Media) and five serial dilutions were made. These dilutions were then plated (50 μL) on the following culture media: Potato Dextrose Agar (PDA, BD Difco™) supplemented with 1 μg/mL of tetracycline for yeast isolation, incubated for five days; Man-Rogosa-Sharpe Agar (MRS, BD Difco™) for lactic acid bacteria (LAB), incubated for 48 h; and Glucose Yeast Extract CaCO3 (GYC) agar (10 % glucose (Extra pure (TM Media), 1 % yeast extract (BD, Bacto™, 2 % calcium carbonate (Merck), 1.5 % agar (BD, Bacto™), pH 6.8) for acetic acid bacteria (AAB), incubated for 96 h. All plates were incubated at 30 °C. Then, typical colonies were selected, isolated, and cultured under identical conditions to obtain pure colonies. These pure cultures were then preserved at −80 °C in Eppendorf tubes containing Luria Bertani broth (LB, BD Difco™) for bacteria and Potato Dextrose Broth for yeast (PDB, BD Difco™), with 20 % glycerol (Sigma >99,5 %) as a cryoprotectant in both isolates.

2.1.1. DNA extraction from microbial isolates

DNA extraction from isolates was performed as proposed by Post [25], with some modifications. Colonies were resuspended in 20 μL of 20 mM NaOH (Merck <100 %) and heated in a microwave (Whirpool) for 90 s. The quantity and quality of the extracted DNA from microbial isolates were verified by spectrophotometry (NanoDrop; Thermo Fisher Scientific, Wilmington, DE, USA).

2.1.2. PCR amplification

The primers 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) were used to amplify the 16S rRNA region of bacterial DNA, as described by Zhang [26]. The PCR mix was prepared in a final volume of 15 μL, containing 7.5 μL of GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 1 μL of each primer, 5.5 μL of ultrapure sterile distilled water, and 1 μL of bacterial DNA. PCR amplification was carried out in a Mastercycler thermocycler (Eppendorf Nexus GSX1, Hamburg, Germany) following the conditions: initial denaturation at 96 °C for 5 min; 35 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min; and a final extension at 72 °C for 3 min, followed by cooling to 4 °C, as suggested by Tigrero-Vaca [27].

For yeast, the universal primers ITS1 (5′CTTGGTCATTTAGAGGAAGTAA3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) were used. The reactions were conducted as mentioned earlier. The amplification conditions included an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Agarose (Ultrapure Agarose Invitrogen) gel electrophoresis confirmed the amplicon presence, and samples were subsequently sent to an external laboratory for Sanger sequencing. Taxonomic identification of microbial isolates was elucidated by aligning the sequence data with the GenBank database using BLAST (Basic Local Alignment Search Tool) in the Geneious Prime program (version 2020.0.3).

2.2. Selection of microorganisms based on tolerance to stress conditions

Following the guidelines from Pereira [28] and Visintin [12] for selecting starter microorganisms, tolerance to different stress conditions was assessed. Previously, the most relevant species in the application and development of starter cultures for cacao fermentation were chosen [10,18,22,29,30].

An inoculum of each microorganism previously conserved at – 80 °C (recovered on their respective selective agar) was prepared in a tube containing 20 mL of 0.85 % saline solution (Merck >99 %) until an optical density of 1 on the McFarland scale (3×10^8 CFU mL^{-1}) was reached. Then, 1 mL was taken from each inoculum suspension for subsequent tests. Simultaneously, 100 mL of culture media were prepared for each microorganism: MRS broth (BD Difco™) for LAB, Soy Trypticasein Broth (TSB, BD, BBL™) with a pH adjusted to 5.6 with 3 N HCl (Merck 37 %) for AAB, and Potato Dextrose Broth (PDB, BD Difco™) for yeast.

2.2.1. Evaluation of tolerance to various stress conditions

Initially, experimental trials were carried out with three temperature levels (30, 37, and 45 °C) to measure the effect of biomass growth on this variable and select the best condition. Subsequently, with the chosen temperature, microbial growth was evaluated by modifying the culture medium at the different levels of the other variables (Table 1): pH, ethanol (Merck <100 %) glucose (Extrapure, TM MEDIA), fructose (TM MEDIA), lactic acid (Sigma 85 %) and acetic acid (Sigma-Aldrich >99 %). 3 N HCl was used to adjust the pH to 2, 3, and 5 of each culture broth for pH tolerance evaluation. Ethanol (Merck <100 %) was added at 6, 10, and 12 % to the culture broths; glucose (Extrapure, TM MEDIA) and fructose (TM MEDIA) were added at 5, 15, and 30 %; and finally, lactic acid (Sigma 85 %) and acetic acid (Sigma-Aldrich >99 %). were added at 1, 3, and 5 %. Each microorganism was inoculated (1 mL) in its respective culture medium in triplicate and incubated under orbital agitation for 24 h.

2.2.2. Quantification of biomass in dry weight

Once the incubation period concluded, biomass was assessed based on dry weight using the methodology recommended by Arana [31], with some modifications: 20 mL from each sample were taken in a Falcon tube and centrifuged at 4500 G for 20 min. The supernatant was removed, and 6 mL of 0.85 % saline solution (Sigma >99 %) was added, followed by vortex (Scientific Industries, vortex – genie 2) homogenization; 1 mL was transferred to a pre-weighed Eppendorf tube, subjected to a second centrifugation at 16000 G for 15 min, and finally, the supernatant was discarded, and the residue was dried by vacufuge (Eppendorf, Concentrator plus) under v-aq mode for 40 min at 60 °C. The biomass's dry weight (gL^{-1}) was determined by *Equation (1)*, where the concentration factor is 20 mL of culture suspension divided by 6 mL of diluent (0.85 % saline).

$$Dry\ weight\ \left(g/L\right) = \frac{Tube\ weight\ with\ dry\ sample - Tube\ weight}{Concentration\ factor} x\ 100 \quad (1)$$

Table 1
Levels used for the modification of the culture media in the tolerance tests for each microorganism.

Conditions		Levels		Number of experiments
Temperature °C	30	37	45	3
pH	2	3	5	3
% Ethanol	6	10	12	3
% Glucose	5	15	30	3
% Fructose	5	15	30	3
% Lactic acid	1	3	5	3
% Acetic acid	1	3	5	3
Total experiments in triplicate *(n = 3)*:				63

2.3. Design and assessment of alternative culture medium to produce starter microorganisms

For each selected species based on stress tolerance, fermentation simulation culture media were prepared (Table 2), following the research by Lefeber [32] and Pereira [28], with some modifications. The dry weight (gL^{-1}) of each microorganism in the culture medium with cacao pulp as substrate (control medium, CM) was compared with a culture medium containing an alternative substrate (alternative substrate medium, ASM): Whey for BAL, molasses for yeast, and orange pulp for AAB.

