Impact of turning, pod storage and fermentation time on microbial ecology and volatile composition of cocoa beans.

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Abstract

Cocoa quality depends on several parameters, such as cocoa variety, environmental growth conditions, cultivation technique, and post-harvest treatments applied to coca beans. In this work, we studied the impact of cocoa post-harvest processing on both microbial communities structure and volatile composition. Cocoa beans samples were fermented in wooden boxes in Ivory Coast at different time intervals with turning and without turning, and derived from pods stored for two different duration times. Cocoa beans were analyzed using a molecular fingerprinting method (PCR-DGGE) in order to detect variations in microbial communities' structure; this global analysis was coupled to SPME-GC-MS for assessing cocoa volatile profiles. The results showed that the main parameter that influenced microbial communities structure was fermentation time, followed by turning, whereas, pods storage duration had a minor impact. Similar results were obtained for aromatic profile, except for pods storage duration that significantly affected volatile compound production. Global statistical analysis using Canonical Correspondence Analysis (CCA), showed the relationship between microbial communities and volatile composition. Furthermore, this study allowed the identification of discriminating microbial and chemical markers of cocoa post-harvest processing.

1. Introduction

Cocoa is ranked among the most traded agricultural products in the world. Cocoa beans production is estimated to 4,552,000 tons in 2016/2017; 73% of which is produced by Africa, mainly by Ivory Coast, which is the cocoa first producer, followed by Ghana, Cameroon and Nigeria (ICCO, 2017).

Cocoa has an intrinsic quality potential that depends on several parameters, such as cocoa variety, age of the cocoa tree, soil chemical composition, post-harvest treatments, and industrial process (Kongor et al., 2016). In particular, postharvest processing applied on beans can enhance or degrade the sanitary and/or organoleptic qualities of cocoa (Saltini et al., 2013). The transformation of cocoa goes through three main stages (pods opening, fermentation, and drying). The crucial step is fermentation, which is a spontaneous process involving microbial flora succession responsible for aroma precursor's production inside the beans through biochemical reactions. Fermentation lasts usually between 5 to 7 days. Yeasts are the first type of microorganism involved followed by Lactic Acid Bacteria (LAB), and then Acetic Acid Bacteria (AAB) (Schwan and Wheals, 2004). Several recent studies tried to understand the role of these microorganism groups in cocoa fermentation and to control this phenomenon by inoculating selected strains previously isolated from cocoa (Lefeber et al., 2012, Crafack et al., 2013). The major results of these works showed that yeast strains have a greater impact on the improvement of cocoa aromatic quality when compared to bacterial strains (Crafack et al., 2013; 2014; Ho et al., 2014; Koné et al., 2016; Hamdouche et al., 2017).

Several studies on the impact of postharvest treatments on cocoa beans composition showed that fermentation types (Papalexandratou et al., 2011; Visintin et al., 2016) and times (Hamdouche et al., 2015) significantly affected the microbiota associated with cocoa beans,

while pods storage (pulp preconditioning) and turning affected chemical composition and physical appearance of cocoa (Nazaruddin et al., 2006; Guehi et al., 2010).

Aroma profile significantly contributes to cocoa quality and thus is crucial for the acceptability of chocolate. This profile varied mainly with fermentation and drying time (Rodriguez-Campos et al., 2011; 2012). Aroma precursors previously formed during fermentation and drying steps were highly developed during roasting. Roasting reduces acidity by volatile acids evaporation and removes bitter, acidic and astringent flavors (Beckett 2000, Ramli et al., 2006). Maillard reactions generate a valuable source of aromatic compounds as pyrazines, pyrroles, furans and thiazoles (Belitz et al., 2009). The 2-methylpyrazine and 2, 3, 5, 6 tetramethylpyrazine are the main volatile compounds present in roasted cocoa. Both are responsible for the sweet and strong cocoa flavor respectively (Lefeber et al., 2012).

The most used techniques for volatile compounds extraction are distillation, direct solvent extraction, solid phase extraction (SPE), headspace methods and solid phase micro-extraction (SPME). After concentration, gas chromatography is the most suitable analytical method. This technique allows the separation of a very large number of compounds on a fused silica capillary column. Different detection methods coupled with gas chromatography allow volatile compounds detection and identification: flame ionization, flame photometry, atomic emission, and mass spectrometry. SPME-GC-MS for the headspace analysis of volatile compounds allowed sensitive and representative analysis of cocoa products with high reproducibility (Ducki et al., 2008).

A recent work revealed that cocoa aroma compounds composition varied according to cocoa variety (Menezes et al., 2016), but no study showed the impact of pod storage and turning on microbial ecology and the associated impact on aroma profiles. The main aims of this original study were: (i) to measure the impact of the variation of three parameters (pod storage,

fermentation time and turning) during cocoa post-harvest processing on microbial and volatile profiles; (ii) to identify discriminating microbial and/or aroma markers of each process; (iii) to establish relationship between microbial communities associated with cocoa beans and volatile compounds produced.

2. Material and Methods

2. 1. Cocoa beans sampling

Four hundred cocoa pods were harvested in November 2014 in Akoupé (Ivory Coast). The number of harvested pods was divided in two batches (200 pods each), the first batch was stored during 2 days and the other one during 8 days. After storage, all pods were opened using a machete. For each pods opening delay, approximately 30 kg of pulp-bean mass were extracted manually, recovered and distributed in two wooden boxes for fermentation during 7 days. Two types of processing were applied on these cocoa beans: fermentation with and without turning. The turning was done at 2 and 4 fermentation days, this choice was made following the artisanal practices applied. One kilogram of cocoa beans was collected at 2, 4, 5, 6 and 7 days of fermentation and sun dried during 7 days on wooden drying racks. During drying step, no turning was done. The sampling was performed in sterile conditions by using sterile gloves and plastic bags. Samples were sent by plane to Cirad laboratory, Montpellier, France and stored at room temperature. The microbial and chemical analyses were carried out immediately.

2. 2. Microbial ecology analysis

2. 2. 1. Microbial DNA extraction

Microbial DNA was extracted from 10 g of fermented and dried cocoa beans. Two technical repetitions were assessed for each sample. The extraction was performed according to the method described previously by Hamdouche et al. (2015). The quantity and purity of

extracted DNA were measured by a UV spectrophotometer (Biospec-Nano), and by electrophoresis through a 0.8% agarose gel in 1X TAE buffer (containing 40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA; Eppendorf) with a 1 Kb molecular weight ladder (Promega). The agarose gels were run at 100 V for 30 min and stained for 15 min in Gel Red solution (50 $mg.mL^{-1}$; Biotium), then observed and photographed on a UV transilluminator, using a black and white CCD camera (Scion Company) and Gel Smart 7.3 system software (Clara Vision).

2. 2. 2. PCR amplification of extracted DNA

For bacterial communities analyses: A 160 bp fragment of the V3 variable region of 16S rDNA was amplified using gc-338f (5'-GCG CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCA TCC TAC GGG AGG CAG CAG-3', Sigma) and 518r (5'-ATT ACC GCG GCT GCT GG-3', Sigma) DNA primers (Leesing, 2005; Hamdouche et al., 2015; 2016). A 40-bp GC-clamp (Sigma) was added to the forward primer in order to ensure that the DNA fragment will remain partially double stranded and that the region screened is in the lowest melting domain (Sheffield et al., 1989). Each mixture (final volume 50 µL) contained about 100 ng of DNA template, DNA primers at 0.2 mM, deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mM $MgCl_2$, 5 µL of 10X of Taq reaction buffer $MgCl_2$ free and 1.25 unit of a-Taq polymerase (Promega). In order to increase the specificity of amplification and to reduce the formation of spurious by-products, DNA was amplified by a "touchdown" PCR previously described by Hamdouche et al. (2015).

For fungal communities analyses: a 250 bp fragment of D1/D2 region of the 26S rDNA was amplified using universal DNA primers gc-NL1 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3', Sigma) (Cocolin et al., 2000). A 30-bp GC-clamp was added to

143 the forward primer. PCR mixture performed as previously described for bacterial DNA
144 analyses. The amplification program was described previously by Hamdouche et al. (2015).

145 Electrophoresis in 2% (w/v) agarose gel with TAE 1X buffer stained with Gel Red (as
146 described above) was used to verify and quantify PCR products using a standard (DNA mass
147 ladder 100 bp, Promega). The migration lasted about 30 min at 100 V.

148 **2. 2. 3. Denaturing gradient gel electrophoresis (DGGE) analysis**

149 PCR products were separated by DGGE, using a Bio-Rad Dcode universal mutation detection
150 system (Bio-Rad Laboratories, USA) according to the procedure described by Muyzer et al.
151 (1993) and improved by Leesing (2005). Forty microliters (µL) of each PCR product were
152 loaded onto 8% (w/v) polyacrylamide gels (acrylamide/N,N´- methylene bisacrylamide,
153 37.5/1, Promega) in 1X TAE.

154 Electrophoresis experiments were performed at 60°C using a denaturing gradient ranging
155 from 30 to 60% (100% corresponded to 7 M urea and 40% (v/v) formamide, Promega). The
156 migration was carried out at 20 V for 10 min and then at 80 V for 12 h. Then the gel was
157 stained for 30 min with Gel Red solution and then photographed as described above in 2.2.1
158 section.

159 **2. 2. 4. Purification and identification of DGGE bands**

160 Detected DNA bands were cut from the DGGE gel with a sterile scalpel. The DNA of each
161 band was then eluted in 100 µL TE buffer (10 mM Tris-HCl; pH 8.0, Promega) at 4°C
162 overnight. The DNA purification was carried out according to the protocol described by
163 Hamdouche et al. (2015; 2016). Bacterial and fungal purified DNA bands were amplified
164 using 338f and NL-1 (without GC clamp) and 518r and LS-2 primers respectively. PCR
165 amplicons were sent for sequencing to GATC Biotech (Germany).

166 The raw sequences data were edited using Bioedit Software and compared to the GenBank
167 database using the BLAST program (Altschul et al., 1997) (http://www.ncbi.nlm.nih.
168 gov/BLAST/). Sequences having a percentage of identity of 97% or greater were considered
169 to belong to the same species (Palys et al., 1997).

170 **2. 2. 5. Image and statistical analysis**

171 In each lane, banding patterns were standardized with two bacterial reference DNA patterns
172 (*Lactobacillus plantarum* and *Escherichia coli* DNAs) for bacteria and two yeast DNA
173 reference patterns of yeasts (*Lodderomyces elongisporus* and *Candida apicola* DNAs). Each
174 band relative positions to reference DNA were measured and recorded.

175 The images of DGGE gels were analyzed using ImageQuant TL software V. 2003
176 (Amersham Biosciences, USA). In DGGE analysis, the revealed banding pattern is considered
177 as an image of all the major microbial populations. An individual band refers to a unique
178 'sequence type' or phytotype (Kowalchuk et al., 1997). DGGE fingerprinting was marked by
179 the presence and absence of co-migrating bands and their intensity using ImagQuant TL
180 software V. 2003.

