



## Research article

## Cocoa bean turning as a method for redirecting the aroma compound profile in artisanal cocoa fermentation

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## ABSTRACT

Two artisanal fermentation processes for *Criollo* cocoa beans with different turning start times (24 h and 48 h) were studied. The aromatic profile of cocoa turned every 24 h (B1) displayed volatile compounds associated with fermented, bready, and fruity aromas. When cocoa beans were fermented with a different turning technique with a start time of 48 h (B2), they provided volatile compounds mainly associated with descriptors of floral, woody, sweet, fruity and chocolate aromas. The turning start time of 48 h stimulated a microbial profile dominated by yeast such as *Hanseniaspora opuntiae*, *Pichia manshurica*, and *Meyerozyma carpophila*, favoring the production of several key aroma markers associated with cocoa bean fermentation quality, such as phenylethyl acetate, 2-phenylacetaldehyde, 3-methylbutanal, 2-phenylethyl alcohol, 2,3-butanedione, 3-methylbutanoic acid, and 2-methylpropanoic acid, while an immediate turning start time (24 h) favored an aerobic environment that stimulated the rapid growth of *Acetobacter pasteurianus*, *Bacillus subtilis* and a higher biodiversity of lactic acid bacteria (LAB) (e.g., *Lactobacillus plantarum* and *Pediococcus acidilactici*), which increased the production of ethyl acetate and 3-hydroxy-2-butanone. Volatile compound generation and microbial populations were evaluated and analyzed by multivariate analysis (principal component analysis and partial least squares discriminant analysis) to find correlations and significant differences. This study shows that the method of turning Criollo cacao beans can lead to the formation of desirable aromatic compounds.

## 1. Introduction

The cocoa aroma potential is one of the most important qualitative attributes that determine the economic value and acceptability of chocolate and chocolate-derived products (Magagna et al., 2017). Cocoa bean aroma depends on genotype (Qin et al., 2017), geographic origin (Marsegli et al., 2020), and postharvest processing (fermentation, drying, and roasting) (Frauendorfer and Schieberle, 2008; Mota-Gutierrez et al., 2018; Rodríguez-Campos et al., 2011), followed by winnowing, nib grinding, refining, conching and tempering (Ascrizzi et al., 2017), all of which impart high variability in the composition and quality of the cocoa bean's flavor, appearance, and texture. Several chocolate brands are interested in controlling each stage in the manufacturing of their

products, with much focus on the principal and crucial process of fermentation (Heimdal et al., 2014; Schwan and Wheals, 2004).

The fermentation of cocoa beans is a traditional artisanal process that involves spontaneous fermentation mediated by a series of microbial biochemical reactions that give rise to precursor compounds of the characteristic aroma of cocoa and its derived products (De Vuyst and Leroy, 2020) and constitutes a source of employment for a large number of people worldwide. The process begins with the manual removal of the cocoa beans from the pod; then, the beans are placed in heaps, boxes (wooden or plastic), or trays and finally covered with banana leaves and left to ferment for 5–7 days, depending on the origin and genotype of cocoa (Guehi et al., 2010; Moreira et al., 2018). A wide range of yeasts and bacteria appear throughout the fermentation process, increasing the

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cocoa pulp mass temperature above 45 °C and producing a liquid rich in ethanol, lactic acid, and acetic acid, which change color and yield several aromatic precursors by proteolysis and carbohydrate hydrolysis (Camu et al., 2008a). The fermentation method is generally based on generation-to-generation experience, resulting in very heterogeneous processes applied worldwide, which implies great variation in microbial growth and the generated metabolites according to cocoa genotype, origin, production method, batch size, pod ripeness, pod storage, and frequency of bean turning (or lack thereof) (Moreira et al., 2018).

Turning is a technique applied during fermentation that consists of mixing the cocoa-pulp mass to homogenize the temperature of the beans to improve the oxygenation of the mass, stimulating higher metabolism among the acetic acid bacteria (AAB), an increase in the maximum fermentation temperature, and thus acceleration of the fermentation (Camu et al., 2008b). The application of two turning times (the first at 48 h and a second at 96 h) during the fermentation of African cocoa beans has been previously studied. In Ghanaian cocoa bean heap fermentation (heaps of approximately 150 kg), Camu et al. (2008b) observed that turning stimulates the production of an unpleasant aftertaste with a strong sour flavor that does not exist in products derived from fermented cocoa beans without turning. Guehi et al. (2010) observed that compared to fermentation without turning, turning favored a lower production of lactic acid and stimulated the oxidation of ethanol into acetic acid and the oxidation of polyphenols, which could decrease the acidity and astringency of fermented mixed-hybrid cocoa beans from the Ivory Coast in wooden boxes and heaps (batch of 100 kg). Hamdouche et al. (2019) reported that turning improves the compositional quality of the volatile compound profile of fermented cocoa beans from the Ivory Coast in wooden boxes (batches of 30 kg), increasing the production of key chemical markers such as benzaldehyde, 2- and 3-methylbutanal and esters such as ethyl acetate, produced via the increased stimulation of lactic acid bacteria (LAB), and that of acetoin and acetic acid, favored by the greater stimulation of AAB. However, these studies have only been conducted in *Forastero* or hybrid cocoa genotypes. Therefore, it is necessary to study how the turning method can affect the artisanal fermentation of *Criollo* cocoa beans. An important reason for this is that the quality of the *Criollo* genotype is considered higher than that of the *Forastero* genotype, and its fermentation time is shorter, varying according to the fermentation grade desired (Castro-Alayo et al., 2019).

Farmers apply different fermentation processes to fresh cocoa pulp mass to improve the aroma profile and decrease the fermentation time. One of these processes involves the consecutive turning of cocoa beans with different turning start times (at 24 h or 48 h). Therefore, the aim of this work was to study the changes in microbial populations and volatile and aroma compounds due to the turning time in *Criollo* cocoa fermentation.

## 2. Materials and methods

### 2.1. Fermentation process, monitoring, and sampling

Samples of *Criollo* cocoa pods were harvested in October 2018 in Chiapas, Mexico (14°59'36.1"N 92°11'10.8"W). Cocoa pulp mass was spontaneously fermented via the wooden box method. The freshly cut pods were opened using a machete at the same site at which they were fermented. The beans were manually extracted and divided into two batches. Each batch was distributed into individual wooden boxes (0.79 m × 0.80 m × 0.75 m each), and the weight of each batch was 100 kg of cocoa pulp mass. Two experimental fermentation methods were evaluated: spontaneous fermentation with a first turning time of 24 h after placing the beans in the fermentation box and consecutive turning every 24 h (B1); and spontaneous fermentation with the first turning time of 48 h after placing the beans in the fermentation box and consecutive turning every 24 h (B2). The selection of the methods was performed following the artisanal practices applied. The turning method was carried out by transferring the cocoa pulp mass from one box by turning to another

empty box and distributing it evenly with a wooden shovel. The total fermentation time was 144 h for both batches. The end of fermentation was based on temperature, pH changes, and the death of the bean embryo, which was evaluated by the cocoa cut test according to ISO 2451: 2017, where the beans were cut lengthwise to expose the maximum cut-surface of the cotyledons. The results obtained in the cut tests for both fermentation boxes are shown in Table 1. Sampling was always carried out at the same time (every 24 h) for each fermentation time (0, 48, 72, 96, 120, and 144 h) and at the same depth (30 cm from the upper surface). The samples were placed in hermetically sterile bags. All samples were frozen at -20 °C and transported to the laboratory. Microbial and chemical analyses were carried out immediately. Environmental temperature, box temperature, pH value, and soluble solids (°Bx) were measured throughout the fermentation process. The pH was measured using a pH meter (pH Hanna HI98108). Soluble solids were measured with a refractometer, while the temperature was measured in the middle of the fermenting mass with a digital thermometer (Fluke t3000 FC).





### 2.2. Volatile compounds analysis

Analysis of volatile compounds were performed using the methodology described by Rodriguez-Campos et al. (2011). The volatile compounds were extracted by Head-Space Solid Phase Micro-Extraction (HS-SPME) using a 50/30- $\mu$ m Divinylbenzene/Carboxene/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco). The cocoa bean sample was ground with a pestle and mortar until obtaining a cocoa flour, then 2 g of the flour were placed in a head-space vial (of 10 mL) (Agilent) and sealed with a PolyTetraFluoroEthylene (PTFE) cap. The sample was equilibrated at 60 °C for 15 min, and then the fiber was exposed for 30 min at the same temperature. The volatile compounds were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent, 6890N), equipped with an Innowax® capillary column (60 m × 0.25 mm id × 0.25  $\mu$ m film thickness). The selective mass detector was a quadrupole (Agilent Technologies, 5975), with an electronic impact ionization system at 70 eV and at 260 °C. The GC oven temperature was programmed as follows: 40 °C for 5 min, with increments of 10 °C/min until reaching 200 °C, finally this temperature was maintained for 30 min. High purity helium was used as carrier gas at a flow of 0.7 mL/min. The injector was operated in splitless mode at 240 °C (0.5 min). Identification of volatile compounds were conducted comparing the mass spectra of the compounds in the samples with the National Institute of Standards and Technology (NIST/EPA/NIH v2.0 d 2004 Library, Gaithersburg, MD, USA) database with a match of at least 80% and through a comparison of the retention indices with injected pure standards (of the main VOCs studied) under the same sample conditions described above. The average relative abundance ( $n = 3$ ) of each compound was reported as a percentage of the normalized area of the corresponding peak. Aroma descriptors for the compounds identified were obtained by the online databases Flavornet (<http://www.flavornet.org/flavornet.html>), The Good Scents Company (<http://www.thegoodscentscompany.com/index.html>), and the literature.