Approximately 10^6 CFU mL^{-1} of each microorganism was inoculated in its culture medium and incubated for 72 h. The media inoculated with LAB and AAB were kept at 37 °C, and the yeast at 28 °C. Every 24 h, 20 mL samples were taken for biomass determination analysis by dry weight, as mentioned earlier.

2.4. Laboratory-scale fermentation

The fermentation experiments were conducted on a laboratory scale, as Pereira [24] suggested, with some modifications, using Nacional cacao obtained from the Agricultural Experimental Farm at ESPOL University. Cacao beans with pulp were pasteurized at 65 °C for 30 min [29], and 100 g were placed in 250 mL Erlenmeyer flasks. These flasks were inoculated with approximately 10^6 CFU mL^{-1} of each microorganism selected based on tolerance tests. The inoculum percentage suspended in the cacao beans was 3 %, meaning 1 mL of each microorganism as a starter culture (SC). A spontaneous fermentation process was considered as a control sample (C). The incubation temperature was adjusted every 12 h to simulate a large-scale fermentation, starting with a temperature of 28 °C until 12 h, where the temperature was raised to 30 °C, at 24 h it was adjusted to 32 °C, 35 °C at 36 h, 38 °C at 48 h, 42 °C at 60 h, 46 °C at 72 h and 48 °C until completing 120 h of the fermentation process. Samples were taken every 24 h for volatile compound analysis and stored at −20 °C until analysis.

2.4.1. Determination of volatile compounds

Volatile compounds were characterized by Headspace solid-phase microextraction (HS-SPME), following the methodology suggested in previous studies [27,33]. One bean from each sample was selected and ground under liquid nitrogen to increase the exposed surface area and enhance the compounds' volatilization. The powder was placed in 50 mL SPME vials and heated in a water bath at 60 °C for 30 min. Subsequently, a Solid phase microextraction (SPME) fiber with a coating of 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), provided by Supelco, was inserted to expose it to the Headspace of each sample for 30 min.

The SPME fiber was injected into a Gas chromatograph GC 7890A coupled to a Mass spectrometer MS5975C (Agilent Technologies, Santa Clara, CA, USA), equipped with a DB5-MS column (30 m × 250 μm × 0.25 μm). The injector and oven temperatures were set at 240 °C and 310 °C, respectively. Additionally, helium was used as the carrier gas at a constant flow rate of 0.8 mL/min. The MS detector was programmed in electron impact mode and positive polarity. The total ion current was recorded over a mass range of 40–750 amu. The spectrum data were retrieved using the ChemStation E.02.02 software (Agilent Technologies, Inc., Santa Clara, CA, USA), and the identity of the aroma compounds was determined by comparing the mass spectra of each compound with the Wiley 9 library and the NIST11 databases.

2.5. Statistical analysis

All tests were conducted in triplicate. The data obtained in the experimental trials were analyzed through a one-factor analysis of variance ANOVA using Minitab 20 Statistical Software (Minitab Inc., State College, PA, USA). These were the evaluation of tolerance, biomass as a function of the time the culture medium developed, and abundance of volatile compounds obtained at 120 h of

Table 2
Formulation of a culture medium for the mass reproduction of LAB, ABB, and yeast.

Microorganisms	Components of the culture medium	Percentage %
LAB	Citric acid	1
	Yeast extract	0,5
	Soy peptone	0,5
	Heptahydrated magnesium sulfate	0,05
	Monohydrated magnesium sulfate	0,02
	Tween 80	0,1
	Substrate (Whey)	20
AAB	Ethanol	0,5
	Yeast extract	1
	Soy peptone	0,5
	Pentahydrated calcium lactate	1
	Substrate (Orange pulp)	20
Yeast	Citric acid	1
	Yeast extract	0,5
	Soy peptone	0,5
	Substrate (Molasses)	20

fermentation. Fisher's test was used to evaluate and identify significant differences, considering a confidence level of 95 % ($p < 0.05$). In addition, a heat map was made to visualize the abundance of volatile compounds developed during spontaneous and starter culture fermentation at different times using XLSTAT 2023 software (Addinsoft, Boston, USA).

3. Results and discussion

3.1. Isolation of microorganisms from spontaneous culture by culture-dependent methods

During the 96 h of spontaneous fermentation, a total of 249 microbial isolates were obtained, corresponding to 12 microbial genera (Fig. 1). The fermentation was predominantly dominated by LAB (63 %), including the genera *Lacticaseibacillus, Lactiplantibacillus, Lentilactobacillus, Levilactobacillus, Liquorilactobacillus* and *Leuconostoc*. Meanwhile, 17 % of the isolates were of the *Saccharomyces* genus, and a minority percentage (2 %) were bacteria from the *Acetobacter* genus. The remaining 18 % of isolates included other spore-forming genera, *Bacillus, Peribacillus,* and *Priestia*, as well as non-spore-forming genera, such as *Staphylococcus*.

The initial fermentation phase was characterized by a varied microbial presence with genera *Priestia* (25 %), *Saccharomyces* (24 %), *Lactiplantibacillus* (18 %) and *Bacillus* (15 %) being the most prominent (Fig. 2a). As fermentation progressed, a shift favoring *Saccharomyces cerevisiae* and LAB, particularly *Lactiplantibacillus plantarum* and *Levilactobacillus brevis*, was noted (Fig. 2b). This change underlines the synergistic relationship between yeasts and LAB, facilitated by the ethanol and carbon dioxide produced by yeasts, which in turn supports LAB growth and activity. This interaction underscores a critical transition towards an environment increasingly dominated by LAB, markedly influencing fermentation's microbial succession and metabolic profile. On the other hand, the presence of *Priestia megaterium* has been reported as an indigenous species in fermentations of Forastero cacao from Ghana, Trinitario cacao from Trinidad, and hybrid cacao from Brazil, and during the aerobic phase of fermentation *B. cereus* and *P. megaterium* species can produce C3–C5 free fatty acids causing the sour taste of the chocolate [34]. While microorganisms belonging to other genera, such as *Leuconostoc*, *Staphylococcus*, and *Acetobacter*, were found in lower proportions (<10 %).

As the fermentation process progresses over time, there are changes in the microbial communities. At 24 h, there is an increase in the species *S. cerevisiae* (37 %), which is responsible for ethanol production and promotes LAB growth (48 %). This effect has been documented by Endo [35], who reported ethanol production in the range of 6.5–25 mg/g of pulp during these stages. The simultaneous growth of yeast and LAB is attributed to their non-competitive interactions, with the increase in LAB possibly favoring the yeast's carbon dioxide production [11]. Furthermore, there was a decrease in the species of the *Bacillus* genus (15 %). It is known that yeasts initiate and accelerate fermentation by degrading pectin in cacao pulp [36]. However, several studies have also demonstrated the ability of the genus *Bacillus* to produce pectinolytic enzymes [37,38]. Ouattara [39] evaluated the pectate lyase activity of several *Bacillus* isolated from spontaneous fermentation of Trinitario, Forastero, and Criollo cacao in Côte d'Ivoire, including species also isolated in this study (*Bacillus cereus* and *Bacillus pumilus*).