181 Pair wise community similarities were quantified using Dice similarity coefficient
182 (Heyndrickx et al., 1996), then generated data were exploited by Principal Component
183 Analysis (PCA) and Canonical analysis of correspondence (CAC) using XLSTAT (V. 2014).
184 The bands intensities (or volume as expressed in "grey level x pixel" unit) measured by
185 ImageQuant were statistically compared using ANOVA.

186 CAC is a method that encompasses several statistical analyses; Ter Braak (1986) developed it
187 for the first time. In our case, it was used to describe the linear relationships that exist between
188 two groups of variables measured on the same individuals. The first group represents the
189 variables to be explained (pod storage duration, fermentation time and turning) and the second

group shows the explanatory variables that are the microbial communities (DNA bands identified by PCR-DGGE).

2. 3. Volatile compound analysis

2. 3. 1. Sample preparation and SPME-HS conditions

The shell and the bean were both analysed, because the objective of the work was to evaluate the volatile composition of exported merchant cocoa (fermented and dried beans). In food industry, before the shelling, fermented and dried beans are roasted in order to develop the chocolate aromas, these aromas depend essentially on the precursors born during fermentation.

Two hundred grams of unshelled dried cocoa beans from Ivory Coast were put in liquid nitrogen and ground using a waring blender (SEB, France), to obtain cocoa flour, which was stored at - 80°C until analysis. Three grams of cocoa powder were introduced into vial (10 mL), 30 µL of internal standard of 1-butanol (202.5 mg/L) (Sigma) were added, and the vial was sealed with a septum cap. The extraction of three technical repetitions was performed using a 50/30 µm divinylbenzene/carboxene/polydimethylsiloxane fiber (DVB/CAR/PDMS, Supelco), and using the technique of solid phase micro-extraction in the headspace (SPME-HS) previously described by Rodriguez-Campos et al. (2012) and modified in our lab by Koné et al. (2016). These authors had conditioned the fiber at 250°C for 3 min then exposed it to the sample headspace at 50°C for 45 min.

2. 3. 2. Gas Chromatography Analytical Conditions

The analysis of the volatile compounds extracted from cocoa powder was performed using an Agilent 6890N gas chromatography–mass spectrometer (GC-MS) in automatic injection mode, on a polar capillary column DBWAX, 30 m length × 0.25 mm internal diameter × 0.25 µm film thickness (Agilent, Palo Alto, CA, USA). The GC-MS conditions have been established in Cirad laboratory, the injection temperature was 250°C. The GC oven temperature was initially set at 40°C for 5 min, increased to 140°C at a rate of 2°C/min and then increased at a rate of 10°C/min to 250°C. The carrier gas was high purity Helium at 1 $mL.min^{-1}$. Splitless injection mode was used at 250°C for 2 min. The selective mass detector was a quadrupole (Hewlett Packard, Model 5973), with an electronic impact ionization system at 70 eV and at the temperature source at 230°C.

2. 3. 3. Volatile compounds identification

For the identification of compounds, pure standards were not used, this is why the identification was just tentative. The identification was done: (i) by comparing the mass spectra with commercial database Wiley275.L, HP product no. G1035A); (ii) and by comparing of the linear retention index calculated (RI) with those found in the literature data. Retention index (RI) was calculated in the same chromatographic conditions using series of n-alkane C8-C20 (Supelco, Bellefonte, USA). Peak areas were used for relative quantification of compounds using the MSD Chemstation software (version E.02.02.1431, Agilent Technologies).

2. 3 .4. Statistical analysis

The chromatographic data obtained by SPME-GC-MS were statistically analyzed using ANOVA and Principal Component Analysis (PCA). As described previously, Canonical analysis of correspondence (CAC) was applied to GC-MS and DGGE data, in order to

describe relationship between microbial communities (explanatory variables) and volatile compounds produced (variables to be explained).

3. Non-volatile organic acids analysis

3.1. Organic acids extraction

The extraction was carried out according to the protocol of Holm et al. (1993). Five grams of cocoa powder were mixed with 50 mL of warm distilled water (75°C) in a 100 mL flask. The resulting suspension was well mixed and then cooled to room temperature. The volume was supplemented with distilled water up to 100 mL, then filtered through a pleated filter paper (Whatman) and a 0.45 µm filter (Sigma-Aldrich), then transferred to an HPLC vial (1 mL) (Thomson).

3.2. Organic acids quantification by HPLC

Concentrations of acetic acid, citric acid, lactic acid, malic acid, oxalic acid and tartaric acid were determined by a High Performance Liquid Chromatography (HPLC) with conductivity using AS11-HC column (Dionex). The mobile phase, at a flow rate of 0.6 $mL.min^{-1}$ consisted of ultrapure water (eluent A), NaOH 0.1 N (eluent B) and NaOH 0.002 N (eluent C). A volume of 0.02 mL for each sample was injected and run together with external standards. All samples were analyzed in triplicate and the data were controlled and processed with ClarityChrom software and analyzed using ANOVA.

3. Results and discussion

3. 1. Microbial community structures

3. 1. 1. Variation in bacterial community structures

Microbial DNA profiles were generated by DGGE from all cocoa fermentations carried out, with and without turning, with a delay of pod breaking of 2 and 8 days, respectively. Bacterial

DNA profile bands diversity at different time intervals of fermentation (Fig. 1.a and b). After two fermentation days, a few DNA bands (Y, B V, W) were observed and identified by sequencing as belonging to *Enterobacteriaceae*, *Lactobacillus fermentum,* and *Acetobacter pasteurianus* taxa respectively (Fig. 1.a,b ; Table 1). At days 4 and 5, additional DNA bands (A, E, F, J, L, M, N) were detected and identified as belonging to *Lactobacillus curvatus, Bacillus megaterium, Bacillus sp, Acetobacter sp and Providencia sp species,* respectively. All eight bacterial taxa remained detected until the last day of fermentation. This is concordant with previously reported data (Lefeber et al., 2011; Hamdouche et al., 2015), which showed that bacterial communities of cocoa undergo drastic changes after 4 days of fermentation and remain pretty similar throughout the process length.

Moreover, at the shortest fermentation time, only *L. fermentum, A. pasteurianus* and species belonging to *Enterobacteriaceae* taxa were detected and confirmed the presence of LAB and AAB in the initial phase of fermentation (Schwan and Wheals, 2004; Papalexandratou et al., 2013; Hamdouche et al., 2015).

Comparison of bacterial DNA profiles of fermented cocoa beans with and without turning showed that the majority of revealed bands were common in the two types of fermentation processing whatever the pod storage time. However, some bands (M, E, F) identified as *Bacillus* genus were mainly revealed in DGGE profiles obtained from cocoa beans fermented with turning (Fig. 1.a,b ; Table 1). Furthermore, some bands (G, H) were detected only in fermentation with turning and were identified as belonging to *Acetobacter sp*. These observations were explained by the better development of aerobic microorganisms due to aeration obtained by turning. The abundance of *Bacillus* genera populations seems to be the consequence of temperature elevation after 2 days and at the turning time, which promoted the development of thermophilic and aerobic species (Lima et al., 2011). Previous works, carried out on fermentation of different cocoa beans masses, showed that the fermentation of

283 the large cocoa bean-pulp mass decreased the aeration, which slows down microbial activities
284 (Camu et al., 2008; Guehi et al., 2010). It is important to note that *A. pasteurianus* species (V,
285 W) was not present during fermentation with turning, at 5, 6 and 7 days (Fig. 1.b-Table 1).
286 This result was unexpected because *A. pasteurianus* was the most prevalent AAB species
287 involved in spontaneous cocoa from Ivory coast, Brazil and Malaysia (Hamdouche et al.,
288 2015, Papalexandratou et al., 2011; 2013) and was tested as starter culture in cocoa
289 fermentation (Moens et al., 2014; Lefeber et al., 2010 ; 2012). First of all, we thought that this
290 unexpected result came from technical error, but the most surprising thing about this
291 observation, was the correlation of this result with the one obtained from volatile compounds
292 analysis. The disappearance of *A. pasteurianus* species was correlated with acetic acid content
293 decreases during fermentations with turning. If the technical error is sparing, the result could
294 be explained by the fact that the temperature increased during fermentation with turning (by
295 increasing activity AAB). In our fermentation assays, the temperatures could have risen such
296 as it became lethal for this thermosensible species. However, since, temperatures were not
297 measured in our studies, we could not conclude about this hypothesis.

298 When comparing DGGE bacterial profiles obtained from stored pods during 2 and 8 days,
299 DNA bands (R, K, X) specific to cocoa pods stored 8 days were detected and identified as
300 belonging to *Klebsiella pneumoniae, Bacillus sp,* and *Acinetobacter sp* (Fig. 1.b compared to
301 Fig. 1.a). These bacterial taxa could be present on pods surface and developed during storage
302 time, which rendered them detectable by PCR-DGGE. To our knowledge, no work has
303 investigated the pod storage effect on cocoa microbial ecology except the study of our
304 colleagues Kedjebo et al. (2016) who studied the effect of post-harvest treatment on the
305 occurrence of ochratoxin A (OTA) in raw cocoa beans from Ivory Coast. They showed that
306 OTA content in raw cocoa beans increased with pods storage duration (0, 4 and 7 days) which
307 reflected the increase of ochratoxinogenic fungi activity (*Aspergillus* strains) during cocoa

pods storage time. Therefore, their conclusion confirmed our results that showed a highest microbial contamination with long pod storage after fermentation.

Principal Component Analysis (PCA) was applied on bacterial DGGE profiles to represent the dataset variance. We observed that fermented cocoa samples tended to group according to fermentation time along the first component axis (F1 representing 53.44% of the total variance). For 2 day storage samples, two main groups containing cocoa beans samples turned fermented during 2 and 4 days on the left side of the axis and those fermented during 5, 6 and 7 days on the right side of the axis could be observed (Fig. 2). The picture is similar for 8 days storage of non-turned samples. Eight-day storage of turned samples appeared as two main groups: 2 day fermented on the left side of the axis and the rest of the samples on the right side. 2 days storage unturned samples tend to group according to the fermentation duration.

The secondary axis F2 (12.58% variance) separated groups of cocoa beans samples fermented by turning (bottom part) from those fermented without turning (upper part, Fig.2). This result showed that bacterial communities varied mainly according to fermentation time followed by processing (turning). PCA did not show an obvious effect of storage time on bacterial ecology of cocoa comparing with the impact of the two other parameters (fermentation time and turning). The strong dynamics and activity of the microbial populations observed during the fermentation does not seem to be linked to any other environmental parameter.