### 2.3. Microbial ecology analysis

For the microbial analysis, a 5 g cocoa bean sample was vortexed with 10 mL of physiological solution for 5 min. The supernatant was placed in a 1.5 mL sterile Eppendorf tube, and different dilutions were then prepared, depending on the expected microbial counts. These were plated on Wallerstein (WL) agar (Sigma-Aldrich) for yeast enumeration, MRS agar (Sigma-Aldrich) for lactic acid bacteria, and GYC agar (25 g/L D-glucose, 5 g/L yeast extract, 10 g/L calcium carbonate, 7.5 g/L agar) for acetic acid bacteria. WL and YPD agar were supplemented with chloramphenicol (100 mg/L), and GYC and MRS agar were supplemented with nystatin (100 mg/L) to inhibit bacterial and fungal growth, respectively. The preparation of the samples, dilutions, and plating methods were performed according to the methodology described by Papalexandratou

**Table 1.** Comparison of results by cocoa bean cutting test.

Treatment	Weight of 300 cocoa beans (g)					Mouldy (%)	Slate beans (%)	Insect-damaged (%)
		Well fermented (%)	Partly purple and partly brown (%)	Violet (%)	Slightly over fermented (%)			
B1	402.9	82	4	0	12	0	2	0
B2	407.1	85	3	0	7	0	5	0

et al. (2019). Incubation was started *in situ* at ambient temperature (24–34 °C) for 24 h. A total colony count and morphological differentiation of the colonies that grew in the culture media were performed in duplicate. Then, a representative sample (square root of the clone colonies) was isolated on the different agar plates. The colony-forming units (CFU) were recorded and isolated. Microbial morphology served as isolation criteria.

#### 2.4. Microorganism identification by MALDI-TOF

Isolates were grown in their respective culture media and incubated at 28 °C for 18 h. Utilizing the direct transfer method, the biological material (single colony) as a thin film was transferred directly onto a spot on a polished steel MALDI target plate and covered with 1 µL of a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA). Each isolate was spotted in duplicate to evaluate reproducibility. Samples were then analyzed in a matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS) Microflex LT spectrometer (Bruker Daltonics) using MALDI Biotyper 3.1 software.

#### 2.5. Data processing and multivariate analysis (PCA and PLS-DA)

The physicochemical analysis was performed in triplicates ( $n = 3$ ). Experimental data are presented as the mean and standard deviation (SD). Analysis of variance (ANOVA) was performed, and Tukey's multiple range tests were conducted to determine the significant differences among the means. Mean differences were considered significant at  $p < 0.05$ . Data analysis was performed using the XLSTAT 2019. Ver. 2.3 software (Addinsoft, Boston, MA, USA).

The variables used in the principal component analysis (PCA) represent the normalized area of the chromatographic peaks obtained by GC-MS and to the CFU corresponding to the main microorganisms identified during fermentation. Each variable corresponds to the average of experimental points (analysis) performed in duplicate for the microbial analysis and triplicate for the analysis of the volatile compounds. A PCA biplot was constructed to identify differences in the dynamics of microbial growth and volatile compounds generated in the two fermentation methods studied. The data set consisted of 12 observations (different fermentation times of both methods) and 60 variables (52 volatile compounds and eight microorganisms). Previously, the complete matrix of volatile compounds (70 volatile compounds) was filtered, selecting only those compounds that were identified at the beginning and the end of the fermentation. Subsequently, the entire set of variables was transformed by mean-centering for the analysis.

Partial least squares discriminant analysis (PLS-DA) was applied to classify the volatile compounds generated according to the fermentation treatment employed. PLS-DA reduces the number of variables utilized in the model, combining the variables to calculate the factors that are most correlated with a class (method in this case). For the analysis, the qualitative dependent variables ( $Y$ ) were the methods (B1 or B2). The quantitative explicative variables ( $X$ ) were the volatile compounds. The qualitative explicative variables were the different fermentation times for both methods. The PLS-DA model was evaluated by exhaustive cross-validation. The data were transformed by mean-centering with a reduction of the aligned data based on a 95% confidence interval.

### 3. Results and discussion

#### 3.1. Volatile compounds profile

Seventy volatile compounds were identified during the evaluated cocoa bean fermentation treatments. The volatile compounds were classified into six chemical classes: volatile acids, alcohols, aldehydes, esters, ketones, and pyrazines. The number of volatile compounds and the relative abundance by chemical group are shown in Figure 1, while the percentage of the individual relative abundance for each compound at different fermentation times and the associated aroma descriptors are presented in Table 2.

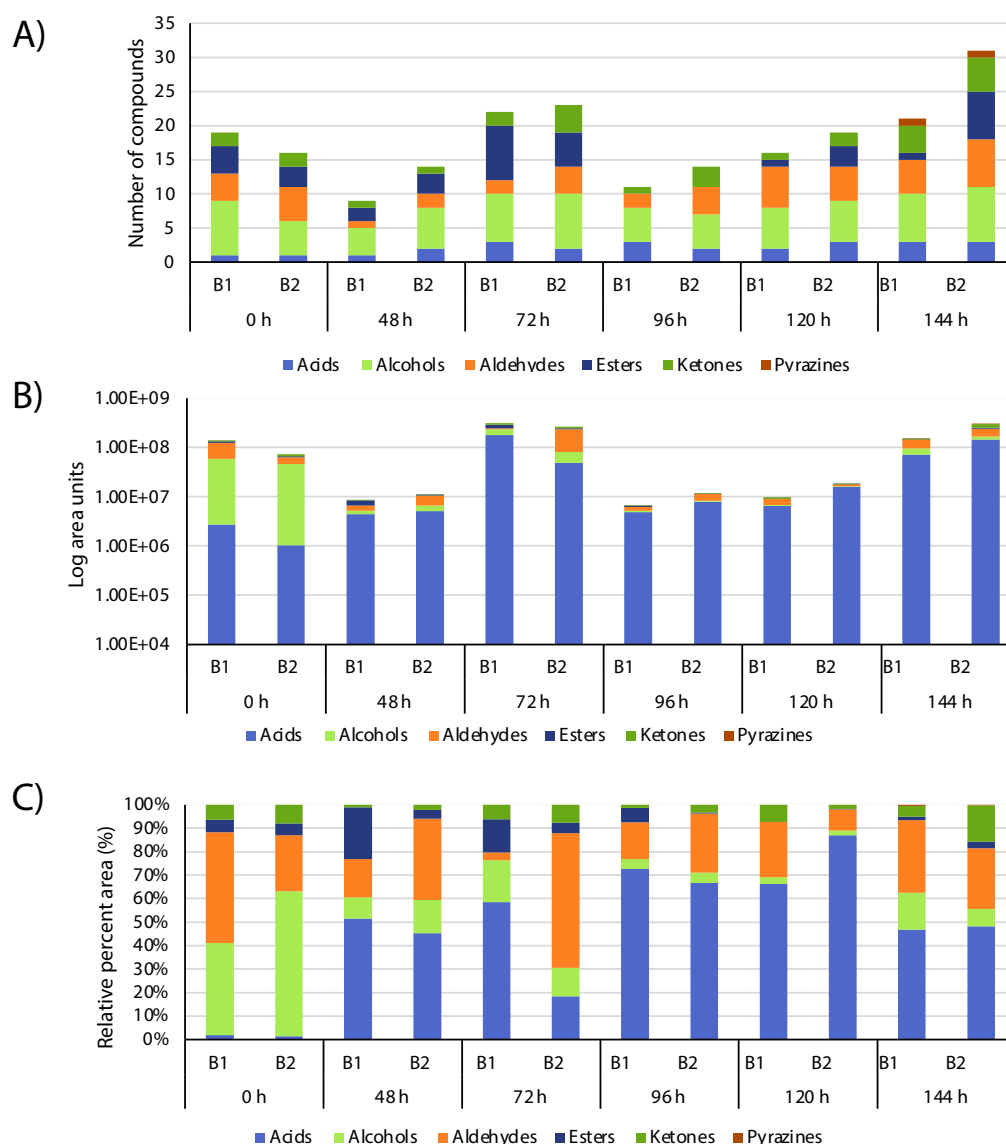
##### 3.1.1. Volatile acids

The studied fermentation processes showed slight differences in the volatile acid profile generated. Volatile acids represented between 46.72 and 48.16% of the final volatile profiles in both fermentation processes. The highest production of acetic acid and 3-methylbutanoic acid was observed in B2. Acids are considered to be key contributors to cocoa flavor (Qin et al., 2017). Acetic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid were the main odor-active volatile acids from fermented *Criollo* cocoa beans responsible for imparting sour, rancid, and intense vinegar-like notes that affect cocoa aroma quality (Frauendorfer and Schieberle, 2008; Magagna et al., 2017). Acetic acid has been associated with the highly acidic notes of chocolates derived from *Criollo* cocoa beans compared to those of the *Trinitario* and *Forastero* genotypes (Acierno et al., 2016). The high volatility of acetic acid causes its concentration to begin to be adversely affected after the increase in temperature of the roasting process, while 3-methylbutanoic acid remains unchanged, representing one of the highest odor activities of the roasted cocoa bean but with only minor influence on the concentrations of rancid (Frauendorfer and Schieberle, 2008). The results indicate that the turning technique that begins after 48 h of fermentation can stimulate the growth of yeasts and higher alcohol production converted into acids such as 3-methylbutanoic acid by AAB (mediated by dehydrogenation) or acetic acid by the degradation of citrate mediated by LAB (Ramos et al., 2020).