The resilience of specific strains to the changing fermentation environment is noteworthy, particularly *L. plantarum* and *L. brevis*, which demonstrated significant tolerance to high acidity and other stress factors. This adaptive capability suggests their potential to develop stress-tolerant starter cultures for more controlled fermentation processes to enhance flavor profiles and ensure consistency in cacao fermentation. Furthermore, exploring alternative media for culturing these resilient strains could offer insights into optimizing fermentation conditions, ultimately contributing to more sustainable and economically viable chocolate production.

At 48 h of fermentation, LAB continued to prevail in a majority (93 %); however, this period also signaled a gradual shift in the microbial landscape, leading to a reduced presence of *Saccharomyces cerevisiae (3 %)* and inhibition of *Bacillus* species, due to the accumulation of ethanol and lactic acid. At this stage, there was also the presence of species of *Staphylococcus* (5 %): *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*; their presence may indicate contamination by human contact with cocoa beans [28]. Next, the amount of *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* decreased considerably at 72 h of fermentation (18 and 29 %, respectively); apparently, the decrease of these competitive bacteria favored the growth of other LAB of the genus *Leuconostoc* (36 %). *Saccharomyces cerevisiae* remained present in low concentrations (4 %), as did *Staphylococcus epidermidis* (7 %).

The decrease in microbial diversity at 96 h of fermentation has been reported by Tigrero-Vaca [27]; this occurs due to the accumulation of various microbial inhibitors, such as the high content of organic acids. Pereira [40] obtained similar results; in analyses by

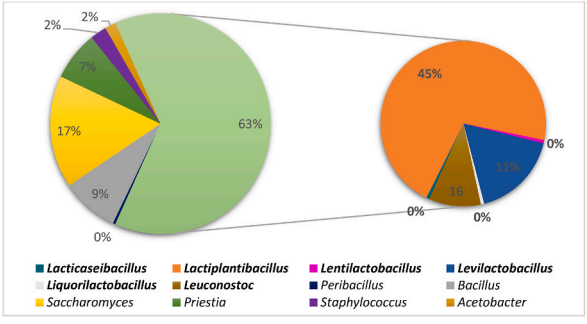

Fig. 1. Microbial genera isolated during spontaneous fermentation.

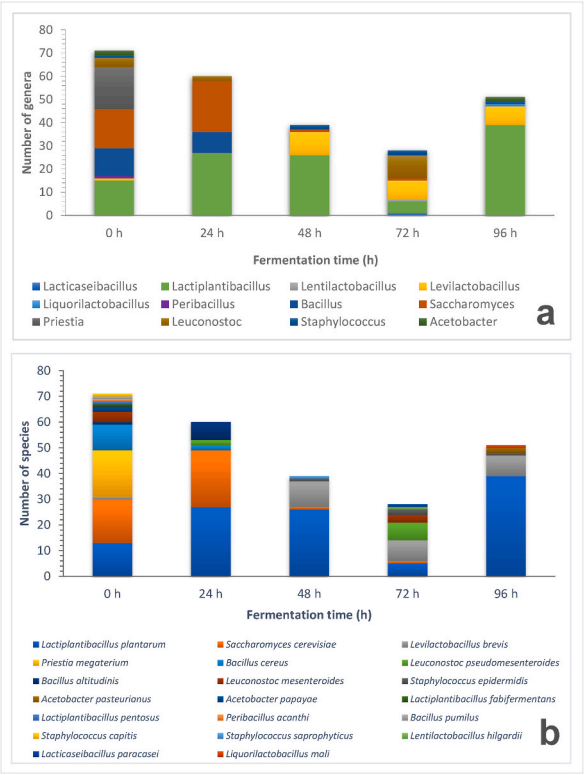

Fig. 2. Growth of microorganisms in a 96-Hour spontaneous fermentation (249 Isolates), classified by genera (a) and species (b).

culture-dependent methods, it was determined that *L. plantarum* dominated the spontaneous fermentation process of hybrid cacao in Brazil. Furthermore, it was reported that the heterofermentative behavior of these LAB allows them to dominate the later stages of cacao fermentation (92 %), primarily due to their increased resistance to stress factors such as acid concentration and higher efficiency in ATP production [41]. Finally, *S. epidermidis* continued to be present in low concentrations (2 %), along with *Acetobacter pasteurianus* species (4 %); the prevalence of this species in the last fermentation stage is due to their ability to oxidize ethanol, lactic acid, and mannitol, as well as their tolerance to acidity and heat [11].

The minority presence of AAB found in this study demonstrates that biological diversity is different according to geographic location and has also been shown to vary between regions or even from one farm to another [42,43]. While the spontaneous fermentation of Forastero cacao in Brazil was dominated by the *Acetobacteraceae* family, especially *Acetobacter* and *Gluconobacter* [44], in West African areas, the growth of acetic acid and lactic acid bacteria was minimal [45].

The observed variance in microbial community composition across different stages of fermentation underscores the dynamic interplay between various microbial species and their adaptive responses to the fermentative environment. These results contribute to a deeper understanding of microbial succession and interactions in cacao fermentation, offering valuable insights for optimizing fermentation processes to enhance desirable chocolate flavor profiles through microbial management strategies.

3.2. Selection of microorganisms based on tolerance to stress conditions

Microorganisms for initial selection were chosen based on their established roles in enhancing cacao fermentation. The LAB species *L. plantarum* and *L. fabifermentans* were selected to assess their stress resistance. *L. plantarum* has been used as a starter culture to improve the production of volatile compounds [18,22]; while *L. fabifermentans* is known for its capacity to grow in low pH, elevated temperatures, and high osmotic pressure [29]. Similarly, the tolerance of the two AAB strains isolated was evaluated. *A. pasteurianus* in the formation of a starter culture for cocoa fermentation improved the composition of volatile compounds [22], in addition, it can activate tolerant mechanisms against high acidity and high-temperature conditions [46], while the tolerance of *A. papayae*, to our knowledge, was assessed for the first time in this research. Finally, *S. cerevisiae*, as a dominant yeast in spontaneous fermentation processes, was directly included for its known contributions to aroma compounds such as esters and pyrazines [23]. On the other hand, *Bacillus* species, despite showing thermotolerance and acid tolerance in prior studies, were excluded due to their limited osmotolerance and ethanol tolerance [47], underscoring the importance of selecting microorganisms with broad-spectrum stress resistance for effective starter culture development.