3. 1. 2. Variation in fungal community structures

During cocoa fermentations, dominant species (1, 10, and 11) were detected on DGGE profiles and identified as belonging to *Hanseniaspora opuntiae, Pichia kudriavzevii and Pichia manshurica* species (Fig. 3 - Table 2). These species had previously been detected in fermented cocoa beans from the same location in Ivory Coast (Hamdouche et al., 2015; Koné et al., 2016) and in other countries and continents (Crafack et al., 2013; de Melo Periera et al.,

2013; Papalexandratou et al., 2013). Minor variations were observed comparing DGGE profiles obtained from cocoa fermented with the two processes due to intrinsic variations in fungal diversity during fermentation (Fig. 3.a.b). Generally, we did not observe specific fungal communities associated to a process probably due to the fact that turning or aeration do not drastically favor fungal populations growth.

In DGGE profiles obtained from fermented cocoa beans previously stored longer (8 days), four specific bands were detected (4, 5, 7, 9) and identified as *Hanseniaspora uvarum* and *Pichia sp* (Fig. 3.b). The results of PCR amplifications coupled to DGGE profiles of both bacterial and yeast communities showed more DNA bands in cocoa pods stored longer (8 days), which probably means that microbial species abundance increased with storage time (as discussed above with bacterial data).

PCA on fungal communities associated with cocoa, represented by F1 and F2 axis (58. 26%) did not allow to discriminate cocoa beans samples, this result was expected, as mentioned before (Fig. 1 in supplementary material).

Canonical analysis of correspondence (CAC) was applied in order to describe the relation between variations in microbial community structures and changes in cocoa post-harvest parameters. CAC results showed that microbial communities associated with turning parameter consisted mainly of bacterial communities: *Bacillus*, LAB and *Acetobacter* species (Fig. 4). This result was expected for AAB because they are aerobic microorganisms, which are usually affected by the aeration. For *Bacillus*, it could be explained by the increasing of the temperature after turning due to the thermophilic aptitude of this genus. No microbial species was found linked to the parameter of pods storage time (Fig. 4) as pods storage duration was not related to the composition of microbiota.

3. 2. Volatile compounds analysis

356 The results obtained on the volatile compounds analysis might not be accurate because of the
357 limit of the identification and quantification method used.

358 Twenty-six volatile compounds were detected in fermented and dried cocoa beans by SPME-
359 GC-MS (Table 3). These compounds were separated in 7 families: aldehydes, ketones, esters,
360 alcohols, acids, pyrazines and phenols.

361

362

363 **3. 2. 1. Variation of aldehyde compounds**

364 Four aldehydes compounds were identified on all fermented cocoa beans analyzed, which
365 were isobutanal, 2 and 3-methylbutanal, benzaldehyde. Benzaldehyde was the dominant
366 aldehyde, followed by 2 and 3-methylbutanal in all cocoa fermentations (Fig. 5.a.g.m and Fig.
367 6.a.g). These aldehydes were previously identified on fermented cocoa beans (Serra-Bonvehi,
368 2005; Rodriguez-Campos et al., 2012; Ho et al., 2014). In all fermentations, aldehydes content
369 increased with fermentation time. These results were correlated with those obtained by
370 Rodriguez-Campos et al. (2012). Statistical test (ANOVA) showed significant differences in
371 aldehydes level ($p<0.05$) between the first stage of fermentation (2 and 4 days) and the last
372 stage (5, 6 and 7 days) (Fig. 5.a.g.m and Fig. 6.a.g).

373 ANOVA test showed significant differences for aldehydes content between cocoa beans
374 fermented with and without turning (Fig. 7.a.b), with the highest level obtained in the case of
375 fermentation with turning at 7 days. These observations were probably related to the presence
376 of LAB that were detected throughout the fermentation stages mostly in the case of turning
377 (Fig. 1.a and b). Aldehydes compounds could be produced by enzymatic reactions from
378 amino acids mainly by LAB (Jinap et al., 1994). The 2 and 3-methylbutanal are usually
379 produced from isoleucine and leucine, while benzaldehyde is produced from phenylalanine by

LAB (Jinap et al., 1994; Smit et al., 2005; Bonnarme et al., 2004). During beans mixing, temperature increased and reached its maximum (Camu et al., 2008), which increased the enzymatic activity. As regard to pod storage time effect, the aldehydes level had not significant difference ($p>0.05$) between the two pod storage delays (Fig. 7.a.b).

3. 2. 2. Variation of ketone compounds

For this family, the detected compounds were 2-heptanone, 2-pentanone, 2-nonanone, acetophenone, and acetoin. This later had been found as the dominant ketone in all cocoa samples (Fig. 5.b.h.n and Fig.6.b.h) as reported by Rodriguez-Campos et al. (2011). In general, acetoin quantity decreased with fermentation time. This compound is mainly produced from pyruvate by alcoholic fermentation and from 2,3-butanediol by yeast (Pretorius, 2000). Acetoin abundance in the first stage of fermentation could be correlated to yeasts intervention in this stage. The quantity of others ketones varied slightly during fermentation. These compounds were naturally present in cocoa beans (Ho et al., 2014) and their production was not affected by fermentation, except for 2-pentanone, which was probably used as substrate by microorganisms during fermentation. Ketones were significantly important ($p<0.05$) in cocoa beans fermented without turning (Fig. 7.a,b). Some ketones that were probably present naturally (Afoakwa *et al.,* 2008, Rodriguez et al., 2012), were degraded and catabolized in the case of the aerated fermentation process, where more diversity and abundance of bacteria were observed by PCR-DGGE (Fig. 1.a). There was no difference in ketones level for the two pod storage durations, except in the case of fermentation with turning, whither the dominance of ketones was observed in pods stored at the shorter time (2 days) (Fig. 7.a.b).

3. 2. 3. Variation of alcohol compounds

403 The alcohols compounds found in cocoa beans samples were: ethanol, 2-pentanol,
404 isopentanol, 2-heptanol, 2,3-butanediol, and 2-phenethylethanol. Among them, 2-
405 phenethylethanol and 2,3 -butanediol were dominant during the four types of fermentations
406 (Fig. 5.c.i.o and Fig. 6.c.i); as already showed by Rodriguez-Campos et al. (2011). Ethanol
407 was surely produced by the majority of yeasts (Ho et al., 2014, Koné et al., 2016). It was
408 probably the same in this study where it was generated by all detected yeasts by PCR-DGGE
409 (Fig. 3). Some alcohols could be elaborated by both bacteria and yeasts such as 2,3-
410 butanediol. Ethanol content presented significant differences ($p<0.05$) between fermentation
411 times (2 and 4 days) and (5, 6, and 7 days) (Fig. 5.c.i.o and Fig. 6.c.i). Its concentration was
412 higher during the first four days of fermentation and then decreased rapidly, because this
413 compound is produced at the first (anaerobic) fermentation stage, which lasted 48 h,
414 subsequently it was oxidized in acetic acid (Schwan and Wheals, 2004) mainly by AAB
415 during the second (aerobic) stage.

416 The abundance of these alcohols was significantly different ($p<0.05$) during the different
417 fermentation process, with the highest level obtained in cocoa fermented beans without
418 turning mainly for ethanol (Fig 7.a,b, Fig. 5.c.i.o and Fig. 6.c.i). In aerated conditions, the
419 contribution of oxygen allows alcohols oxidation. For example, most alcoholic compounds
420 are oxidized to ester compounds, as phenethylethanol that is a precursor of aldehyde and is
421 oxidized to phenylethyl acetate with the presence of oxygen by LAB (Smit et al., 2005).
422 Furthermore, alcohol compounds could be evaporated with the aeration brought by turning
423 process. This result agreed with those obtained by Camu et al. (2008) that showed the
424 evaporation of ethanol during the aeration of cocoa beans mass, as well as favoring the AAB
425 growth that oxidized ethanol into acetic acid.

426 Total alcohols level was significantly higher in cocoa obtained from pods stored longer (Fig
427 7.a.b) as 2,3-butanediol (Fig 5.c.i.o and Fig 6.c.i). This may be explained by the studies of

Afoakwa et al. (2013a; 2013b) that showed the conversion of total sugars with pod storage in alcohol. Furthermore, Meyer et al. (1989) observed that increasing pod storage time before fermentation improved subsequent microbial aeration, prevented LAB development/growth and increased yeasts abundance and activity. This result could be explained by the fact that alcoholic fermentation may begin during the long storage of the pods injured during the harvest, which are naturally or subsequently contaminated by yeasts.

3. 2. 4. Variation of ester compounds

The six detected esters were ethyl acetate, isobutyl acetate, isoamyl acetate, phenylethyl acetate, methyl isopentanoate, and methyl isovalerate (Fig .5.d.j.p and Fig. 6.d.j). Ethyl acetate was the dominant ester in all fermentations. According to Rodriguez-Campos et al. (2012), esters concentration decreased with fermentation time, but in our case, no significant difference was observed ($p>0.05$) depending on fermentation time except for ethyl acetate, which dominated in the first stage of fermentation (2 days) (Fig .5.d.j.p and Fig. 6.d.j). This ester seems to be produced only at the beginning of cocoa fermentation as reported previously (Rodriguez-Campos et al., 2011), and also in wine fermentation (Rojas et al., 2003) mainly in the first day of fermentation. This may be due to the abundance of glucose and ethanol during this stage, because they are the main substrates for ethyl acetate synthesis (Yong et al., 1981). Moreover, Ethyl acetate is a product of esterification from acetic acid and ethanol (Pretorious, 2000); this concept explains the correlation between the amount of this ester, acetic acid and ethanol quantities mainly in the case of fermentation with aeration, in which a logical evolution of the quantities was observed for the most of volatile compounds detected in this study. A large quantity of these compounds was observed at the beginning of fermentation and decreased sharply after 2 days (Fig. 5.c.i.o.e.k.q and 6.c.i.e.k). The concentration of this ester was significantly higher in cocoa fermented without turning (Fig 7.a,b). Esters were synthetized by yeasts from alcohols during anaerobic phase (Rojas et al., 2002), which

explained their dominance in fermentation without aeration. This observation could explain the high level of esters in this process. Concerning pods storage time, esters were higher as mainly methyl isovalerate in cocoa obtained from pods, which were stored less time (Fig. 7.a.b). That could be explained by the decrease of sugar and protein during cocoa pod storage (Guehi et al., 2010, Afoakwa et al., 2013) that could be substrates for esters synthesis. Substrates reduction during long storage (8 days) allowed probably the decrease of the esters content.