##### 3.1.2. Alcohols

The final volatile profile of the B1 fermentation process showed a higher percentage of alcohol compared to B2. High alcohol content is desirable to obtain cocoa products with flowery and sweet notes (Rodríguez-Campos et al., 2012).

The final profile of B1 was characterized by a higher content of 3-methyl-1-butanol, guaiacol (2-methoxyphenol), and 2-phenylethyl alcohol, which constitutes one of the most important volatile compounds within the profile of fermented *Criollo* cocoa beans because they are odor-active compounds that denote fruity, smoky, and floral notes, respectively (Frauendorfer and Schieberle, 2008). The final concentration of 2-phenylethyl alcohol in the fermented cocoa beans does not increase after the roasting process but instead remains nearly constant, indicating that its production level during fermentation is crucial in the floral notes characteristic of fermented *Criollo* cocoa beans (Castro-Alayo et al., 2019; Frauendorfer and Schieberle, 2008). The final profile of the B2 fermentation process was characterized by a higher content of



**Figure 1.** Volatile compounds identified by HS-SPME GC-MS during the fermentation of both boxes. A) Number of compounds in major classes of volatile in cacao samples; B) Logarithm of area units of major classes of volatile compounds; and C) Relative concentration of major classes of volatile compounds.

2-pentanol, 2,3-butanediol, 4,5-octanediol, and benzyl alcohol. 2-Pentanol has been reported to contribute to the aroma of fermented *Criollo* cocoa beans imparting floral notes (Qin et al., 2017), but its content is negatively affected by the roasting process (Mota-Gutierrez et al., 2019). 2,3-Butanediol confers sweet and floral notes in samples of *Criollo* cocoa and participates in condensation reactions with amino acids for the formation of methyl-substituted pyrazines (Heimdal et al., 2014). Benzyl alcohol, only detected under B2 fermentation, is associated with sweet and fruity notes of fermented cocoa beans, whose content can significantly increase during the roasting process (Heimdal et al., 2014). Mota-Gutierrez et al. (2018) observed a positive correlation between *A. pasteurianus* and 3-methyl-1-butanol and *H. opuntiae* and 2-pentanol in the fermentation of *Forastero* cocoa beans from Cameroon. Ho et al. (2015) reported the production of 2,3-butanediol by LAB from the citric acid of cocoa pulp. The production of L-phenylalanine by strains of *B. subtilis* has been reported (Castro-Alayo et al., 2019), and the conversion of phenylalanine into 2-phenylethyl alcohol can be mediated by yeast (Mota-Gutierrez et al., 2019). This could suggest that by stimulating the growth of AAB and *B. subtilis*, higher production of 3-methyl-1-butanol and 2-phenylethyl alcohol is favored in B1. In B2, the longer anaerobic time stimulated greater growth of yeast such as *H. opuntiae* and

therefore greater production of alcohols such as 2-pentanol and 2,3-butanediol. Moreover, yeasts participate in the esterification of alcohols (Cevallos-Cevallos et al., 2018). Therefore, the increase in yeast growth in the B2 fermentation process could have favored the increased conversion of these alcohols to their derived esters.

### 3.1.3. Aldehydes

The percentage of aldehydes in the final volatile profile was different between treatments (Table 2). The final volatile profile of the cocoa beans fermented via the B1 method showed a higher percentage of aldehydes than was obtained via the B2 method. The final profile of the B1 method was characterized by phenylacetaldehyde, nonanal, and pentanal. Among these compounds, only phenylacetaldehyde is a key aroma marker of chocolate, as it is one of the volatiles strongly related to the characteristic aroma of fermented *Criollo* cocoa beans, imparting notes such as honey (Magagna et al., 2017; Qin et al., 2017). The volatile profile of the B2 method was characterized by benzaldehyde, 2-methylpropanal, butanedial, 2-methylbutanal, and 3-methylbutanal. 3-Methylbutanal, 2-methylbutanal, and 2-methylpropanal are the main odor-active compounds of fermented *Criollo* cocoa beans associated with malty and fruity notes and related to their characteristic sugary taste

**Table 2.** Volatile compounds identified by HS-SPME/GC-MS in the treatments B1 and B2 at the different fermentation times (0, 24, 48, 72, 96, 120, and 144 h).