In the evaluation of thermotolerance presented in Table 3, it was found that *L. plantarum* and *L. fabifermentans* exhibit optimal growth at 30 °C, achieving respective dry weight increases of 4.05 gL^{-1} and 0.92 gL^{-1}. As temperatures increased, both species

experienced significant declines in growth, culminating in a complete absence of growth at 45 °C. This pattern of thermosensitivity mirrors the observations of Korcari [29], who also reported that these bacteria tolerate temperatures up to 37 °C but show markedly reduced growth at 42 °C. On the other hand, in examining thermotolerance (Table 3), AAB displayed a lower growth rate than LAB. At an incubation temperature of 30 °C, *A. papayae* and *A. pasteurianus* exhibited growth rates of 0.15 gL^{-1} and 0.44 gL^{-1}, respectively. With increasing temperatures, a modest growth enhancement was observed in both AAB strains. Despite the lack of statistical significance, this trend indicated thermotolerance in both species: *A. papayae* recorded growth rates of 0.30 gL^{-1} at 37 °C and 0.33 gL^{-1} at 45 °C, while *A. pasteurianus* achieved dry weights of 0.31 gL^{-1} at 37 °C and 0.37 gL^{-1} at 45 °C. Although the differences in growth between temperatures were not statistically significant, the demonstrated thermotolerance of these bacteria is paramount, as it directly influences the physicochemical reactions throughout various fermentation stages. Notably, elevated temperatures ranging from 42 °C to 50 °C have been reported in later fermentation stages, correlating with a substantial increase in AAB growth [12,40,48]. The ability of these microorganisms to adapt and grow under varied thermal conditions directly could impact the quality and characteristics of cacao fermentation, making the understanding of their thermotolerance not just a matter of scientific interest but also practical importance for cacao fermentation practices. For subsequent experiments, the established control growth rate at 30 °C in MRS broth (pH 6.4 for LAB) and TSB broth (pH 5.6 for AAB) served as a benchmark. Furthermore, to determine whether a microorganism exhibits tolerance to various stress conditions, we have adopted the criteria proposed by Visintin [12], which suggest that if a microorganism does not achieve a growth rate exceeding 10 % relative to the control, it should be considered as not having grown, indicating that it is not tolerant.

Likewise, in Table 4, the results regarding pH tolerance are depicted. Among LAB, the species *L. plantarum* exhibited the highest tolerance growth at pH 5, surpassing the control by 38 % with a growth of 5.60 gL^{-1}, whereas *L. fabifermentans* showed lower growth, only reaching 15.2 % of the control (0.14 gL^{-1}). Notably, the stability of LAB was compromised at acidic pH levels of 2 and 3, where growth remained below 10 % compared to the respective controls. In contrast, AAB displayed remarkable growth capacity across all pH levels tested. *A. papayae* demonstrated superior tolerance compared to *A. pasteurianus*, with growth increasing proportionally with rising pH levels, achieving 0.25 gL^{-1}, 0.45 gL^{-1}, and 0.45 gL^{-1} at pH 2, 3, and 5, respectively, exceeding their respective controls. While *A. pasteurianus* growth at pH 5 was comparable to the control (0.39 gL^{-1}), its growth significantly declined at pH 2 and 3, dropping by 66 % compared to the control. Nevertheless, it maintained stability under these acidic conditions. Furthermore, the ability of starter cultures to withstand fluctuations in pH is crucial for their efficacy in cacao fermentation processes, where maintaining stable pH conditions is essential for the progression of fermentation and the development of desired flavor profiles.

During fermentation, significant fluctuations occur in the concentrations of ethanol, glucose, fructose, and acids. These changes are critical in shaping the microbial dynamics involved in cacao fermentation. The effects of these fluctuations on the behavior of LAB and AAB are of particular interest.

For instance, in the case of LAB, when *L. plantarum* was cultivated in MRS broth containing 6 % ethanol, it exhibited robust growth, reaching a dry weight of 6,09 gL^{-1}, which surpassed the control sample (Table 5). In contrast, *L. fabifermentans* showed deficient growth (0.16 gL^{-1}) under the same conditions. Moreover, neither species tolerated ethanol concentrations of 10 % and 12 %. On the other hand, AAB displayed differential responses to ethanol concentrations. For example, *A. pasteurianus* exhibited the highest growth in TSB broth containing 6 % ethanol, achieving a dry weight of 0.14 gL-1, which exceeded the control.

Additionally, there was a 13 % increase in growth compared to the control (0.06 gL^{-1}) when exposed to 10 % ethanol. However, neither AAB strain tolerated the 12 % ethanol concentration, as evidenced by the absence of growth. Similarly, *A. papayae* did not surpass the control and showed no tolerance to ethanol concentrations of 12 %. These observations underscore the importance of understanding microbial responses to ethanol fluctuations during cacao fermentation.

Considering the effects of glucose concentrations on LAB and AAB behavior, notable differences were observed in their growth patterns. For instance, when *L. plantarum* was subjected to MRS broth with varying glucose concentrations, it exhibited significant growth at concentrations of 5 % glucose (3.15 gL^{-1}) and fructose (1.64 gL^{-1}), representing 77 % and 40 % growth compared to the control, respectively (Table 6). This ability to thrive at moderate glucose levels aligns with findings by Hernandez [49], who noted similar tolerance in different serotypes of *L. plantarum*. However, the tested *L. plantarum* strain did not exhibit tolerance at 30 % sugar concentrations, suggesting a limit to its osmotolerance capacity. Conversely, *L. fabifermentans* demonstrated minimal growth (<10 %) across all tested glucose concentrations, indicating negligible osmotolerance capacity. In contrast, AAB strains showed varying degrees of osmotolerance to glucose concentrations. For example, *A. pasteurianus* displayed robust growth in TSB broth containing 5 % glucose (0.26 gL^{-1}) and fructose (0.29 gL^{-1}), representing 59 % and 65 % growth compared to the control. Remarkably, *A. pasteurianus* also tolerated concentrations of 15 % and 30 % glucose, highlighting its high osmotolerance capacity. Although *A. papayae* exhibited lower growth than *A. pasteurianus*, it showed tolerance to 5 % glucose (0.07 gL^{-1}) and fructose (0.09 gL^{-1}) concentrations, with 46 % and 60 % growth, respectively. However, its tolerance was markedly reduced at concentrations of 15 % and 30 %, falling below 20 %.

Table 3
Growth (gL^{-1}) of LAB in MRS broth and BAA in TSB broth at different incubation temperatures.

	L. plantarum	L. fabifermentans	A. papayae	A. pasteurianus
30 °C	4,05 ± 0, 21[a]	0,92 ± 0,05[a]	0,15 ± 0,03[a]	0,44 ± 0,03[a]
37 °C	2,59 ± 0,15[b]	0,40 ± 0,11[b]	0,30 ± 0,13[a]	0,31 ± 015[a]
45 °C	0,21 ± 0,09[c]	0,05 ± 0,02[c]	0,33 ± 0,16[a]	0,37 ± 0,14[a]

The data are presented as mean ± standard deviation. Different lowercase letters in the columns for each bacterium indicate significant differences ($p \leq 0.05$).