3. 2. 5. Variation of acid compounds

Three acids were detected in fermented cocoa beans, of which acetic acid was largely dominant comparing with isobutyric and isovaleric acids. Acetic acid quantity decreased significantly during fermentation realized with turning (Fig. 5.e.k.q and Fig. 6.e.k). In the case of fermentation without turning, the evolution of acetic acid was irregular because its quantity was not stable, decreased and increased during fermentation (Fig. 7.a,b). The illogical evolution of acetic acid amount in this case, could be explained by the fact that there was no beans turning, therefore no homogenization in the metabolites produced and beans composition. Whatever the pods storage delay, a normal evolution of acetic acid amount during fermentation with turning was observed. According to the literature, acetic acid increased and reached its maximum around 72h of cocoa fermentation then decreased after 88 h (Shwan and Wheal, 2004; Lefeber and al., 2012). This data confirmed our result, which showed that turning of cocoa beans allowed to realize fermentation with a normal evolution over time. The concentration of this acid was significantly higher in cocoa beans fermented without turning. To explain this, one possibility is that residual or minimal acetic fermentation (with AAB growth and/or activity) occurs in unmixed cocoa, and the rise in temperature plus the aeration favors the volatilization nutrients depletion (Camu et al., 2008) during fermentation with turning.

During fermentation, assimilation of citric acid by LAB generates a slight rise in pH to 4.5 and temperature to 37°C of cocoa pulp (Camu et al., 2007) which create a favorable environment for the growth of AAB involved in the second phase of fermentation (acetic fermentation). During this phase, AAB are responsible of the oxidation of ethanol to acetic acid (Schwan and Wheals, 2004). Our results on acetic acid bacterial communities structure was related to acetic acid production, because low acid acetic level was observed in parallel with the disappearance of the main AAB, which was *A. pasteurianus* (band V and W) (Fig. 1) in the absence of aeration.

We also observed that volatile acids content decreased among time in cocoa beans and were very low after a pod storage of 8 days (Fig. 7.a.b). This observation is in agreement with the works of Tomlins et al. (1993) and Jinap et al. (1994), who observed a lower acetic acid content in stored cocoa compared with un-stored cocoa pods. As mentioned previously from the literature, acetic acid is produced by oxidation of the ethanol during the second fermentation phase (aerobic). These results were consistent with those obtained on ethanol (section 3.2.3) which was low after pod storage of 8 days. Therefore, acetic acid content, which is volatile, could vary according to the ethanol content and time.

3. 2. 6. Variation of others volatile compounds

Pyrazines: Tetramethylpyrazine was the unique compound belonging to pyrazine family that was detected (Fig. 5.f.l.r and Fig. 6.f.l). In most cases, tetramethylpyrazine compound has been reported to produce characteristics notes of roasted cocoa that is important for flavor quality (Afoakwa et al., 2008; Serra-Bonvehi, 2005). Furthermore, various studies reported on the microbial origin of pyrazines in fermented food as cocoa and soybeans (Selamat et al., 1994; Besson et al., 1997). In our case, tetramethylpyrazine was probably produced by *Bacillus* as *Bacillus megaterium* species that was detected by PCR-DGGE (Fig. 1). They play an important role in producing alkylypyrasines in association with *B. subtilis* during cocoa

503 fermentation. Our results showed that tetramethylpyrazine level had no significant difference
504 during fermentation times, pods storage times and between fermentation processing. Pyrazine
505 compounds were not affected by any of the three processes.

506 *Methoxyphenol:* This volatile phenolic compound was only detected in fermented cocoa beans
507 obtained from pods stored for 8 days and it was significantly ($p<0.05$) higher in the case of
508 fermentation with turning (Fig. 5.f.l.r, Fig. 6.f.l). Some phenolic compounds were previously
509 detected at the end of cocoa fermentation (6 and 8 days) (Rodriguez-Campos et al., 2012).
510 The presence of this compound only in fermented cocoa beans obtained from long pods
511 storage when more microbial diversity was detected, could be explained by the increase of
512 nutrient degradation, including phenolic acids, thus 2-methoxyphenol elaboration. We also
513 connected the production of this compound to the natural heating during sun drying. In
514 general, phenolic acids are degraded thermically or decomposed by microorganisms into
515 phenols, as 2-methoxyphenol that results from a heat degradation of ferulic acid during coffee
516 and cocoa roasting or kiln drying (Berlitz, 2009).

517 **3. 3. Organic acids composition**

518 Five non-volatile organic acids were detected and quantified: citric, lactic, malic, oxalic and
519 tartric acids. The total of non-volatile acids was significantly ($p <0.05$) higher on cocoa beans
520 fermented without turning (when compared to turned cocoa), especially at the end of
521 fermentation (5, 6 and 7 days) (Fig. 8). This confirmed the results obtained on volatile acids,
522 which are less abundant during fermentation with aeration. The amount of acids is
523 significantly greater ($p <0.05$) on cocoa beans with short pod storage durations (2 days) (Fig.
524 8). This is consistent with the results obtained on volatile acids by Afoakwa et al. (2013a;
525 2013b) who showed consistent decreases in non-volatile acidity during pod storage.

The concentration of volatile and non-volatile acids identified was affected during fermentation probably under microbial activities. Those naturally present in cocoa beans were degraded, mainly citric acid, while others were produced during fermentation, such as acetic and lactic acids, which could be considered as cocoa quality markers.

3. 4. Correlations between microbial ecology and volatile compounds produced in cocoa fermentation

In order to measure the impacts of turning during fermentation (fermentation time and pods storage time), PCA was applied on all volatiles compounds detected in fermented cocoa beans (Fig. 9). Statistical analysis showed a global (on the volatile groups) and specific (on volatile compounds) effects. We showed that ketones, alcohols, esters and acids contents were higher in cocoa fermented without turning, while aldehydes were significantly abundant in the process with turning. Pod storage duration had an effect on all chemical families except for aldehydes and pyrazines. ACP separated 4 samples groups according to fermentation time, fermentation process and pod storage time. Principal axis F1 (38.86%) separated cocoa beans samples fermented (5, 6 and 7 days) without turning from the beans fermented with turning (Fig. 9). ACP analysis allowed identifying discriminant compounds that were affected by studied parameters. Discriminant and significant compounds in fermentation without turning were acetic acid, isobutyric acid, 2,3-butanediol, acetoin, phenethyl acetate, isoamyl acetate. In the case of turning, all aldehydes compounds detected and some esters were identified as discriminant compounds. The same axis F1 regrouped samples according to fermentation time, the higher level of acetic acid, ethyl acetate, isobutyl acetate and ethanol at the first stage of fermentation (2 and 4 days) discriminated beans fermented at short term from the other samples. F2 axis (18.45%), separated the two pods storage times starting from 5 days of fermentation, by 2-methoxyphenol and acetophenone that were significant (abundant) in long storage (8 days).

551 Variations in volatile composition were compared with literature and with microbial ecology
552 analyses carried out in our study. Some hypotheses were given about the relationship that
553 could exist between microbial community structures and volatile composition of cocoa beans.
554 CAC was applied on all data obtained in order to determine these relationships (Fig. 10).
555 Microbial taxa detection by DGGE and their correlation with volatile compounds had a
556 similar evolution whatever the fermentation time and the processing applied. It was not
557 possible or rather difficult to relate each volatile group and/or compound to a given microbial
558 taxa, which is explained by the fact that several yeasts or bacteria could produce the same
559 compounds (Ho et al., 2014; Koné et al., 2016). Moreover, the CAC described the relation
560 between *A. pasteurianus* (V, W) acetic acid, and acetoin and between *H. opuntiae* to ethyl
561 acetate and ethanol (Fig. 10). This suggested that acetic acid level was related to *A.*
562 *pasteurianus* abundance. This hypothesis could be verified by the identification of volatile
563 profile for each microbial species detected during fermentation.

564 **4. Conclusion**

565 This study measured the effect of three cocoa process parameters on both microbial
566 community structures and volatile composition of cocoa beans, and showed that fermentation
567 time has a major impact on the aroma, followed by turning. In our study, pod storage duration
568 (2 days vs 8 days) had a minor impact on cocoa microbial communities. Contrariwise, the
569 effect was pronounced on volatile composition when it was associated to turning. Fermented
570 cocoa beans with desired flavors were obtained with short pod storage times, which promoted
571 a significant presence of esters. Consequently, as expected a short fermentation without
572 aeration could have a negative impact on the cocoa volatile composition, because of the high
573 levels of undesirable compounds such as acetic acid and acetoin.

574 The molecular approach coupled to volatile and statistical analysis provided insight into the
575 relationship between the microbial communities' structure and aroma compound production

in fermenting cocoa beans. To better describe the relationship, it would be interesting to carry out the study in a specific way by identifying the aromatic profile of each microbial strains involved in the processes. Consequently, this global analysis allowed the identification of molecular and volatile markers, which could be good indicators of cocoa post-harvest processing. Acetic acid, acetoin, benzaldehyde, 2 and 3-methylbutanal content are related to cocoa fermentation time and aeration type, whereas, the evaluation of esters concentration could be used to distinguish pods storage duration of fermented beans.

At this stage, the impact of post-harvest processing was measured on cocoa volatile composition but not specifically on aroma quality. That aspect yet remains to be explored in the future.