Rt	Compound name	Odor descriptor <sup>b</sup>	Percentage (%) <sup>a</sup> per fermentation time												
			0 h		24 h	48 h		72 h		96 h		120 h		144 h	
			B1	B2	B1	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2
<b>Acids</b>															
18.672	Acetic acid	Sour, vinegary	1.90 ± 0.09	1.41 ± 0.04	6.43 ± 0.04	51.48 ± 8.72	45.27 ± 2.13	58.12 ± 4.58	17.46 ± 1.33	69.70 ± 8.91	65.98 ± 1.36	64.28 ± 0.52	86.07 ± 14.01	35.85 ± 6.40	35.86 ± 3.28
19.928	Propanoic acid	Fruity, pungent	n.d.	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.01	n.d.	0.20 ± 0.02	n.d.	n.d.	n.d.	0.66 ± 0.02	0.42 ± 0.02
20.294	2-Methylpropanoic acid	Rancid	n.d.	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.01	0.29 ± 0.01	2.84 ± 0.15	0.77 ± 0.01	1.94 ± 0.16	0.83 ± 0.01	3.14 ± 0.46	3.15 ± 0.07
20.959	Propanedioic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00	n.d.	n.d.	n.d.
21.571	3-methylbutanoic acid	Rancid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.07 ± 0.56	8.74 ± 0.21
21.582	3-methylpentanoic acid	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.57 ± 0.04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	<b>total percentage</b>		<b>1.90 ± 0.09</b>	<b>1.41 ± 0.04</b>	<b>6.43 ± 0.04</b>	<b>51.48 ± 8.72</b>	<b>45.27 ± 3.74</b>	<b>58.55 ± 4.52</b>	<b>18.42 ± 1.34</b>	<b>72.73 ± 9.07</b>	<b>66.75 ± 1.37</b>	<b>66.30 ± 0.66</b>	<b>86.90 ± 14.02</b>	<b>46.72 ± 6.82</b>	<b>48.16 ± 3.37</b>
<b>Alcohols</b>															
9.195	Ethanol	Ethanol-like	16.88 ± 0.74	32.89 ± 1.99	n.d.	6.24 ± 0.01	9.94 ± 0.72	12.95 ± 0.69	n.d.	n.d.	n.d.	n.d.	0.21 ± 0.03	n.d.	n.d.
12.59	2-Methyl-1-propanol	Wine	0.96 ± 0.04	n.d.	n.d.	n.d.	n.d.	0.19 ± 0.00	0.23 ± 0.04	n.d.	n.d.	0.08 ± 0.01	n.d.	0.10 ± 0.00	0.08 ± 0.00
13.134	2-Pentanol	Light, seedy, sharp	2.85 ± 0.20	6.88 ± 0.51	n.d.	n.d.	0.87 ± 0.00	n.d.	1.20 ± 0.21	0.67 ± 0.06	n.d.	n.d.	0.25 ± 0.04	0.20 ± 0.02	0.49 ± 0.01
14.785	3-Methyl-1-butanol	Malty, chocolate	3.91 ± 0.27	n.d.	11.49 ± 1.25	n.d.	n.d.	0.57 ± 0.11	1.94 ± 0.08	n.d.	n.d.	n.d.	n.d.	0.62 ± 0.02	0.16 ± 0.00
14.826	1-Pentanol	Sweet, pungent	1.48 ± 0.11	2.88 ± 0.35	n.d.	1.18 ± 0.13	1.31 ± 0.24	n.d.	n.d.	0.96 ± 0.02	0.19 ± 0.02	0.67 ± 0.06	0.32 ± 0.02	n.d.	0.34 ± 0.01
14.965	2-Methyl-5-hexen-3-ol	-	n.d.	n.d.	n.d.	n.d.	n.d.	0.48 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16.611	2,3-Butanediol	-	0.62 ± 0.03	1.20 ± 0.08	n.d.	0.27 ± 0.01	0.44 ± 0.07	n.d.	n.d.	0.58 ± 0.00	0.14 ± 0.01	0.33 ± 0.00	0.15 ± 0.00	n.d.	0.17 ± 0.00
16.621	2-Heptanol	Citrusy, fresh, lemon grass-like	0.64 ± 0.04	0.98 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.00	n.d.	n.d.	n.d.	n.d.
16.631	3-Methyl-2-hexanol	-	n.d.	n.d.	1.76 ± 0.17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17.164	3-Methyl-2-butanol	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00
17.667	2-Methyl-5-hexen-3-ol	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.00	n.d.
19.134	2-Ethyl-1-hexanol	-	1.00 ± 0.05	n.d.	1.42 ± 0.01	n.d.	n.d.	n.d.	0.71 ± 0.07	n.d.	n.d.	n.d.	n.d.	1.05 ± 0.10	n.d.
19.462	2-Decanol	-	n.d.	n.d.	1.80 ± 0.11	n.d.	n.d.	0.06 ± 0.00	0.22 ± 0.01	n.d.	n.d.	n.d.	n.d.	0.34 ± 0.02	n.d.
19.472	2-Pentadecanol	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.30 ± 0.11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21.113	4,5-Octanediol	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00	n.d.	n.d.	0.15 ± 0.00
23.616	1-Phenyl-1-decanol	-	0.23 ± 0.01	0.44 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23.626	α-Methyl-benzene methanol	-	n.d.	n.d.	0.26 ± 0.00	n.d.	n.d.	0.22 ± 0.00	0.27 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24.488	Guaiaicol	Spicy	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00	n.d.	n.d.	0.20 ± 0.00	1.09 ± 0.05	0.16 ± 0.00	0.05 ± 0.00	2.75 ± 0.26	0.78 ± 0.03
24.652	Benzyl alcohol	Sweet, fruity	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.02
25.288	2-Phenylethyl alcohol	Honey-like	10.64 ± 0.86	14.58 ± 2.95	8.82 ± 0.07	1.37 ± 0.09	1.51 ± 0.00	3.27 ± 0.04	6.26 ± 0.82	1.72 ± 0.24	2.75 ± 0.11	1.53 ± 0.06	1.19 ± 0.06	10.70 ± 0.16	4.93 ± 0.23
	<b>total percentage</b>		<b>39.13 ± 0.83</b>	<b>61.65 ± 3.48</b>	<b>25.50 ± 1.20</b>	<b>9.06 ± 0.24</b>	<b>14.15 ± 1.31</b>	<b>17.76 ± 0.77</b>	<b>12.10 ± 1.12</b>	<b>4.13 ± 0.32</b>	<b>4.45 ± 0.19</b>	<b>2.78 ± 0.14</b>	<b>2.17 ± 0.18</b>	<b>15.78 ± 0.58</b>	<b>7.39 ± 0.29</b>
<b>Aldehydes</b>															
6.867	Butanal	-	0.76 ± 0.04	0.118 ± 0.04	0.59 ± 0.02	n.d.	n.d.	0.06 ± 0.00	0.15 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7.052	2-Methylpropanal	Green, pungent	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.01	0.02 ± 0.00	0.33 ± 0.03	1.16 ± 0.09
8.795	2-Methylbutanal	Malty, cocoa, chocolate, almond-like	0.83 ± 0.14	5.80 ± 0.82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.40 ± 0.02	0.72 ± 0.02
8.826	2,3-Dimethylpentanal	-	n.d.	n.d.	1.39 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8.898	Butanedial	-	2.74 ± 0.53	n.d.	n.d.	n.d.	5.14 ± 0.07	n.d.	n.d.	n.d.	n.d.	0.07 ± 0.00	n.d.	n.d.	0.11 ± 0.00
8.918	3-Methylbutanal	Malty, cocoa, chocolate	4.23 ± 0.34	5.51 ± 0.17	2.77 ± 0.50	n.d.	n.d.	0.20 ± 0.01	35.93 ± 1.59	n.d.	0.80 ± 0.03	1.70 ± 0.18	n.d.	n.d.	1.19 ± 0.08
8.997	Pentanal	Almond-like, malt	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.36 ± 0.52	n.d.
17.903	2-Methylpentanal	-	n.d.	n.d.	1.35 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.34 ± 0.01	n.d.	n.d.
17.985	Nonanal	Fatty, waxy, pungent	1.27 ± 0.12	1.31 ± 0.34	1.13 ± 0.13	n.d.	n.d.	n.d.	0.44 ± 0.06	n.d.	n.d.	n.d.	n.d.	0.39 ± 0.02	0.25 ± 0.01
20.098	Benzaldehyde	Almond, burnt sugar, bitter	n.d.	9.59 ± 1.32	n.d.	n.d.	n.d.	n.d.	3.65 ± 0.11	n.d.	1.80 ± 0.13	3.48 ± 0.39	0.53 ± 0.00	1.23 ± 0.09	3.03 ± 0.07
21.595	Phenylacetaldehyde	Honey-like	37.61 ± 5.66	0.50 ± 0.02	38.58 ± 0.65	16.32 ± 2.29	29.37 ± 0.93	3.16 ± 0.23	17.20 ± 1.87	14.97 ± 1.37	22.27 ± 1.77	17.59 ± 1.81	7.50 ± 0.13	23.14 ± 4.50	19.46 ± 0.38
23.79	Benzenebutanal	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.57 ± 0.01	0.13 ± 0.02	0.63 ± 0.02	0.61 ± 0.00	n.d.	n.d.
	<b>total percentage</b>		<b>47.36 ± 1.80</b>	<b>23.89 ± 1.66</b>	<b>45.81 ± 0.70</b>	<b>16.32 ± 2.29</b>	<b>34.51 ± 0.07</b>	<b>3.42 ± 0.24</b>	<b>57.37 ± 3.54</b>	<b>15.54 ± 1.31</b>	<b>25.02 ± 1.95</b>	<b>23.54 ± 1.79</b>	<b>8.99 ± 0.14</b>	<b>30.85 ± 4.66</b>	<b>25.91 ± 0.65</b>
<b>Esters</b>															
7.257	Methyl acetate	-	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.02	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.00	n.d.	0.20 ± 0.02

(continued on next page)

Table 2 (continued)

Rt	Compound name	Odor descriptor <sup>b</sup>	Percentage (%) <sup>a</sup> per fermentation time													
			0 h		24 h		48 h		72 h		96 h		120 h		144 h	
			B1	B2	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2	B1	B2
8.241	Ethyl acetate	Fruity, pineapple-like	3.39 ± 0.28	n.d.	n.d.	19.79 ± 1.40	2.55 ± 0.26	10.25 ± 0.46	n.d.	n.d.	n.d.	n.d.	0.09 ± 0.00	n.d.	n.d.	
11.102	Isobutyl acetate	Fruity	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 ± 0.01	0.17 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.		
12.262	2-Pentanol acetate	Green, fruity	n.d.	n.d.	n.d.	2.18 ± 0.10	1.03 ± 0.04	0.08 ± 0.00	0.16 ± 0.01	n.d.	n.d.	0.09 ± 0.01	0.09 ± 0.00	n.d.	0.16 ± 0.01	
15.370	Ethyl hexanoate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.14 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.		
17.164	2-Hydroxy-ethyl propanoate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.73 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.		
17.855	2,3-Butanediol diacetate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.21 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.		
18.41	Ethyl octanoate	Fruity, flowery	1.10 ± 0.02	1.60 ± 0.24	n.d.	n.d.	n.d.	0.10 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	0.39 ± 0.04		
19.462	2-Methoxymethyl-2-butanolate	-	n.d.	n.d.	n.d.	n.d.	0.28 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.61 ± 0.05		
21.103	Ethyl decanoate	-	n.d.	n.d.	n.d.	n.d.	0.36 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
21.482	Isopropyl propanoate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.28 ± 0.74	n.d.	n.d.	n.d.	n.d.		
21.493	Isopropyl methanoate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.49 ± 0.02	n.d.	n.d.	n.d.		
22.58	Benzyl-3-hydroxy propanoate	-	0.11 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06 ± 0.00		
23.318	1-Phenylethyl acetate	Fruity, sweet	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.46 ± 0.01	n.d.	n.d.	n.d.	n.d.	0.25 ± 0.02		
23.906	<b>2-Phenylethyl acetate</b>	Flowery, honey-like	n.d.	n.d.	n.d.	n.d.	n.d.	2.19 ± 0.14	1.73 ± 0.02	n.d.	n.d.	n.d.	n.d.	1.53 ± 0.07	1.09 ± 0.07	
27.141	Isopropyl myristate	-	n.d.	n.d.	n.d.	n.d.	n.d.	0.51 ± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
32.052	Methyl hexanoate	-	n.d.	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
33.011	Ethyl palmitate	Waxy, green	0.46 ± 0.05	3.51 ± 0.84	n.d.	n.d.	n.d.	0.37 ± 0.01	0.37 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.		
48.313	Ethyl linoleate	-	0.14 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
	<b>total percentage</b>		<b>5.20 ± 0.35</b>	<b>5.11 ± 1.08</b>	<b>0.00</b>	<b>21.97 ± 1.50</b>	<b>3.86 ± 0.30</b>	<b>14.15 ± 0.71</b>	<b>4.33 ± 0.08</b>	<b>6.28 ± 0.74</b>	<b>0.49 ± 0.02</b>	<b>0.09 ± 0.01</b>	<b>0.25 ± 0.01</b>	<b>1.53 ± 0.07</b>	<b>2.76 ± 0.21</b>	
<b>Ketones</b>																
7.411	4-Hydroxy-2-butanone	-	n.d.	n.d.	0.68 ± 0.14	n.d.	n.d.	n.d.	4.27 ± 0.16	n.d.	n.d.	n.d.	n.d.	n.d.		
10.344	<b>2,3-Butanedione</b>	Buttery, creamy	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.87 ± 0.10	7.29 ± 0.10	0.99 ± 0.02	n.d.	8.69 ± 0.20		
11.102	3-Methyl-2-butanone	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.16 ± 0.00	n.d.	n.d.	n.d.	0.34 ± 0.02	0.11 ± 0.00		
13.523	3-Penten-2-one	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17 ± 0.01		
14.529	2-Heptanone	Sweet, fruity	0.56 ± 0.05	0.82 ± 0.11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
14.508	4-Methyl-2-hexanone	-	0.35 ± 0.00	0.69 ± 0.07	5.71 ± 0.08	n.d.	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.00	n.d.	n.d.	0.27 ± 0.03		
16.457	<b>3-Hydroxy-2-butanone</b>	Buttery, creamy	n.d.	n.d.	8.99 ± 0.58	n.d.	n.d.	5.48 ± 0.69	4.37 ± 0.67	n.d.	n.d.	n.d.	2.73 ± 0.12	3.68 ± 0.05		
17.903	2-Nonanone	Milk, green, fruity	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.50 ± 0.06	n.d.	n.d.	n.d.	n.d.	0.53 ± 0.01		
21.739	Acetophenone	Must-like, flowery, sweet, almond	5.50 ± 0.05	6.43 ± 1.78	6.09 ± 0.94	1.16 ± 0.16	2.20 ± 0.30	0.64 ± 0.04	2.85 ± 0.01	1.32 ± 0.12	1.35 ± 0.04	0.00	0.71 ± 0.01	1.01 ± 0.19	2.57 ± 0.14	
	<b>total percentage</b>		<b>6.41 ± 0.05</b>	<b>7.94 ± 1.78</b>	<b>22.26 ± 1.74</b>	<b>1.16 ± 0.16</b>	<b>2.20 ± 0.30</b>	<b>6.12 ± 0.73</b>	<b>7.78 ± 0.74</b>	<b>1.32 ± 0.12</b>	<b>3.29 ± 0.14</b>	<b>7.29 ± 0.10</b>	<b>1.70 ± 0.03</b>	<b>4.61 ± 0.34</b>	<b>15.50 ± 0.42</b>	
<b>Pyrazines</b>																
17.09	<b>2,5-Dimethylpyrazine</b>	Earthy, chocolate, nutty	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.51 ± 0.04	0.28 ± 0.01	
	<b>total percentage</b>		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<b>0.51 ± 0.04</b>	<b>0.28 ± 0.01</b>	