Table 4
Growth (gL^{-1}) of LAB and BAA in modified MRS and TSB broths at different pH concentrations compared to unmodified cultivation broth (control).

	L. plantarum	*L. fabifermentans*	*A. papayae*	*A. pasteurianus*
Control	4,05 ± 0,21[a]	0,92 ± 0,05[a]	0,15 ± 0,03[a]	0,44 ± 0,03[a]
pH 2	0,08 ± 0,07[b]	0,02 ± 0,02[b]	0,20 ± 0,03[ab]	0,15 ± 0,03[b]
pH 3	0,11 ± 0,06[b]	0,05 ± 0,02[b]	0,25 ± 0,02[b]	0,18 ± 0,03[b]
pH 5	5,60 ± 0,41[c]	0,14 ± 0,02[c]	0,45 ± 0,03[c]	0,39 ± 0,03[a]

The data are presented as mean ± standard deviation. Different lowercase letters in the columns for each bacterium indicate significant differences ($p \leq 0.05$).

Table 5
Growth (gL-1) of LAB and BAA in modified MRS and TSB broths at different ethanol concentrations compared to alcohol-free cultivation broth (control).

	L. plantarum	*L. fabifermentans*	*A. papayae*	*A. pasteurianus*
Control 0 %	4,05 ± 0,21[a]	0,92 ± 0,05[a]	0,15 ± 0,03[a]	0,44 ± 0,03[a]
Ethanol 6 %	6,09 ± 0,18[b]	0,16 ± 0,08[b]	0,06 ± 0,03[b]	0,14 ± 0,03b
Ethanol 10 %	0,36 ± 0,23[c]	0,05 ± 0,03[c]	0,04 ± 0,02[b]	0,06 ± 0,03c
Ethanol 12 %	0,04 ± 0,01[d]	0,02 ± 0,03[c]	0,02 ± 0,02[b]	0,02 ± 0,02 c

The data are presented as mean ± standard deviation. Different lowercase letters in the columns for each bacterium indicate significant differences ($p \leq 0.05$).

Table 6
Growth (gL^{-1}) of LAB and BAA in modified MRS and TSB broths at different carbohydrate concentrations compared to unmodified cultivation broth (control).

	L. plantarum	*L. fabifermentans*	*A. papayae*	*A. pasteurianus*
Control	4,05 ± 0,21[a]	0,92 ± 0,05[a]	0,15 ± 0,03[a]	0,44 ± 0,03[a]
Glucose 5 %	3,15 ± 0,29[b]	0,05 ± 0,02[bc]	0,07 ± 0,02[b]	0,26 ± 0,02[b]
Glucose 15 %	1,56 ± 0,22[c]	0,05 ± 0,02[bc]	0,03 ± 0,00[b]	0,13 ± 0,02[c]
Glucose 30 %	0,06 ± 0,05[d]	0,06 ± 0,03[b]	0,01 ± 0,02[b]	0,07 ± 0,02[d]
Fructose 5 %	1,64 ± 0,10[c]	0,06 ± 0,00[b]	0,09 ± 0,00[b]	0,29 ± 0,03[b]
Fructose 15 %	0,64 ± 0,10[e]	0,01 ± 0,02[c]	0,03 ± 0,00[b]	0,16 ± 0,02[c]
Fructose 30 %	0,09 ± 0,08[d]	0,07 ± 0,02[b]	0,02 ± 0,02[b]	0,13 ± 0,02[c]

The data are presented as mean ± standard deviation. Different lowercase letters in the columns for each bacterium indicate significant differences ($p \leq 0.05$).

Regarding the response to acids, only the two AAB species exhibited notable tolerance, attributed to their role in converting lactic acid, produced by LAB during fermentation, into acetic acid [49]. Notably, *A. papayae* displayed superior tolerance compared to *A. pasteurianus* (Table 7). A. papayae exhibited robust growth in 1 % lactic acid (60 %) and acetic acid (46 %), followed by considerable growth at concentrations of 3 % of these acids (46 % in lactic acid and 40 % in acetic acid). Conversely, A. pasteurianus showed its highest tolerance at 1 % lactic acid (38 % growth), with significantly lower growth rates observed in other concentrations (<29 %). This discrepancy elucidates the superior performance of A. papayae under low pH conditions, where it exhibited enhanced growth compared to the control culture media modified with a pH below 5.

Based on the previously analyzed results of the microbial responses to various stress conditions, it was observed that *L. plantarum* exhibited greater tolerance across different culture medium conditions, indicating its versatility and adaptability. While both *A. pasteurianus* and *A. papayae* displayed similar tolerances to high temperatures (45 °C), ethanol concentrations up to 10 %, and acidic

Table 7
Growth (gL^{-1}) of LAB and BAA in modified MRS and TSB broths at different concentrations of lactic acid and acetic acid compared to unmodified cultivation broth (control).

	L. plantarum	*L. fabifermentans*	*A. papayae*	*A. pasteurianus*
Control 0 %	4,05 ± 0,21[a]	0,92 ± 0,05[a]	0,15 ± 0,03[a]	0,44 ± 0,03[a]
Lactic acid 1 %	0,19 ± 0,03[b]	0,09 ± 0,03[b]	0,09 ± 0,00[b]	0,17 ± 0,02[b]
Lactic acid 3 %	0,17 ± 0,12[b]	0,07 ± 0,02[b]	0,07 ± 0,02[b]	0,13 ± 0,02[c]
Lactic acid 5 %	0,12 ± 0,05[b]	0,06 ± 0,03[bc]	0,03 ± 0,00[b]	0,08 ± 0,02[d]
Acetic acid 1 %	0,23 ± 0,05[b]	0,07 ± 0,03[b]	0,07 ± 0,02[b]	0,13 ± 0,02[d]
Acetic acid 3 %	0,19 ± 0,09[b]	0,04 ± 0,05[bc]	0,06 ± 0,00[b]	0,07 ± 0,02[d]
Acetic acid 5 %	0,17 ± 0,11[b]	0,01 ± 0,02[c]	0,04 ± 0,02[b]	0,07 ± 0,02[d]

The data are presented as ± standard deviation. Different lowercase letters in the columns for each bacterium indicate significant differences ($p \leq 0.05$).

conditions, *A. pasteurianus* demonstrated superior osmotolerance. Furthermore, the recovery and growth of *A. pasteurianus* consistently outperformed *A. papayae* in the tests conducted. Therefore, considering their robust performance and tolerance profiles, *L. plantarum*, *A. pasteurianus*, and *S. cerevisiae* were selected as the optimal microorganisms for the starter culture in cacao fermentation.