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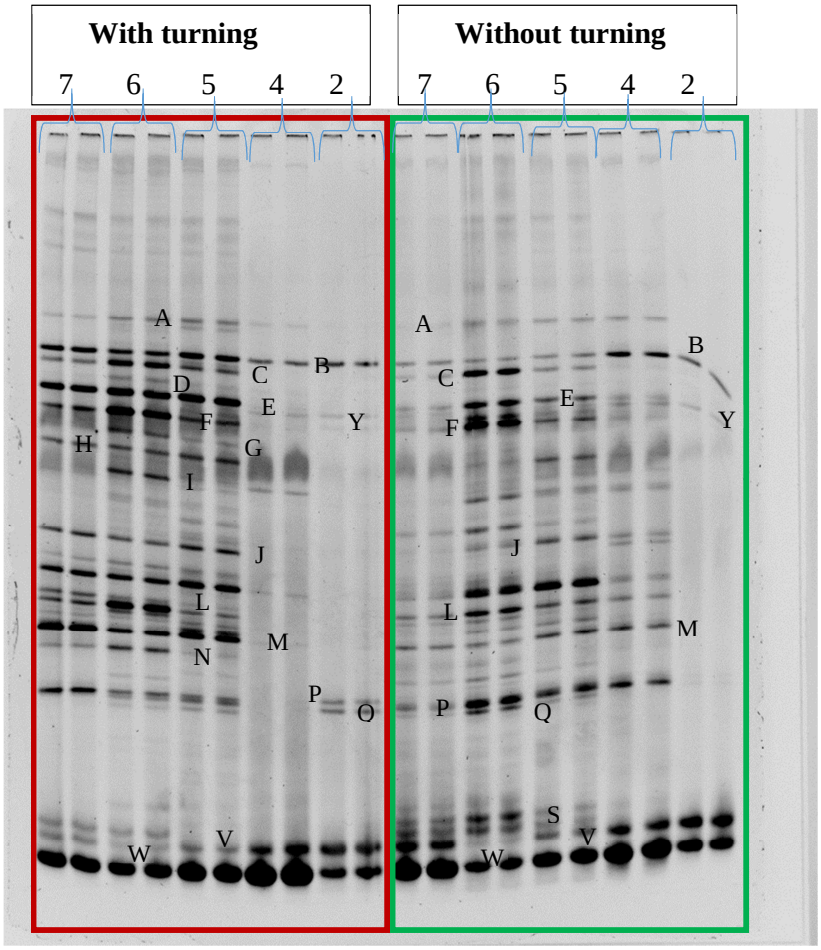
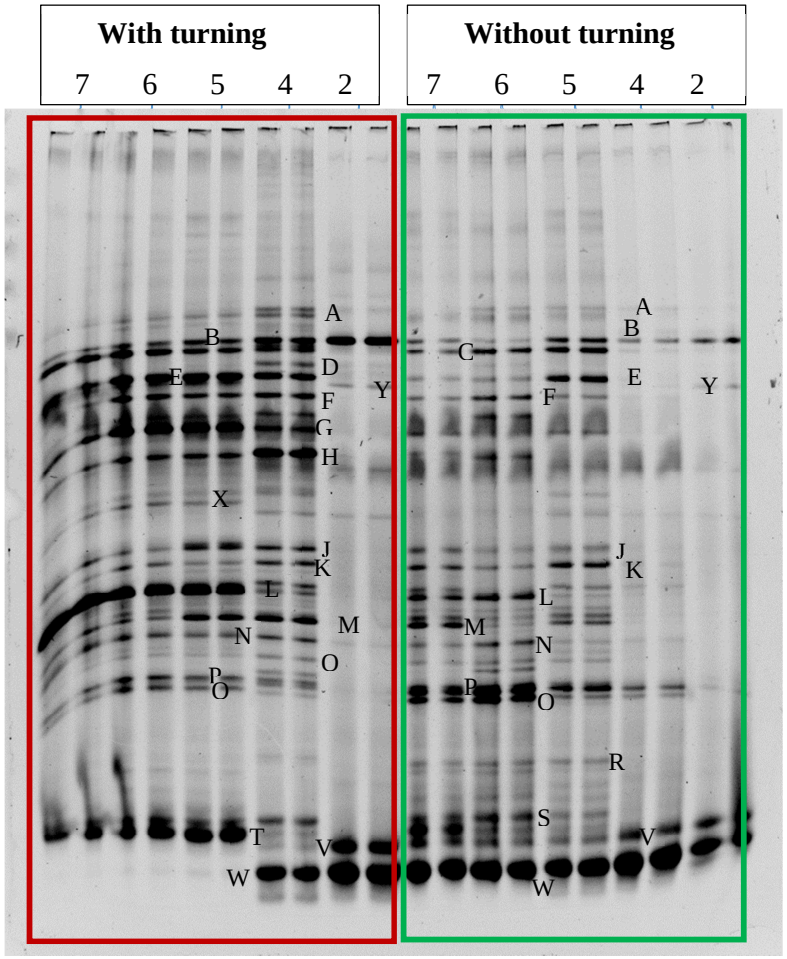


(a)



(b)

Figure 1: Bacterial DNA DGGE profiles obtained from fermented cocoa beans (with and without turning) during 2, 4, 5, 6 and 7 days fermentation, previously stored during 2 days (a) and 8 days (b). Identification of DGGE bands for bacterial species are given in Table 1.

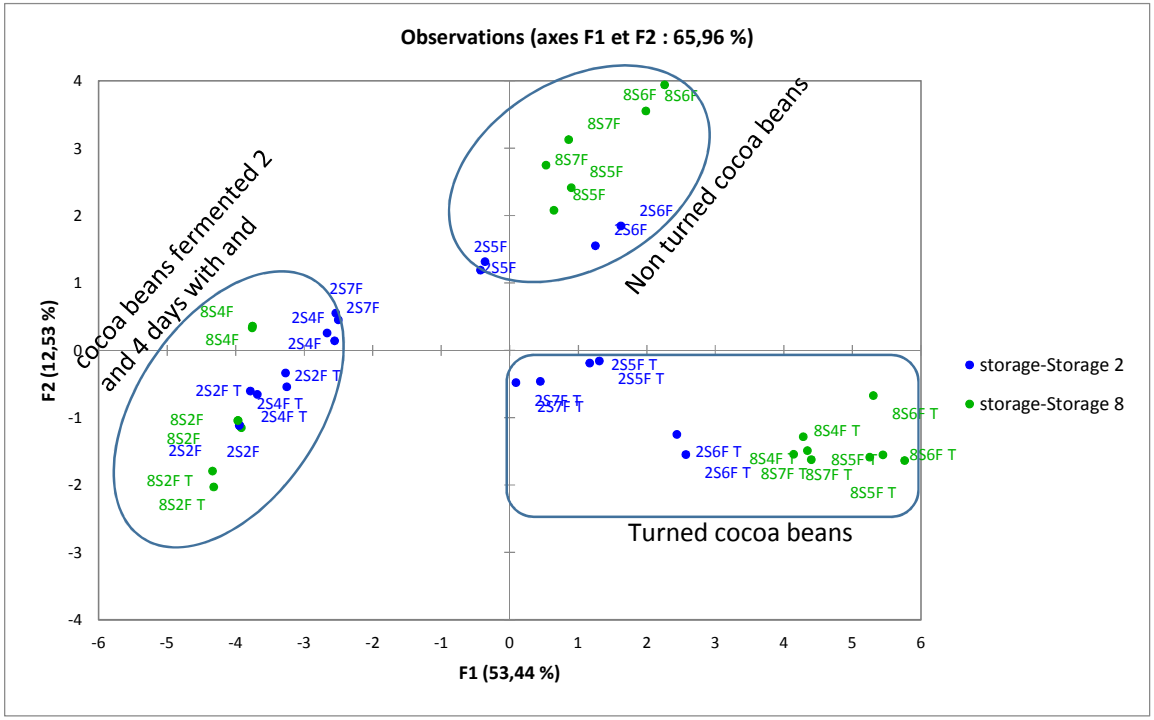
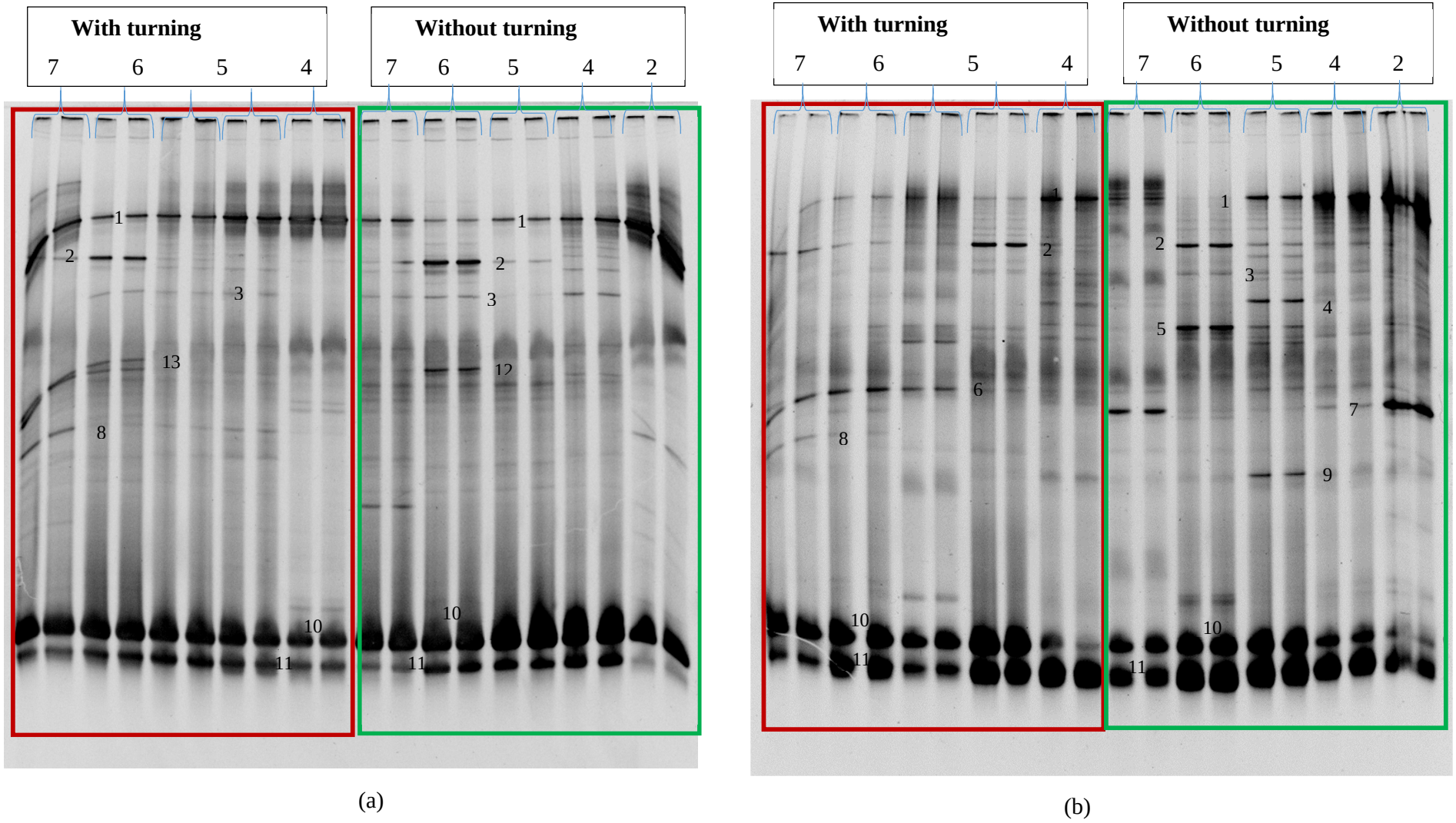


Figure 2: Principal component analysis (PCA) of bacterial 16S rDNA DGGE band profiles obtained from cocoa beans stored during 2 days, fermented with turning (2S2F T, 2S2F T, 2S4F T, 2S4F T, 2S5F T, 2S5F T, 2S6F T, 2S6F T, 2S7F T and 2S7F T) and without turning (2S2F , 2S2F , 2S4F , 2S4F , 2S5F , 2S5F , 2S6F , 2S6F , 2S7F and 2S7F) during 2, 4, 5, 6 and 7 days fermentation and from cocoa beans stored during 8 days, fermented with turning (8S2F T, 8S2F T, 8S4F T, 8S4F T, 8S5F T, 8S5F T, 8S6F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F , 8S2F , 8S4F , 8S4F , 8S5F , 8S5F , 8S6F , 8S6F , 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.



(a) (b)

Figure 3: Yeasts DNA DGGE profiles obtained from fermented cocoa beans (with and without turning) during 2, 4, 5, 6 and 7 days fermentation, previously stored during 2 days (a) and 8 days (b) . Identification of DGGE bands for yeast species are given in Table 2.