Rt= Retention time in minutes. n.d = not detected.

<sup>a</sup> Percentage of compound based on the area normalization.<sup>b</sup> Odor descriptor shows according Magagna et al. (2017), Rodriguez-Campos et al. (2011), Hinneh et al. (2019); and Ascriczzi et al. (2017).

(Castro-Alayo et al., 2019; Frauendorfer and Schieberle, 2008). These compounds are generated from isoleucine and leucine by LAB (Hamdouche et al., 2019). Phenylacetaldehyde production has been related to the genus *Acetobacter* (Mota-Gutierrez et al., 2019). This could indicate that the rapid aeration of the cocoa pulp mass under the B1 method stimulated the growth of AAB, which favored the production of phenylacetaldehyde. The anaerobic environment generated under the B2 method stimulated the growth of LAB, favoring the production of a greater number of odor-active aldehydes, which are characteristic of the fermented *Criollo* cocoa bean.

#### 3.1.4. Esters

At the end of fermentation, the B2 method produced a volatile profile with a higher percentage and higher number of esters than the B1 method, which only presented 2-phenylethyl acetate (Table 2). Esters are characteristic volatile compounds of products derived from *Criollo* cocoa beans. This is due to their attractive sweet, flowery, and fruity aroma descriptors, such as 2-phenylethyl acetate, which are associated with notes similar to honey and are therefore recognized as key aroma markers of cocoa (Koné et al., 2016; Magagna et al., 2017; Moreira et al., 2018). The production of esters is important in cocoa fermentation since the ester concentration produced in this process is maintained even after roasting the cocoa bean, which strongly contributes to the final aroma profile of the derived cocoa product (Frauendorfer and Schieberle, 2008). Esters are produced from the esterification of alcohols mediated by yeasts (Cevallos-Cevallos et al., 2018; Ouattara et al., 2020). Yeasts such as *H. opuntiae* have been associated with 2-phenylethyl acetate and ethyl hexanoate production (Hu et al., 2018). Esters are mostly produced by the synergy between yeasts and LAB in the anaerobic stage of fermentation (da Silva Vale et al., 2019; Hamdouche et al., 2019). The results of this study could indicate a greater production of esters under the B2 method due to the lower aeration incorporated into cocoa-pulp mass as compared to the B1 method.

#### 3.1.5. Ketones

The B2 fermentation method presented a higher percentage and number of ketones, highlighting the presence of 2,3-butanedione, followed by 3-hydroxy-2-butanone and acetophenone than the B1 fermentation method. Ketones such as 2,3-butanedione and 3-hydroxy-2-butanone are recognized technological markers of cocoa processing and are important contributors to the buttery and creamy notes of chocolate (Magagna et al., 2017). Ketones are compounds that are naturally present in cocoa beans, and their content decreases throughout fermentation by degradation or microbial catabolism (Hamdouche et al., 2019). However, some ketones, such as acetophenone, are produced by *H. opuntiae* (Hu et al., 2018), and 3-hydroxy-2-butanone can be produced by bacteria of the genus *Bacillus* spp., favoring increases in these compounds under anaerobic conditions and high temperatures (Ouattara et al., 2020), as occurred under the B2 fermentation method.

#### 3.1.6. Pyrazines

Only the presence of 2,5-dimethylpyrazine was detected in the last stages of both fermentation methods, and a slightly higher production of 2,5-dimethylpyrazine was observed under the B1 method than in B2. Pyrazines in cocoa beans are formed only during the roasting process. However, some methylpyrazines can originate during fermentation due to the enzymatic activity of *Bacillus* spp. (Koné et al., 2016). Therefore, the high production of 2,5-dimethylpyrazine could be associated with the growth of *Bacillus* during the B1 fermentation process.

Table 3 shows the volatile compounds identified that presented greater correlations with the specific time of the fermentation stage, associated with their aroma descriptor. It is interesting that the T0 for B1 and B2 highlight different volatile compounds despite coming from the same batch (same origin) of harvest, but this is due to the natural variability of spontaneous fermentation. These variations may depend on the type of cocoa, farm, maturity of the pods, variations in the pulp/bean

ratio, climatic conditions, as has been described in other studies (Camu et al., 2008b; Hamdouche et al., 2019). This difference is contrasted with the microbial communities that were similar in both experiments (T0), this being key for the generation of aromas. It was considered important to contrast both fermentation processes of cocoa beans at 72 h because at that time, both boxes already had cocoa turns, and their microbiological and physicochemical environment was significantly different. The final profile of volatile compounds showed that the B1 method produced notes associated with fermentation and fruit trees, while the B2 method produced a greater diversity of notes, such as chocolate, fruit, floral, and woody notes.

### 3.2. Microbial community dynamics during fermentation processing

The evolution of the CFU of yeasts, LAB, AAB, and spore-forming bacteria under the studied fermentation methods is presented in Figure 2. The initial yeast counts were 6.71 and 7.28 log CFU/g under the B1 and B2 fermentation methods, respectively, and a yeast population domain characterized by *Hanseniopsis opuntiae* and *Pichia manshurica* was observed. The initial yeast counts were similar to those reported by Papalexandratou et al. (2019) in the fermentation of Nicaraguan *Criollo* cocoa beans (6.00–7.50 log CFU/g). *H. opuntiae* and *P. manshurica* are among the main yeasts isolated at the beginning of cocoa bean fermentation, possibly due to their tolerance to low pH levels and high citrate concentrations (Hamdouche et al., 2019; Papalexandratou et al., 2019). In the subsequent fermentation stages, the yeast population was characterized by pronounced differences between methods. The B2 method favored greater yeast growth compared to the B1 method. Additionally, under the B2 method, a higher taxonomic group and greater yeast biodiversity, represented by *H. opuntiae*, *P. manshurica*, and *Meyerozyma carpophila*, was observed from 48–120 h (Figure 2B). Under the B1 method, only *P. manshurica* was maintained until 120 h, *H. opuntiae* growth only occurred from 0–72 h, and *M. carpophila* was not detected (Figure 2A). Yeasts play an important role at the beginning of cocoa bean fermentation due to the reduction of pectin in sugars for the production of ethanol, decreasing the viscosity of the mass and causing cocoa pulp drainage at the bottom of the fermentation box (De Vuyst and Weckx, 2016; Garcia-Armisen et al., 2010). Prolonging the start time of the cocoa pulp mass turning process can promote an anaerobic environment and more substrates available for the growth and biodiversification of yeast (Figuroa-Hernández et al., 2019; Koné et al., 2016; Serra et al., 2019). The rapid temperature rise above 45 °C under the B1 method (Figure 3) could inhibit the growth of some yeast species relative to the B2 method, in which the temperature peak was reached at a later time (Daniel et al., 2009). The growth of *Kodamaea ohmeri* was observed during the last stages of B1 fermentation (Figure 2A), where *H. opuntiae* and *P. manshurica* were no longer detectable, and the last species was not identified in B2. This may be associated with the high concentration of acetic acid, which acts as a yeast growth inhibitor, to which *K. ohmeri* presents demonstrated resistance, taking advantage of the sugars available in the medium (Sharma et al., 2018).