3.3. Design of an alternative culture medium to produce starter microorganisms

In biotechnological processes, scaling solutions to an applicable level poses a significant challenge. However, a promising strategy to address this challenge and enhance the production process of starter cultures involves utilizing agro-industrial wastes as a medium for production. This approach offers several advantages, including cost reduction using readily available waste materials. This study evaluated an alternative substrate medium (ASM) for each microorganism, incorporating whey, orange pulp, and molasses, aligning with a sustainable approach to biotechnology. The effects of different carbon sources on starter culture production (dry weight) are shown in Fig. 3. The growth of *L. plantarum* in the ASM was compared to that in CM over 72 h. In the initial 24 and 48 h of fermentation, *L. plantarum* exhibited higher growth in ASM, 4.91 gL^{-1} and 5.16 gL^{-1}, respectively, compared to CM (1.42 gL^{-1} and 1.31 gL^{-1}, respectively). This trend continued at 72 h, with significant growth observed in both ASM (6.95 gL^{-1}) and CM (2.58 gL^{-1}), indicating a time-dependent enhancement of *L. plantarum* growth in whey-based medium (Fig. 3a). Whey, known for its efficacy as a carbon source, played a pivotal role in enhancing the development of *L. plantarum*. It has previously been utilized for biomass production of thermophilic LAB [50] and probiotic *Lactobacilli* [51].

Furthermore, yeast extract was added to the medium to provide nitrogen, complementing whey's carbon content. Other components, such as citric acid (present in cacao pulp) and Tween 80 (facilitating nutrient absorption and acting as an anti-foaming agent), further supported LAB growth within the culture medium. Despite the promising potential of utilizing agro-industrial wastes as a medium for production, the growth profile of *A. pasteurianus* over the 72 h in the ASM medium is notably lower than that observed in CM. This observation persists despite supplementing the ASM medium with peptone and yeast extract, based on the recommendations

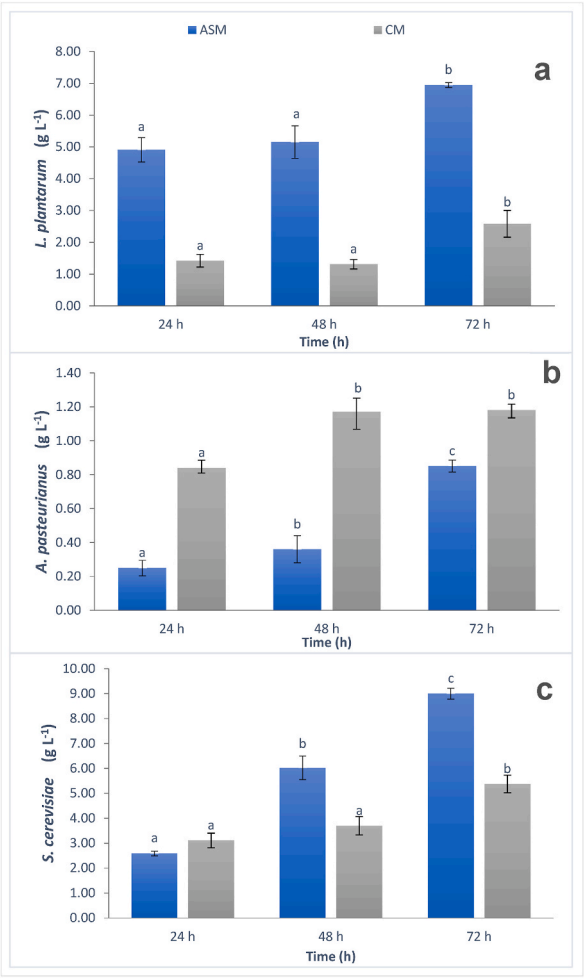

Fig. 3. Growth of microorganisms in a medium using cocoa pulp as control substrate (CM) and a medium with an alternative substrate (ASM). Different lowercase letters indicate significant differences ($p \leq 0.05$).

of Kurosumi [52], who demonstrated that adding a nitrogen source to the culture medium increased BAA production. Consequently, the ideal substrate for *A. pasteurianus* growth was found to be cacao pulp, as evidenced by a final growth at 72 h of 1.18 gL^{-1} in CM, compared to 0.85 gL^{-1} in ASM (Fig. 3b). This effect can be associated with cacao pulp composition that offers essential nutrients for bacterial growth such as glucose, fructose and sucrose, with a total content of 10–15 % and an optimal initial pH for AAB of 3.3–4.0, which is relatively low due to a high concentration of citric acid (1–3 %) [15]. Nevertheless, orange pulp has been used for bacterial cellulose production through *Acetobacter xylinum* [52] and *A. pasteurianus* [53].

The growth of *S. cerevisiae* was observed from 24 h of fermentation in ASM and continued to grow significantly at 48 and 72 h, reaching a microbial dry weight of 6.02 gL^{-1} and 8.9 gL^{-1}, respectively, while CM had significant growth (5.38 gL^{-1}) after 72 h of fermentation (Fig. 3c). The fermentation of *S. cerevisiae* in molasses has been extensively studied for ethanol production [54,55]. Its affinity for sugarcane molasses is due to the rich nutrient composition supporting the growth of this heterofermentative species [56]. Similarly, soy peptone and yeast extract ensured nitrogen availability, trace elements, and citric acid, also present in cacao pulp at the beginning of fermentation.

The utilization of agro-industrial wastes as alternative substrates in biotechnological processes holds immense promise for enhancing the production efficiency of starter cultures. However, it is crucial to delve deeper into additional optimization methods for fermentation media to realize the full benefits of starter culture development. This underscores the significance of ongoing research endeavors aimed at refining fermentation techniques explicitly tailored to the needs of starter culture cultivation, thus advancing biotechnological practices toward more efficient and sustainable production methodologies.

3.3.1. Identification of volatile compounds during fermentation with starter culture and spontaneous fermentation

The GC-MS analysis revealed 30 compounds detected during spontaneous fermentation (Control C) and with the starter culture (SC), in the heat map (see Fig. 4) are divided into two main groups: the first is formed by a group of alcohols, esters, and aldehydes where alcohols predominate at the beginning of fermentation (0 h) and the second group of compounds that are produced in the later stages of fermentation in a period of 96 h (from 24 to 120 h). The prominent aroma descriptors were sweet, fruity, and floral (Table 8). The dominant aromatic groups in both fermentation processes were alcohols with 12 compounds, 7 esters, 7 aldehydes and ketones, and 1 terpene. Additionally, compounds from other hydrocarbons and ethers (3), whose aroma descriptors have not been reported, were determined.

Predominant volatile compounds in fresh cacao (0 h) were alcohols 4-penten-2-ol with fruity aroma descriptor and 2-methylbut-3-en-2-ol of herbal aromas, which were only present in this initial stage, together with 2-Heptanol, an endogenous compound in Nacional cacao beans [57], which is attributed with fruity citrus aromas and remained throughout fermentation. These results coincide with the findings reported by Erazo [58], where 2-heptanol was the most abundant compound in fresh Nacional cacao. Similarly, in this stage, the ester with green flavor descriptors 2-heptanol-acetate also predominated; Hegmann [59] reported this compound as the primary ester found in the fresh pulp of fine-aroma cacao varieties.