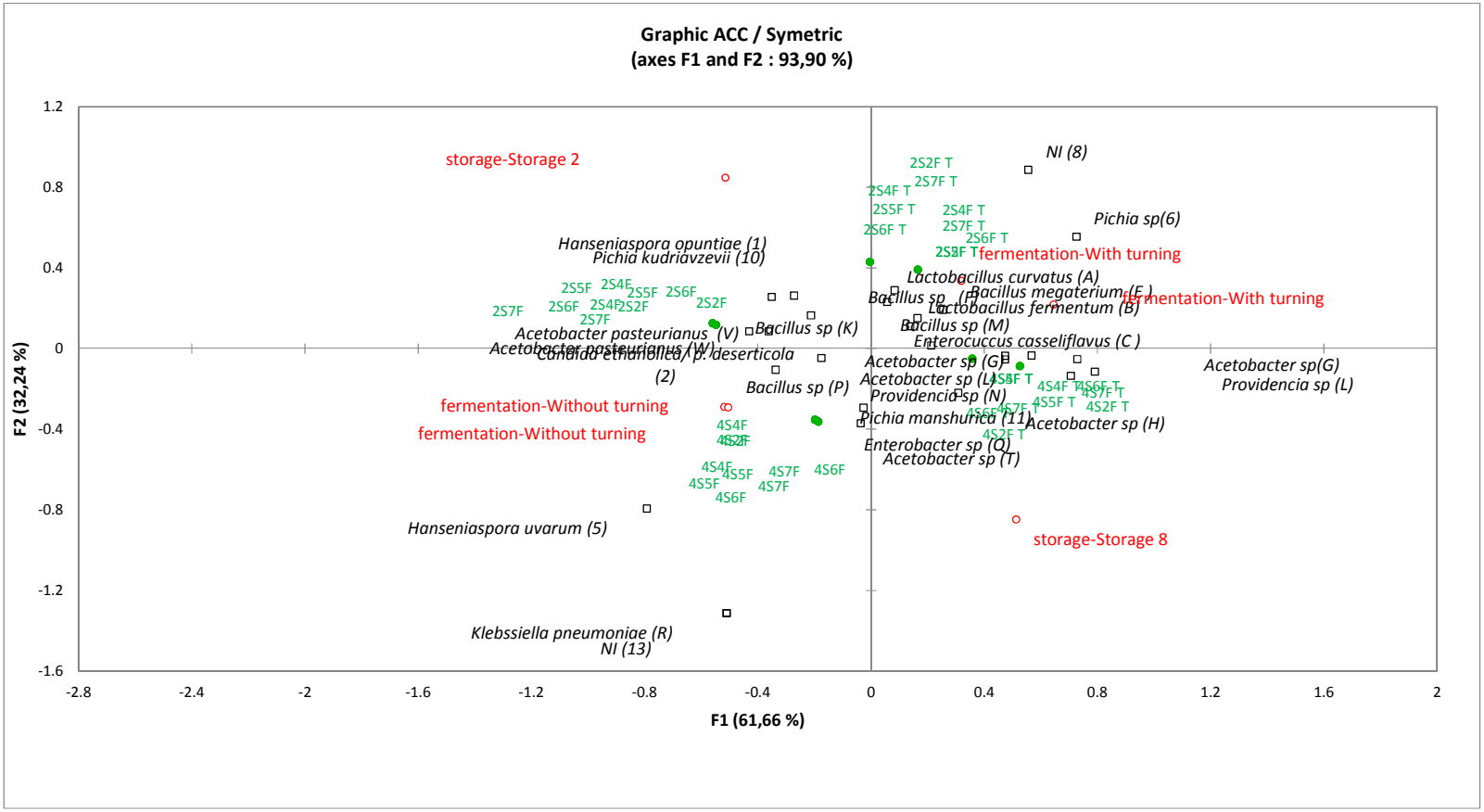
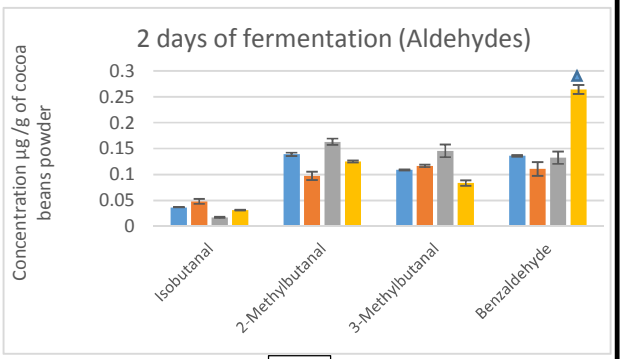
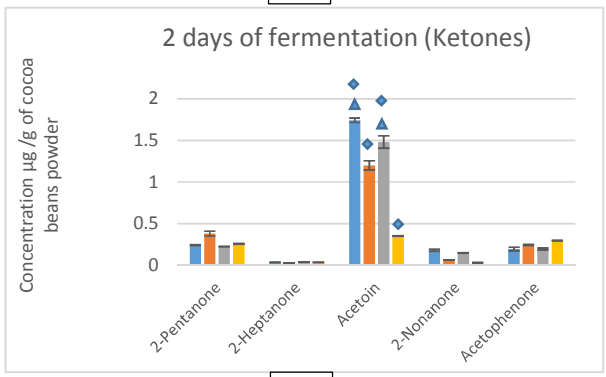


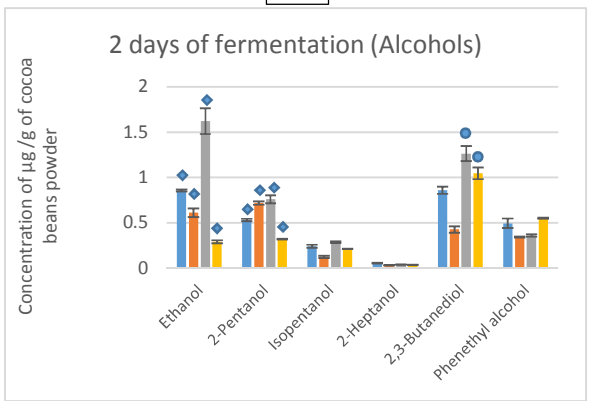
Figure 4: Canonical analysis of correspondence (CAC) of bacterial and yeast DGGE band profiles obtained from cocoa beans stored during 2 days, fermented with turning (2S2F T, 2S2F T, 2S4F T, 2S4F T, 2S5F T, 2S5F T, 2S6F T, 2S6F T, 2S7F T and 2S7F T) and without turning (2S2F , 2S2F , 2S4F , 2S4F , 2S5F , 2S5F , 2S6F , 2S6F , 2S7F and 2S7F) during 2, 4, 5, 6 and 7 days fermentation and from cocoa beans stored during 8 days, fermented with turning (8S2F T, 8S2F T, 8S4F T, 8S4F T, 8S5F T, 8S5F T, 8S6F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F , 8S2F , 8S4F , 8S4F , 8S5F , 8S5F , 8S6F , 8S6F , 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.


2 days of fermentation (Aldehydes)
Concentration µg /g of cocoa beans powder
0.3
0.25
0.2
0.15
0.1
0.05
0
Isobutanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde

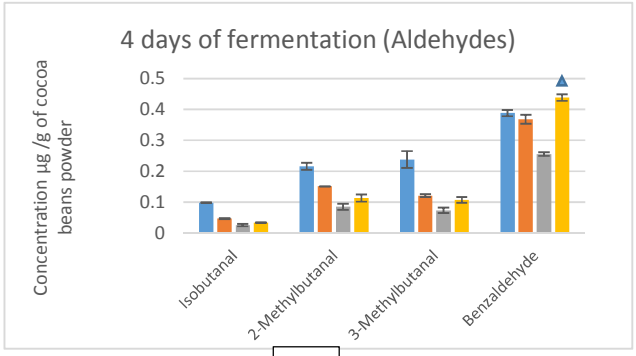
a


2 days of fermentation (Ketones)
Concentration µg /g of cocoa beans powder
2
1.5
1
0.5
0
2-Pentanone
2-Heptanone
Acetoin
2-Nonanone
Acetophenone

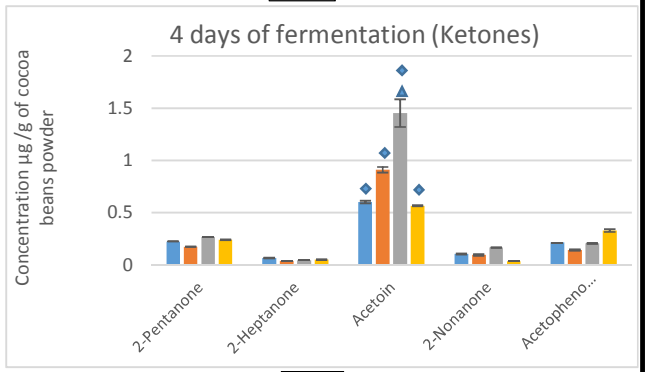
b


2 days of fermentation (Alcohols)
Concentration of µg /g of cocoa beans powder
2
1.5
1
0.5
0
Ethanol
2-Pentanol
Isopentanol
2-Heptanol
2,3-Butanediol
Phenethyl alcohol

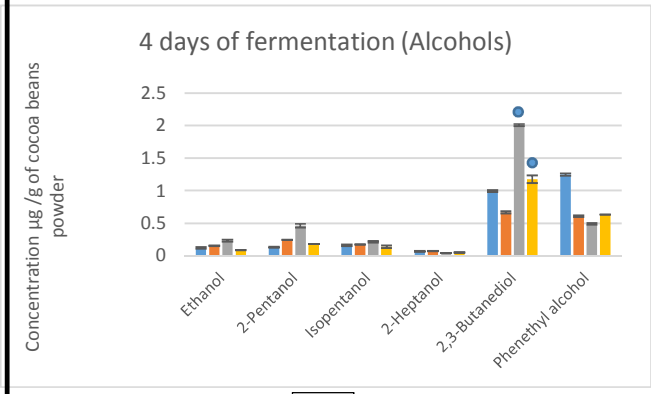
c


4 days of fermentation (Aldehydes)
Concentration µg /g of cocoa beans powder
0.5
0.4
0.3
0.2
0.1
0
Isobutanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde

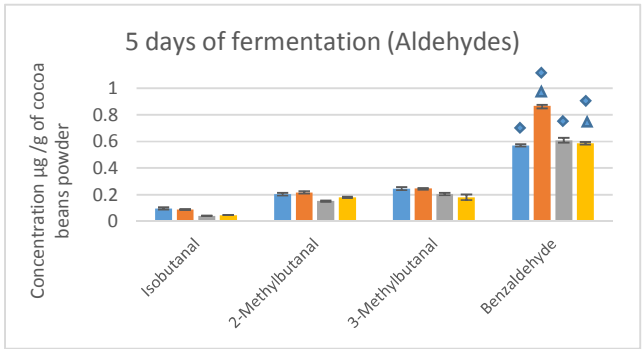
g


4 days of fermentation (Ketones)
Concentration µg /g of cocoa beans powder
2
1.5
1
0.5
0
2-Pentanone
2-Heptanone
Acetoin
2-Nonanone
Acetopheno...