Interestingly, it was observed that in both fermentation processes, one of the common yeasts involved in cocoa bean fermentation, *Saccharomyces cerevisiae*, was not identified. The results are in accord with those reported by Hamdouche et al. (2019) in the fermentation of cocoa beans from the Ivory Coast using a culture-independent technique (PCR-DGGE) for microbial identification and by Pacheco-Montealegre et al. (2020) in the fermentation of Colombian cocoa beans using a high-throughput sequencing technique. Although these studies employed more advanced technologies to screen microbial communities, the yeast *S. cerevisiae* was not detected. This could indicate that its absence is more related to an effect of the environment or cocoa genotype, rather than to the microbial identification technique. Arana-Sánchez et al. (2015) identified *S. cerevisiae* in the fermentation of *Forastero* cocoa beans from the Mexican state of Tabasco, while our study was carried out with *Criollo* cocoa from the Mexican state of Chiapas. This seems to indicate that this

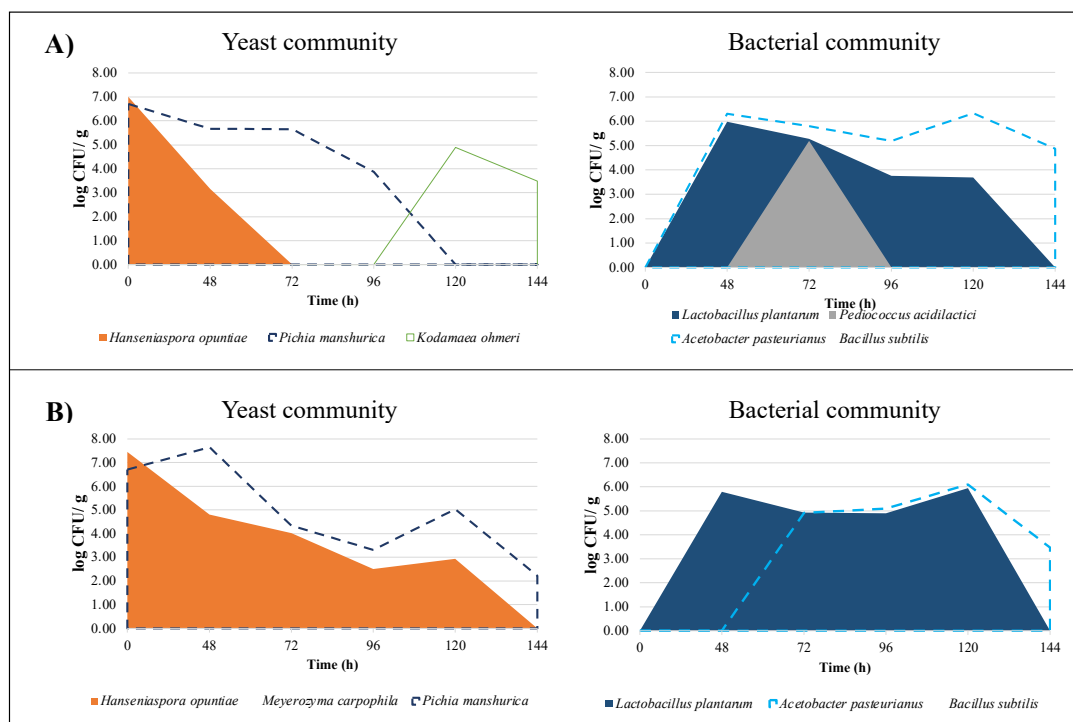
**Table 3.** Volatile compounds identified in the initial stages (0 h), when both fermentation boxes had cocoa bean turns (72 h) and at the end of fermentation (144 h), associated with their aroma descriptors.

Volatiles compounds	Fermentation time	B1	Aroma descriptor	B2	Aroma descriptor
Volatiles compounds	Beginning (0 h)	Nonanal 2,3-butanediol 2-methyl, 1-propanol 1-pentanol 2-pentanol	Waxy, aldehydic, rose Creamy, fruity Ethereal, winey Fermented, sweet Fermented, green	Butanal 2-heptanol 2-heptanone	Chocolate, cocoa Fresh, lemon, grass Fruity, spicy, sweet
	Turning (72 h)	Isopropyl myristate Methyl hexanoate Ethanol	Oily, fatty Fruity, pineapple, ether Alcohol	Ethyl benzene acetate Benzaldehyde Acetophenone 2-Methylbutanal Ethyl hexanoate	Floral, honey, rose Bitter, almond Sweet, almond, acacia Chocolate, cocoa Pineapple, fruity
	Final (144 h)	2-decanol 2-methyl,5-hexen-3-ol Pentanal	Fermented, bready, fruity	Guaiacol 2-pentanol acetate 2,5-dimethyl pyrazine 2-methyl propanoic acid Methyl acetate 3-methylbutanoic acid 2,3-butanedione 3-methyl, 2-butanol 2-methylpropanal Benzyl alcohol	Woody, spicy Herbal, green, musty Chocolate Acidic, sour, cheesy Ethereal, sweet, fruity Cheesy, sweet, fruity Buttery, creamy Fruity, musty, cocoa Floral, fresh Floral, rose

phenomenon is strongly associated with the cocoa genotype and/or indigenous microorganisms (De Vuyst and Leroy, 2020). However, further studies are necessary to confirm this phenomenon.

After the yeast group, the bacterial community appeared at 48 h under both studied fermentation processes but showed different dynamics and a different biodiversity profile. Under the B1 method (Figure 2A), the bacterial community initially showed the predominance of LAB, including *Lactobacillus plantarum* and *Pediococcus acidilactici*, AAB represented by *Acetobacter pasteurianus*, and spore-forming bacteria such as *Bacillus subtilis*. under the B2 method (Figure 2B), the microbial population was dominated by *L. plantarum* at 48 h, followed by *A. pasteurianus* after 72 h and finally *Bacillus subtilis* at the end of

fermentation. *P. acidilactici* was not identified in B2. LAB and AAB constitute the microbial groups that dominate the typical fermentative process of cocoa beans after yeasts. *L. plantarum* was the main LAB identified during cocoa bean fermentation and is involved in the assimilation of glucose, citric acid, and fructose for the production of ethanol, lactic acid, and mannitol (Nielsen et al., 2013). Camu et al. (2008b) observed that during fermentation in heaps of Ghanaian cocoa beans, turning (with two turns at 48 h and 72 h) did not have a clear influence on LAB growth, but they suggested that it favors the growth of *Lactobacillus fermentum* rather than that of *L. plantarum*. However, *L. fermentum* was not identified in this work, and *L. plantarum* was instead the dominant LAB in both fermentation processes studied. This difference in the

**Figure 2.** Relative abundance expressed on a logarithmic scale of yeast community (left side) and bacterial community (right side) at different fermentation times (0, 48, 72, 96, 120, and 144 h) of both fermentation methods studied: A) fermentation of fresh cocoa beans and with first turning at 24 h; and B) with first turning at 48 h.



results could be associated with the behavior of the cocoa bean genotype and/or the autochthonous microbiota.

*A. pasteurianus* is an AAB that participates in the oxidation of ethanol into acetic acid (Illegheems et al., 2015; Ramos et al., 2020) and has been identified as the predominant AAB population in fermentation processes in heaps with turning compared to nonturned heaps (Camu et al., 2008b). This indicates that *A. pasteurianus* plays a very significant role in cocoa bean fermentation with turning, regardless of the cocoa bean genotype. *Bacillus* spp. is involved in depectinizing the cocoa-pulp mass, increasing the permeability of the beans that connects the reactions occurring in the outer part of the bean (by microbial activity), and triggering reactions inside the bean (Ouattara et al., 2017). *A. pasteurianus* and *Bacillus* spp. growth is favored under aerobic conditions and high temperatures (Camu et al., 2008b; Hamdouche et al., 2019; Illegheems et al., 2015). Therefore, the greater oxygenation under the B1 fermentation method may have rapidly stimulated the growth of AAB and *Bacillus* spp. relative to B2. An antagonistic interaction has been reported in the growth of co-cultures of yeast strains (*Pichia* spp.) and *B. subtilis* in simulated cocoa-pulp media (Ouattara et al., 2020), which could explain the lower growth of the *Bacillus* genus under the B2 method, in which yeasts dominated the fermentation. *A. pasteurianus* is a competitive species in an acid- and ethanol-rich environment that favors the poor or non-growth of *L. plantarum* (Camu et al., 2008b). This explains the decrease in LAB in later stages, during which the maximal peak of AAB appeared under the B1 method (Figure 2A), while the AAB peak appeared after 48 h under the B2 method, favoring greater LAB growth in the final fermentation stages (Figure 2B). The growth of *P. acidilactici* under the B1 method could have increased due to the higher frequency of cocoa bean turning than in B2. However, its participation in fermentative bacterial succession seems to be antagonized by *L. plantarum* (Thu et al., 2011).