From the first fermentation phase (24 h), 2-heptanol remained the most abundant compound along with ketones, which also increased in this phase; besides the ketone with fruity, coconut, cheese, floral aroma descriptors: 2-heptanone and the metabolite produced by *S. cerevisiae*, ethanol, were predominated. During alcoholic fermentation, yeasts synthesize short-chain alcohols from simple sugars, which can also be synthesized from a pyruvate molecule in all organisms. Oxidation of primary alcohols produces aldehydes, and oxidation of secondary alcohols produces ketones [60]. Similarly, 2-heptanone was the predominant compound at 48

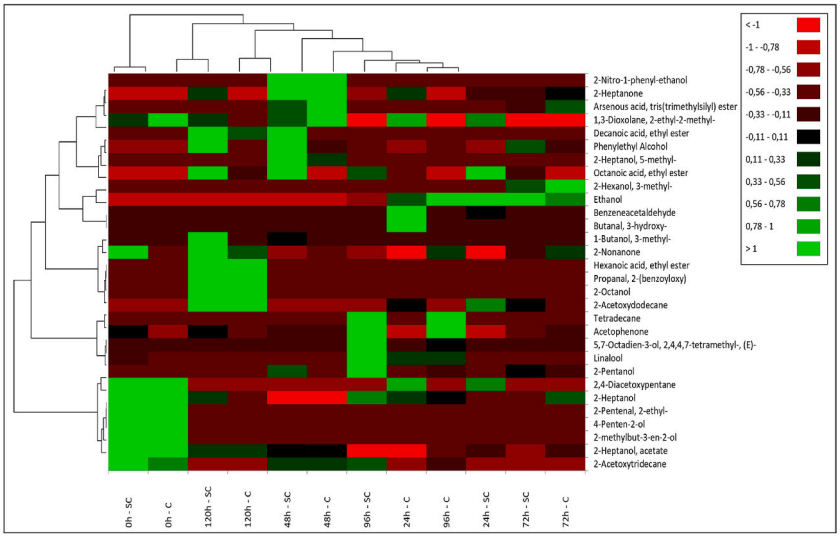

Fig. 4. Heat Map of volatile compound analysis in a spontaneous culture as control (C) and with starter culture (SC) during 120 Hours of fermentation.

Table 8
Volatile compounds and aroma descriptors identified during spontaneous fermentation and with starter culture.

Chemical group	Compounds	[a]Aroma descriptors	Fermentation time
Alcohols	2-methylbut-3-en-2-ol	Herbal	0 h
	4-Penten-2-ol	Fruity, floral	0 h
	2-Heptanol	Citrusy, fruity	0, 24, 72, 96 and 120 h
	Ethanol	Alcoholic	24, 72 and 96 h
	2-Pentanol	Green	48, 72 and 96 h
	1-Butanol, 3-methyl-	Sweet fruity	48 and 120 h
	2-Heptanol, 5-methyl-	NF	48 h
	2-Nitro-1-phenyl-ethanol	NF	48 and 120 h
	Phenylethyl Alcohol	Floral	48, 72 and 96 h
	2-Hexanol, 3-methyl-	NF	72 h
	5,7-Octadien-3-ol, 2,4,4,7-tetramethyl-, (E)-	NF	96 h
	2-Octanol	Fresh spicy green woody herbal earthy	120 h
Aldehydes	2-Pentenal, 2-ethyl-	Spicy green apple	0 h
	Butanal, 3-hydroxy-	Fruity green banana	24 h
	Benzeneacetaldehyde	Green	24 h
	Propanal, 2-(benzoyloxy)-, (R)-	NF	120 h
Ketones	Acetophenone	Sweet spicy hawthorn mimosa almond acacia chemical	0, 48, 72, 96 and 120 h
	2-Nonanone	fresh sweet green weedy earthy herbal	0, 48, 72, 96 and 120 h
	2-Heptanone	fruity spicy sweet herbal coconut woody	24, 48, 72, 96 and 120 h
Esters	2-Heptanol, acetate	Green	0, 24, 48, 72, 96 and 120 h
	2-Acetoxytridecane	NF	0, 48 and 96 h
	Hexanoic acid, ethyl ester	Fruity	120 h
	Octanoic acid, ethyl ester	Waxy sweet musty pineapple fruity with a creamy and milky undertone	24, 48, 72, 96 and 120 h
	2-Acetoxydodecane	Waxy	24, 72 and 120 h
	Arsenous acid, tris(trimethylsilyl) ester	NF	48 and 72 h
	Decanoic acid, ethyl ester	Sweet, waxy, fruity, apple	48 and 120 h
Terpene	Linalool	Floral, citrus	0, 24, 96 and 120 h
Ether	1,3-Dioxolane, 2-ethyl-2-methyl-	NF	0 and 120 h
Other hydrocarbons	2,4-Diacetoxypentane	NF	0, 24 and 48 h
	Tetradecane	NF	96 h

[a] Aroma descriptors from The Good Scents Company, www.thegoodscentscompany.com/index.html. Accessed April 5, 2024

and 72 h of fermentation, mainly in the control sample. Other alcoholic compounds that stood out at 72 h include ethanol and 2-heptanol, more abundant in the Ccontrol and phenylethyl alcohol, responsible for floral aromas, found in similar amounts in both cultures and associated with floral aromas.

However, the ethanol concentration decreased at 96 h of SC fermentation. A noteworthy increase in the abundance of alcohol metabolites 2-pentanol (green aroma), 2-heptanol, and terpene with an alcohol group linalool was observed, suggesting an accelerated carbohydrate metabolism for alcohol production and subsequent formation of secondary alcoholic metabolites by *L. plantarum*. In a biochemical study of the floral aroma of Nacional cacao conducted by Colonges [61], the compound Linalool was determined to be present in both roasted and unroasted fermented cacao, although it has also been reported in other cacao varieties such as CCN51, Criollo, Forastero and Trinitario [33,62].

Finally, at 120 h of fermentation, 15 aromatic compounds were determined. Certain alcoholic compounds with sweet, citric, fruity, and floral aroma descriptors predominated at this stage; notably, there was a significant increase in the abundance of compounds such as 1-butanol, 3-methyl-, phenylethyl alcohol, and 2-heptanol in cacao fermented with SC compared to C (Fig. 5a.). The presence of the sweet fruity aroma compound 1-butanol, 3-methyl- has been described after 72 h of fermentation of Nacional cacao inoculated with *S. cerevisiae* [27]. In the same way, there was a significant increase of the ketones responsible for the aroma's fresh, sweet green, weedy, earthy herbal, and fruity spicy-sweet herbal coconut woody: 2-heptanone and 2-nonanone that increased 67-fold and 2.4-fold, respectively, the abundance in SC compared to C, Viesser [14] also reported the presence of these ketones in coffee fermented with *L. plantarum*. In deficient amounts in both cultures was found the ketone acetophenone, produced by *A. pasteurianus* according to previous research [27], and the only aldehyde found at this stage was propanal, 2-(benzoyloxy)-, (R)- (Fig. 5b.).