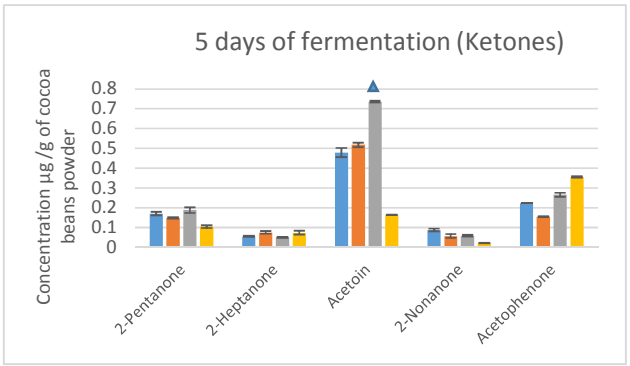
h


4 days of fermentation (Alcohols)
Concentration µg /g of cocoa beans powder
2.5
2
1.5
1
0.5
0
Ethanol
2-Pentanol
Isopentanol
2-Heptanol
2,3-Butanediol
Phenethyl alcohol

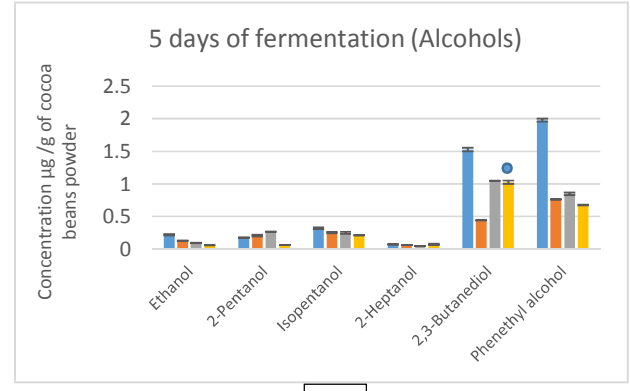
i


5 days of fermentation (Aldehydes)
Concentration µg /g of cocoa beans powder
1
0.8
0.6
0.4
0.2
0
Isobutanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde

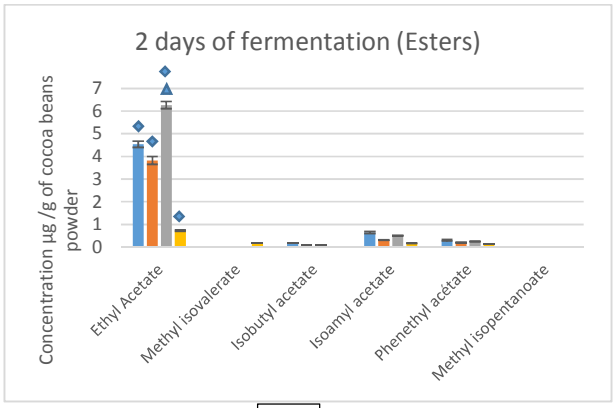
m


5 days of fermentation (Ketones)
Concentration µg /g of cocoa beans powder
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2-Pentanone
2-Heptanone
Acetoin
2-Nonanone
Acetophenone

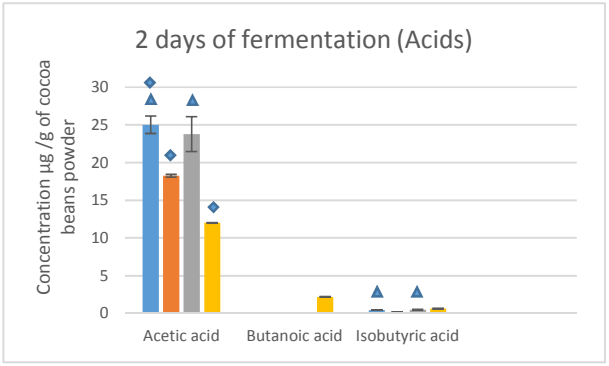
n


5 days of fermentation (Alcohols)
Concentration µg /g of cocoa beans powder
2.5
2
1.5
1
0.5
0
Ethanol
2-Pentanol
Isopentanol
2-Heptanol
2,3-Butanediol
Phenethyl alcohol

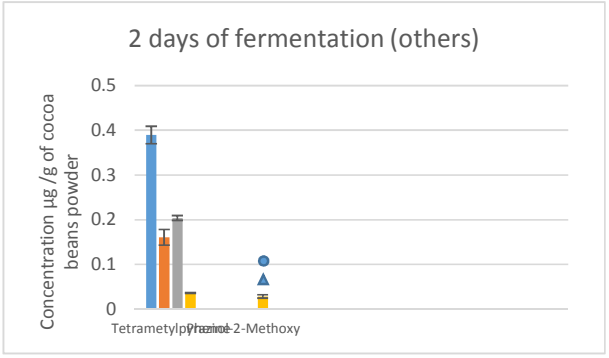
o


2 days of fermentation (Esters)
Concentration µg /g of cocoa beans powder
Ethyl Acetate
Methyl isovalerate
Isobutyl acetate
Isoamyl acetate
Phenethyl acétate
Methyl isopentanoate

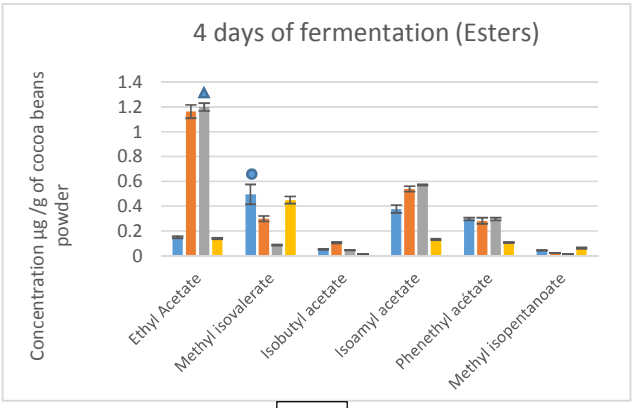
d


2 days of fermentation (Acids)
Concentration µg /g of cocoa beans powder
Acetic acid
Butanoic acid
Isobutyric acid

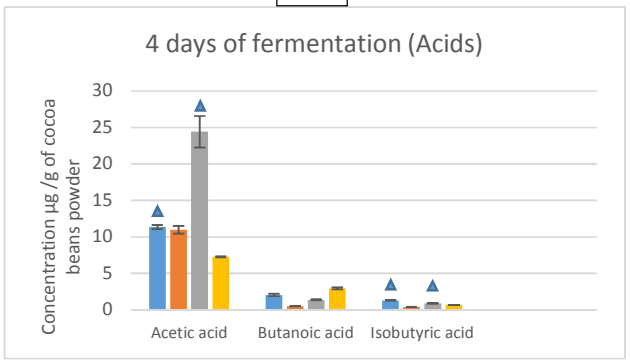
e


2 days of fermentation (others)
Concentration µg /g of cocoa beans powder
Tetrametylpyrazine
Phenol-2-Methoxy

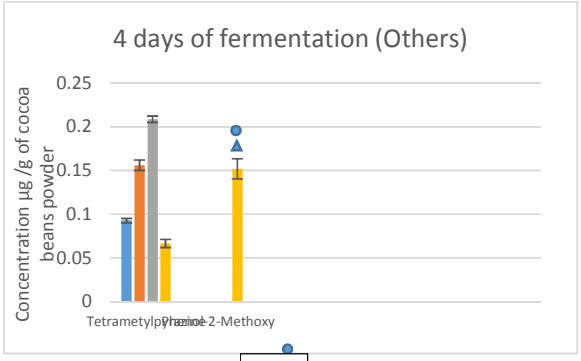
f


4 days of fermentation (Esters)
Concentration µg /g of cocoa beans powder
Ethyl Acetate
Methyl isovalerate
Isobutyl acetate
Isoamyl acetate
Phenethyl acétate
Methyl isopentanoate

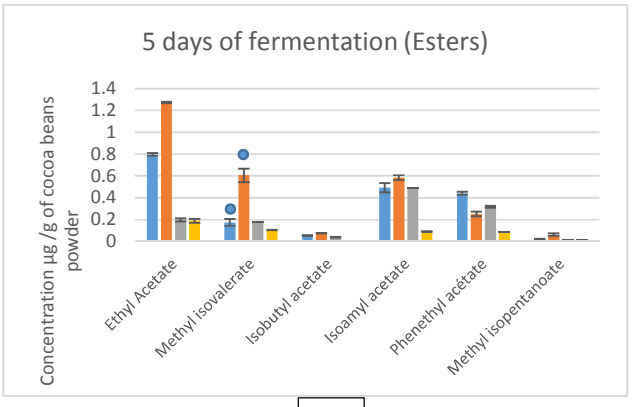
j


4 days of fermentation (Acids)
Concentration µg /g of cocoa beans powder
Acetic acid
Butanoic acid
Isobutyric acid

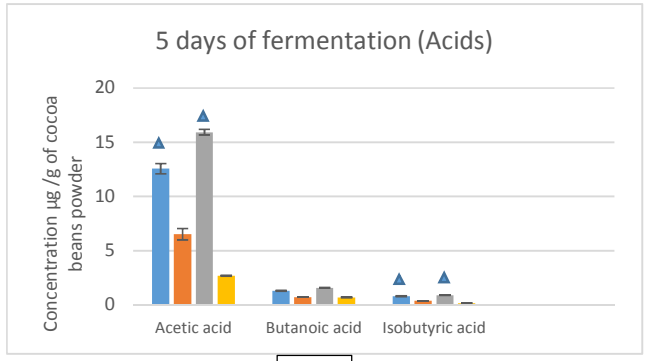
k


4 days of fermentation (Others)
Concentration µg /g of cocoa beans powder
Tetrametylpyrazine
Phenol-2-Methoxy

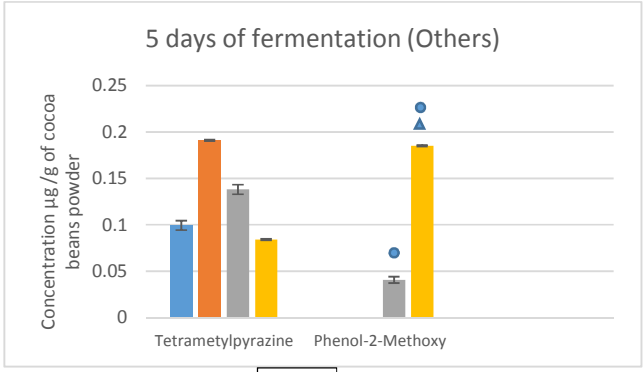
l


5 days of fermentation (Esters)
Concentration µg /g of cocoa beans powder
Ethyl Acetate
Methyl isovalerate
Isobutyl acetate
Isoamyl acetate
Phenethyl acétate
Methyl isopentanoate

p


5 days of fermentation (Acids)
Concentration µg /g of cocoa beans powder
Acetic acid
Butanoic acid
Isobutyric acid

q


5 days of fermentation (Others)
Concentration µg /g of cocoa beans powder
Tetrametylpyrazine
Phenol-2-Methoxy