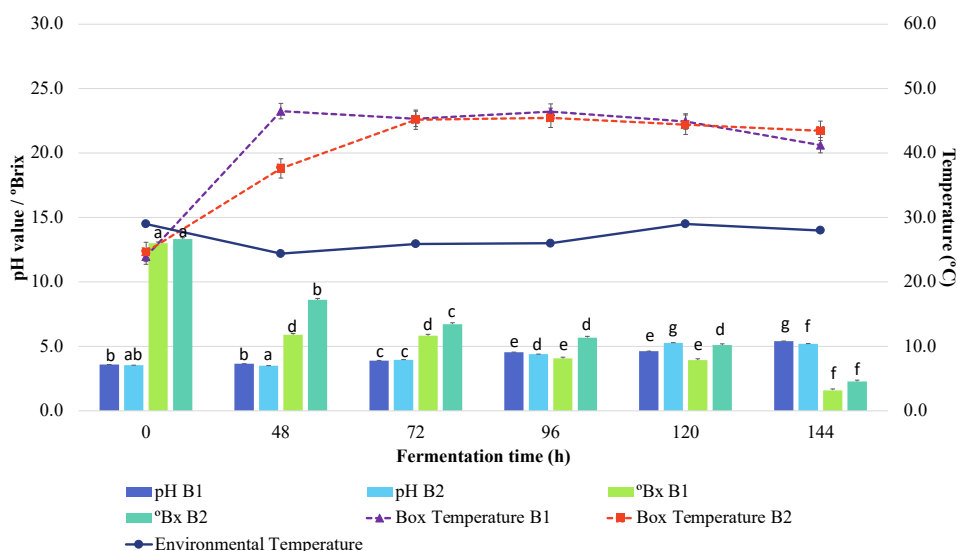
### 3.3. Physicochemical changes

The soluble solids ( $^{\circ}$ Brix), pH, environmental temperature, and temperature inside the fermenting cocoa-pulp mass were monitored during fermentation (Figure 3). The physicochemical parameters at the beginning of fermentation were not significantly different between batches ( $p > 0.05$ ). After the opening of the cocoa pods, the initial ranges of  $^{\circ}$ Brix and pH values were 13.30–13.33 and 3.53–3.59, respectively. These parameters are critical for microbial enzymatic activity generated during cocoa pulp mass fermentation (Koné et al., 2016). The soluble solids of cocoa pulp are related to the sugar content (10–15%), citric acid

(1–3%), and pectin (1–1.5%) (Santander Muñoz et al., 2019). The initial acidic pH of cocoa pulp is associated mainly with the citric acid content (Lagunes-Gálvez et al., 2007). The pH values and box temperature increased throughout the two fermentation processes, while the  $^{\circ}$ Brix decreased. Under the B1 method, the  $^{\circ}$ Brix decreased at a higher rate relative to B2, possibly due to the greater number of turns applied under the B1 method. Turning favors the deposition of a mucilaginous liquid of cocoa beans rich in sugars at the bottom of the fermentation box, decreasing the amount of substrate available for ethanol-producing yeasts (Schwan and Wheals, 2004), which could explain the lower stimulation of yeast growth and biodiversity under the B1 method (Figure 2A). The microbial activity and metabolites generated during cocoa bean fermentation led to increases in temperature and pH (Moreira et al., 2018). The temperature inside the fermenting cocoa-pulp mass reached a maximum value of 46.5  $^{\circ}$ C at 48 h under the B1 method; under the B2 method, the highest temperature was observed at 72 h. While the environmental temperature was similar for both fermentation processes (between  $17 \pm 2$   $^{\circ}$ C), the results were associated with the oxygenation incorporated with the turning at 24 h under the B1 method, which could stimulate the growth of AAB, which were characterized by highly exothermic metabolism resulting in an increase in temperature above 45  $^{\circ}$ C (Papalexandratou et al., 2019; Ramos et al., 2020). The pH of the cocoa pulp mass increased throughout the fermentation processes. Under the B2 method, the highest pH value ( $5.59 \pm 0.03$ ) was observed at 120 h, while under the B1 method, it was observed at 144 h (Figure 3). These pH increases were associated with the consumption of citric acid from fresh pulp, an alternative energy source for yeasts and LAB (Nielsen et al., 2013), which explains the higher rate of the decrease in pH observed under the B2 method. Although acetic acid is also produced during fermentation, it occurs within the bean cotyledon, exerting a minimal impact on the pH of the pulp (Camu et al., 2008a).

### 3.4. Multivariate analysis of the volatile compounds produced during the cocoa bean fermentations

Principal component analysis (PCA) was performed to evaluate the effect of the first turning time on the growth dynamics of the main microorganisms (yeasts, LAB, AAB, and spore-forming bacteria) and the volatile compounds generated throughout the fermentation. Figure 4 presents the first two principal components (PCs), which together explained 56.15% of the total variance (30.80% for PC1 and 25.35% for PC2). PCA allowed a natural grouping of the observations separating the initial time, intermediate times, and final time of both fermentation



**Figure 3.** pH, soluble solids ( $^{\circ}$ Bx), ambient temperature, and box temperature values at different fermentation times (0, 48, 72, 96, 120, and 144 h) of both fermentation methods evaluated: Fermentation of fresh cocoa beans with first turning at 24 h (B1), and fermentation with first turning at 48 h (B2). The pH and  $^{\circ}$ Bx are displayed in bars with values corresponding to the scale on the left side, while box and ambient temperature values are shown in lines with scale values from the right side. Different letters between pH and  $^{\circ}$ Bx values indicate significant differences ( $p < 0.05$ ).

processes in different quadrants of the biplot. PC2 separated the initial fermentation times at the bottom of the plot (with negative scores), mainly related to 2-heptanol, 2-heptanone, 2-hydroxyacetophenone, butanal, and the microorganisms *H. opuntiae* and *B. subtilis*. The fermentation time of 72 h and the final time of 144 h were placed at the top of the biplot, with positive scores of PC2 related to 2-methylpropanal, 2,3-butanedione, 3-methyl-2-butanol, 4-methyl-2,3-pentandiol, 4,5-octanediol, phenylalcohol, methyl-2-methoxy-butanoate, 2-methylpropanoic acid, 3-methylbutanoic acid, and 2,5-dimethylpyrazine. PC1 allowed the separation of the final profile of each fermentation method. B1 fermentation was located in the upper quadrant of the plot with negative scores related to ethyl acetate and *P. acidilactici*. B2 fermentation was associated with positive scores, mainly related to butanal, nonanal, phenylacetaldehyde, acetophenone, 2-heptanone, 4-methyl-2-hexanone, 2-phenylethyl alcohol, 2-pentanol, 2,3-butanediol, 2-heptanol, and ethyl octanoate, as well as with the microorganisms *H. opuntiae* and *B. subtilis*.

Subsequently, supervised classification was carried out. A partial least squares discriminant analysis (PLS-DA) model was constructed to classify the volatile compounds generated with the fermentation method applied to maximize separation between classes and to find the most relevant compounds for each turning method (B1 and B2). PLS-DA biplot scores of volatile compounds are depicted in Figure 5. Seventeen compounds (including 3-methylpentanoic acid, 2-nonanone, 3-methyl-2-butanone, acetophenone, 4-hydroxy-2-butanone, phenylacetaldehyde, benzaldehyde, 3-methylbutanal, 1-phenylethyl acetate, 2-pentanol acetate, 2,3-butanediol diacetate, ethyl hexanoate, 2-hydroxy-ethyl propanoate, ethyl decanoate, 2-phenylethyl alcohol, 2-pentadecanol, and 2-decanol) were highly correlated with B2 due to their placement farther from the center, particularly between the inner and outer circles in the biplot. PLS-DA permits the visualization of the compounds associated with key aroma markers of cocoa beans (e.g., phenylacetaldehyde, 3-methylbutanal, 2-phenylethyl alcohol, 2-phenylethyl acetate, 2,5-dimethylpyrazine, 3-methylbutanoic acid, 2-methylpropanoic acid, and 2,3-butanedione), which are mostly correlated with B2, while B1 exhibited a higher correlation of the key aroma marker 3-hydroxy-2-butanone.

The results may be associated with the fact that the higher aeration frequency in into the cocoa pulp mass under the B1 artisanal fermentation method gave rise to an aerobic environment that caused a decline in yeasts and increases in AAB and spore-forming bacteria of the genus *Bacillus* spp. (Guehi et al., 2010; Hamdouche et al., 2019; Ouattara et al., 2020). The latter microbial genus has been reported to be a producer of 3-hydroxy-2-butanone in simulated cocoa-pulp media (Ouattara et al., 2020). Additionally, *P. acidilactici* was identified under B1 fermentation, which is a species that shows higher production of ethyl acetate when inoculated with yeasts of the genus *Pichia* spp. than when grown in isolation (da Silva-Vale et al., 2019).

On the other hand, B2 fermentation prolonged anaerobic conditions, stimulating the growth of yeasts and LAB, which favored their total dominance during the whole fermentation process. A high production of acetate esters has been associated with better synergy between the coinoculation of yeasts and LAB (da Silva-Vale et al., 2019). Some yeasts isolated from cocoa bean fermentation, such as *H. opuntiae* and *Pichia* spp., have been recognized as producers of ethanol, 2-phenylethyl alcohol, esters (such as 2-phenylethyl acetate, ethyl hexanoate, and 2-pentanol acetate), aldehydes (such as 3-methyl butanal and phenylacetaldehyde), and acetophenone (Cevallos-Cevallos et al., 2018; Hu et al., 2018; Ouattara et al., 2020). Species of the genus *Hanseniaspora* have been reported as producers of key aromatic compounds in fermented cocoa beans, such as 2-phenylethyl alcohol (Mota-Gutierrez et al., 2018). Likewise, a positive correlation was observed between *H. opuntiae*, *A. pasteurianus*, and *Bacillus* spp. with 2-pentanol, and phenylethyl alcohol (Mota-Gutierrez et al., 2019). Subsequently, by incorporating aeration into the cocoa pulp mass, the aerobic conditions favored the growth of AAB, from which alcohols generated by yeasts produced a high content of acids such as propanoic acid,

3-methylpropanoic acid, and 3-methyl butanoic acid (Ramos et al., 2020). All of the aforementioned compounds were correlated with artisanal B2 fermentation.