Regarding ester compounds at the same stage of 120 h of fermentation, they were found in significantly equal amounts in both cultures (Fig. 5c.), with 2-heptanol-acetate predominating. The abundance of the other esters was lower, with fatty sweat cheese, sour notes, and waxy and moldy aroma descriptors, which are considered less desirable in cacao. Esters result from the esterification reaction, a reversible chemical reaction synthesizing an ester molecule and a water molecule from an alcohol molecule and an acid molecule [63].

In this research, no acidic volatile compounds were developed; apparently, *A. pasteurianus* did not influence the production of its primary metabolites (acetic acid) for the subsequent formation of acidic compounds, probably due to the minority percentage of isolated species in spontaneous fermentation and low growth in the recovery for following tests. However, it has been reported that an excess of acetic acid in cotyledons after fermentation results in chocolate with undesirable flavor [64], contributing to unpleasant sour,

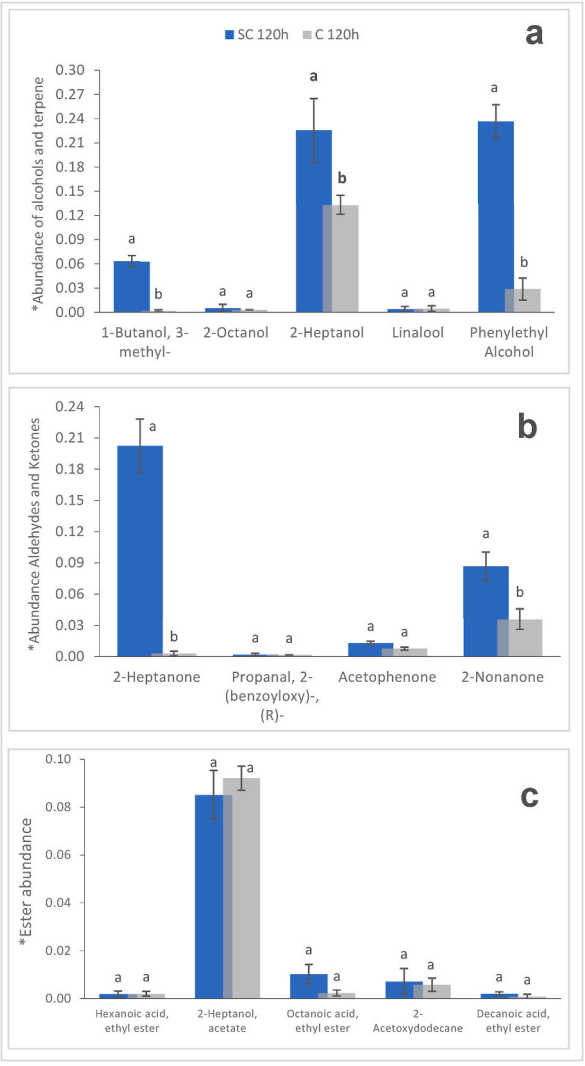

Fig. 5. Aromatic alcoholic compounds (a), aldehydes and ketones (b), and esters (c) produced at 120 Hours of fermentation with starter culture (SC) and spontaneous fermentation as control (C). * Abundance is the normalized peak area (unitless) of each compound.

astringent, and rancid notes [17,65]. In previous research with national cacao, similar results were obtained with the absence of acid compounds in a spontaneous fermentation of 96 h [61]; even with the inoculation of *A. pasteurianus*, no aroma-descriptor compounds were found [27].

This comprehensive analysis highlights the influence of bacterial starters on the volatile compound profile of cacao during fermentation. The changes observed in the volatile compounds contribute to the desired or undesirable aromatic properties of cacao, providing valuable insights for quality control and flavor enhancement in cacao processing.

4. Conclusions

The results elucidated a well-defined microbial interaction throughout the stages of cacao fermentation. Initially, yeasts such as *S. cerevisiae* dominated, followed by the sustained presence of LAB, particularly *L. plantarum*, throughout the fermentation process. The limited isolation of AAB suggests their minor role in this cacao variety or in the region where spontaneous fermentation occurred. The selection of bacterial strains like *L. plantarum* and *A. pasteurianus* as starter cultures was based on their demonstrated resilience to various stress conditions, complementing the automatic selection of *S. cerevisiae* as the sole yeast species isolated in spontaneous fermentation. The formulation of media with byproduct substrates from agroindustry is an economical alternative to produce *L. plantarum* and *S. cerevisiae*, as the culture media used for their isolation and production can be scarce and expensive.

The cacao fermented with the formulated starter culture (*S. cerevisiae, L. plantarum*, and *A. pasteurianus*) exhibited a notable enrichment in alcoholic compounds, with 2-pentanol, 2-heptanol, and phenylethyl alcohol being among the most predominant

throughout fermentation. This study successfully augmented the abundance of these alcohols, resulting in an enhanced profile of volatile organic compounds characterized by citrusy, green, fruity, and floral aromas. Furthermore, extended fermentation (120 h) facilitated improved production of the ketone 2-Heptanone and the ester 2-Heptanol acetate, contributing to favorable aromatic attributes such as fruity, spicy, sweet, herbal, coconut, woody, and green notes.

The utilization of *L. plantarum* and *S. cerevisiae* as starter cultures is advocated to accentuate the synthesis of aroma precursor metabolites in Nacional cacao, giving the limited contribution of *A. pasteurianus* to the production of acidic compounds and its sparse presence in spontaneous fermentation.

This research not only elucidates the complex microbial dynamics during cacao fermentation but also underscores the significance of selecting appropriate starter cultures to modulate aroma profiles effectively. The findings highlight the potential of tailored fermentation strategies in enhancing cacao products' quality and sensory attributes. This emphasizes the ongoing importance of research in optimizing fermentation processes for the cacao industry, paving the way for innovation and sustainable production practices.

Ethics approval

This article does not contain any studies with humans or animals.

Data availability statement

Data associated with this study has not been publicly availed in any repository. The data has been included in the Article/Supplementary Material/Referenced in article.

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CRediT authorship contribution statement

Maria Pilar Constante Catuto: Writing – original draft, Methodology, Formal analysis, Data curation. **Joel Tigrero-Vaca:** Methodology, Formal analysis, Data curation. **Mirian Villavicencio-Vasquez:** Methodology, Formal analysis, Data curation. **Diana Coello Montoya:** Writing – review & editing, Conceptualization. **Juan Manuel Cevallos:** Writing – review & editing, Funding acquisition, Conceptualization. **Jonathan Coronel-León:** Writing – review & editing, Visualization, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jonathan Corone Leon reports financial support, administrative support, article publishing charges, travel, and writing assistance were provided by CEDIA. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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