#### 4. Conclusion

The turning technique in artisanal cocoa bean fermentation is important for the development of specific microbial populations that generate a unique volatile compound profile. The turning technique initiated after 48 h of fermentation favors anaerobic conditions that stimulate the growth of yeasts and *L. plantarum* and is associated with high production of acetate esters, aldehydes (such as 2-phenylacetaldehyde and 3-methylbutanal), alcohols (2-phenylethyl alcohol), acetophenone, and volatile acids (3-methylpentanoic acid), which are recognized as key aroma markers associated with fermented *Criollo* cocoa bean quality. The turning technique that begins after 24 h of fermentation seems to stimulate a synergistic effect between AAB and spore-forming bacteria of the genus *Bacillus* spp. in the production of odor-active compounds such as ethyl acetate and 3-hydroxy-2-butanone, which are associated with the desirable fruity and sweetish sensory notes of chocolates.

The results shown here provide the basis for a better understanding of the effect of turning techniques applied in the *Criollo* cocoa fermentation process, principally according to the profiles of volatile compounds and the microbial dynamics involved. At the same time, the study of the interactions between microorganisms and the volatile compounds generated allowed us to visualize a panorama of potential natural synergism between microorganisms and the production of key compounds of the cocoa aroma that can guide the selection and study of starter cultures for the improvement, modernization, and standardization of artisanal fermentation processes of *Criollo* cacao. However, more studies are needed to establish that the turning technique can improve the sensory attributes of the fermented cocoa bean and its impact as a raw material in the processes of chocolate production. Additionally, the applications of new technologies used for screening microbial populations, such as new generation sequencing (NGS) techniques, is suggested to provide a better overview of the microbial ecology involved in the artisanal methods of cocoa bean fermentation.

#### Declarations

##### Author contribution statement

Velásquez-Reyes Dulce: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gschaedler Anne, Kirchmayr Manuel: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Avendaño-Arrazate Carlos: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Rodríguez-Campos Jacobo: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Calva-Estrada Sergio de Jesús: Analyzed and interpreted the data.

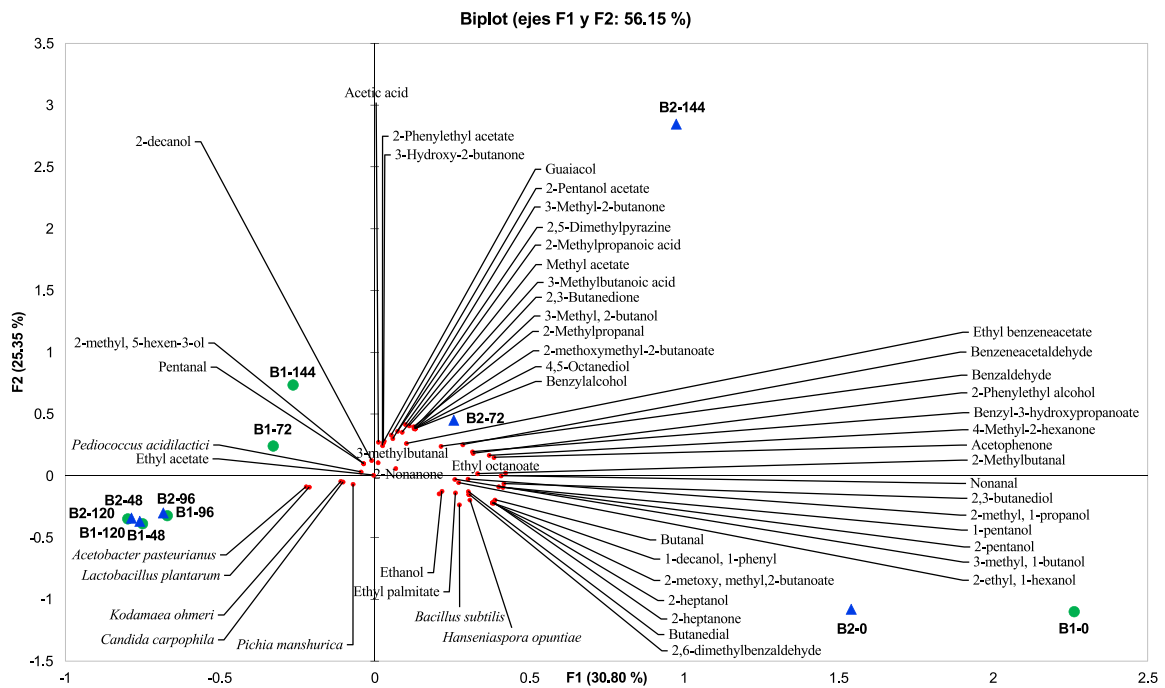
Lugo-Cervantes Eugenia: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

##### Funding statement

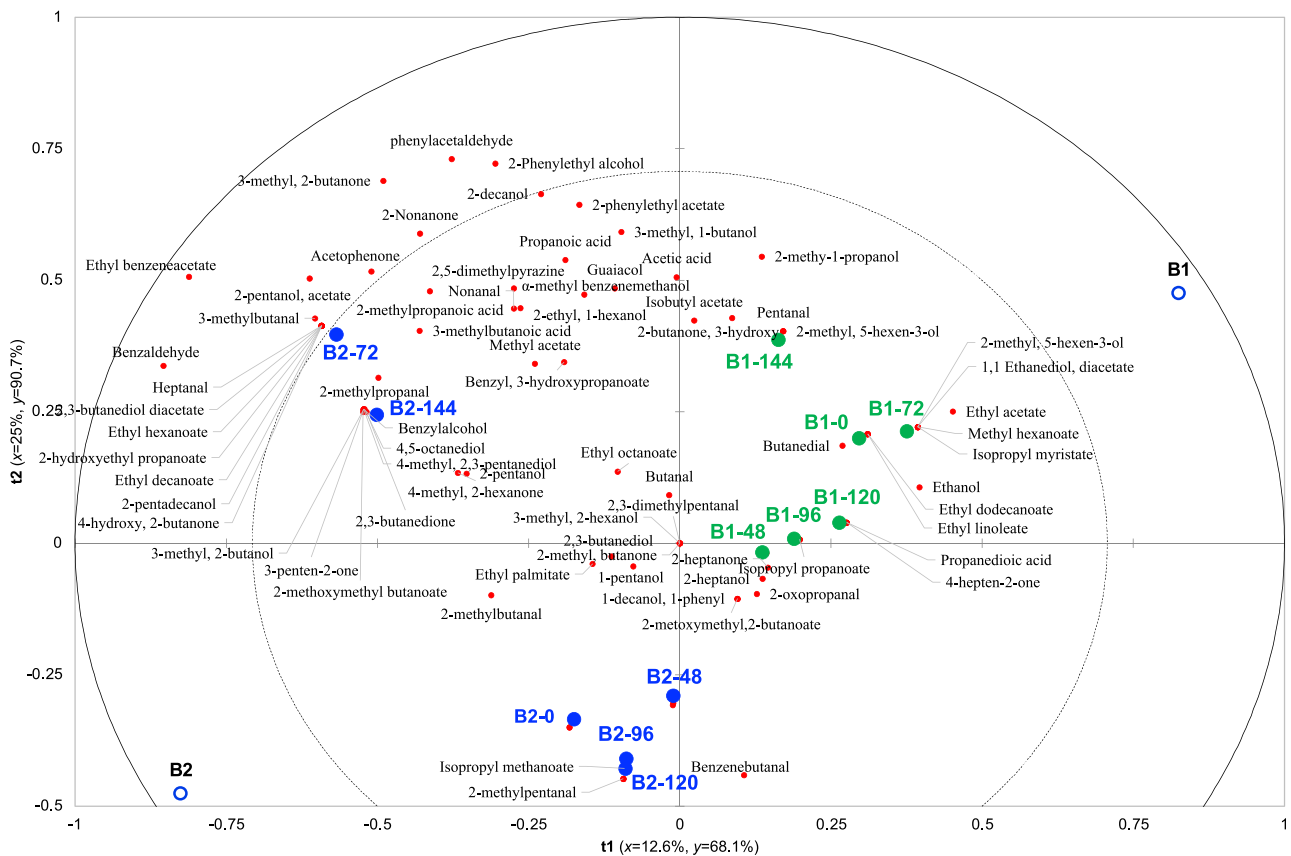
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##### Data availability statement

Data included in article/supplementary material/referenced in article.



**Figure 4.** Principal Component Analysis (PCA) of the overall volatile profile and CFU of the main microorganism at different fermentation times (0, 48, 72, 96, 120, and 144 h) on both processes evaluated (B1 = green, and B2 = blue). Values in parentheses represent the percentage of variance explained by each component.



**Figure 5.** Partial least squares discriminant analysis (PLS-DA) score biplot of the volatile compound distribution according to the fermentative treatments (B1 = green, and B2 = blue) at different fermentation times (0, 48, 72, 96, 120, and 144 h).

## Declaration of interests statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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