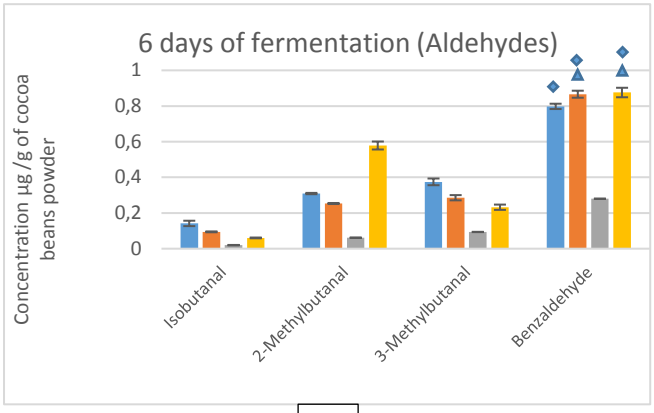
r


2F (Storage 2 days)
2F+T (Storage 2 days)
2F (Storage 8 days)
2F+T (Storage 8 days)

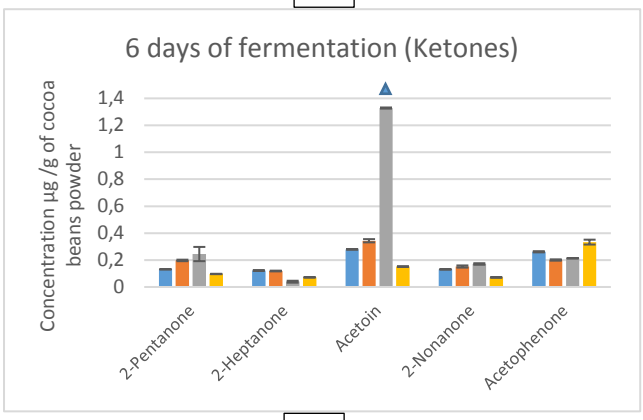

4F (Storage 2 days)
4F+T (Storage 2 days)
4F (Storage 8 days)
4F+T (Storage 8 days)


5F (Storage 2 days)
5F+T (Storage 2 days)
5F (Storage 8 days)
5F+T(Storage 8 days)

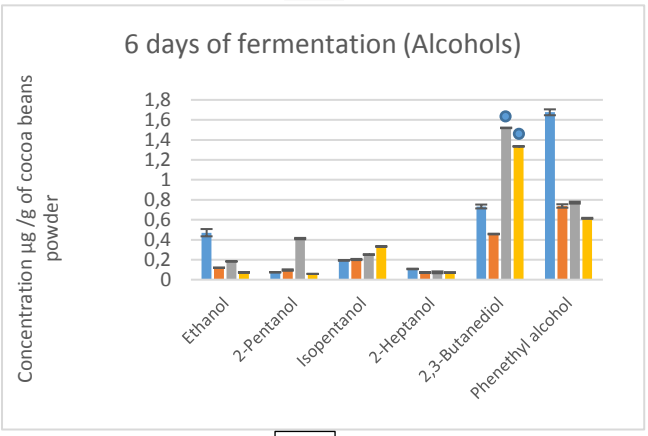
Figure 5: Concentration of aldehydes (a,g,m), ketones (b,h,n), alcohols (c,i,o), esters (d,j,p), acids (e,k,q) and other volatile compounds (f,l,r) produced in cocoa beans previously stored during 2 and 8 days, fermented 2 days (2F), 4 days (4F) , 5 days (5F) with turning (+T) and without turning. ▲ indicates significant differences between fermentation with and without turning. ⬭ indicates significant differences between the two pods storage duration. ◆ indicates significant differences between fermentation times.


6 days of fermentation (Aldehydes)
Concentration µg /g of cocoa beans powder
1
0,8
0,6
0,4
0,2
0
Isobutanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde

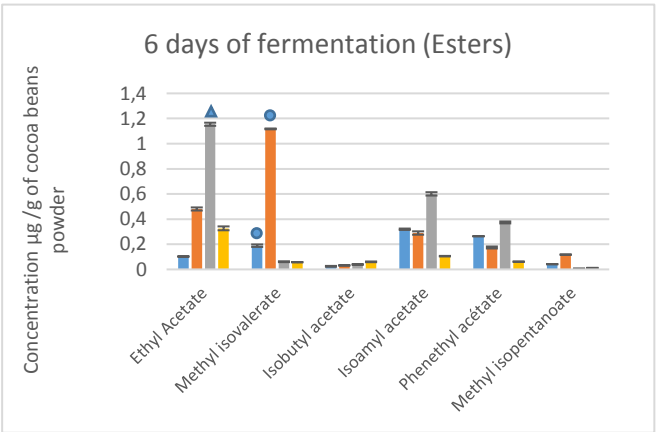
a


6 days of fermentation (Ketones)
Concentration µg /g of cocoa beans powder
1,4
1,2
1
0,8
0,6
0,4
0,2
0
2-Pentanone
2-Heptanone
Acetoin
2-Nonanone
Acetophenone

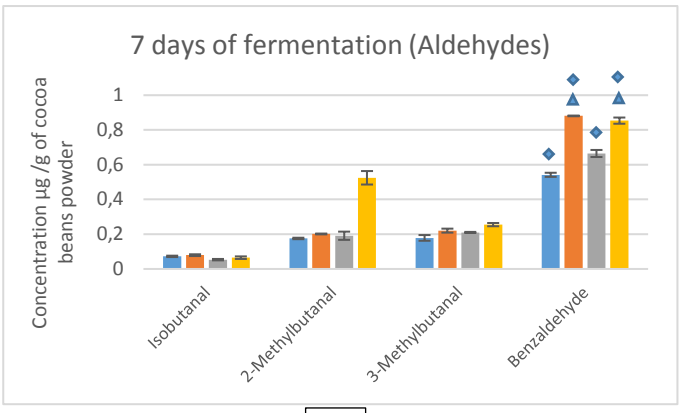
b


6 days of fermentation (Alcohols)
Concentration µg /g of cocoa beans powder
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ethanol
2-Pentanol
Isopentanol
2-Heptanol
2,3-Butanediol
Phenethyl alcohol

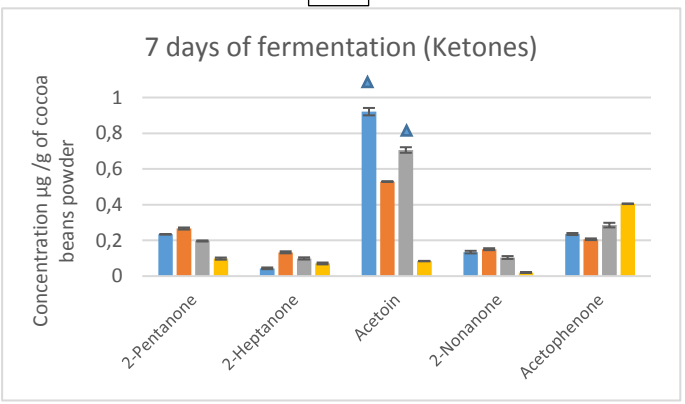
c


6 days of fermentation (Esters)
Concentration µg /g of cocoa beans powder
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ethyl Acetate
Methyl isovalerate
Isobutyl acetate
Isoamyl acetate
Phenethyl acétate
Methyl isopentanoate

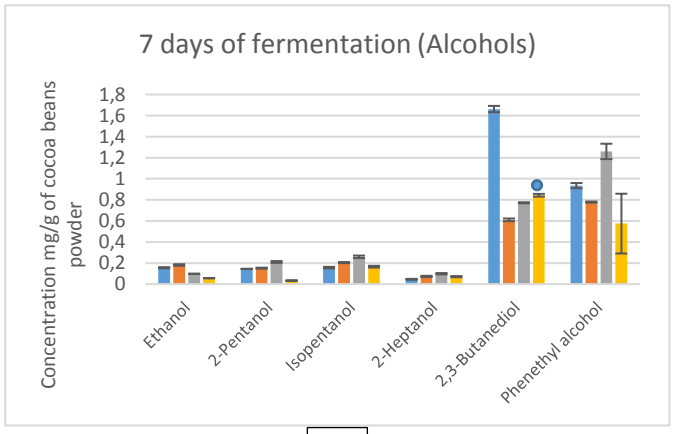
d


7 days of fermentation (Aldehydes)
Concentration µg /g of cocoa beans powder
1
0,8
0,6
0,4
0,2
0
Isobutanal
2-Methylbutanal
3-Methylbutanal
Benzaldehyde

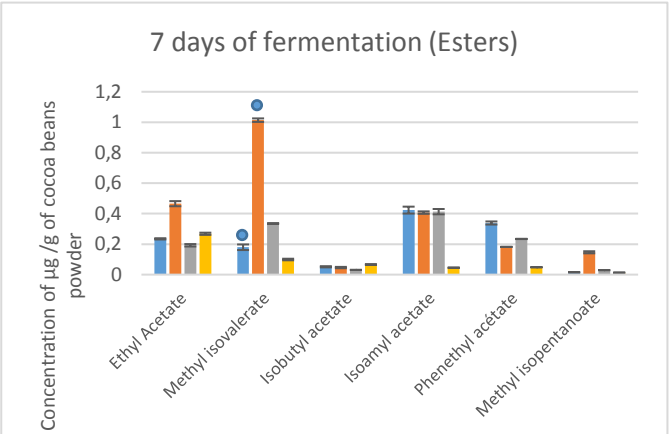
g


7 days of fermentation (Ketones)
Concentration µg /g of cocoa beans powder
1
0,8
0,6
0,4
0,2
0
2-Pentanone
2-Heptanone
Acetoin
2-Nonanone
Acetophenone

h


7 days of fermentation (Alcohols)
Concentration mg/g of cocoa beans powder
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
Ethanol
2-Pentanol
Isopentanol
2-Heptanol
2,3-Butanediol
Phenethyl alcohol

i


7 days of fermentation (Esters)
Concentration of µg /g of cocoa beans powder
1,2
1
0,8
0,6
0,4
0,2
0
Ethyl Acetate
Methyl isovalerate
Isobutyl acetate
Isoamyl acetate
Phenethyl acétate
Methyl isopentanoate

j

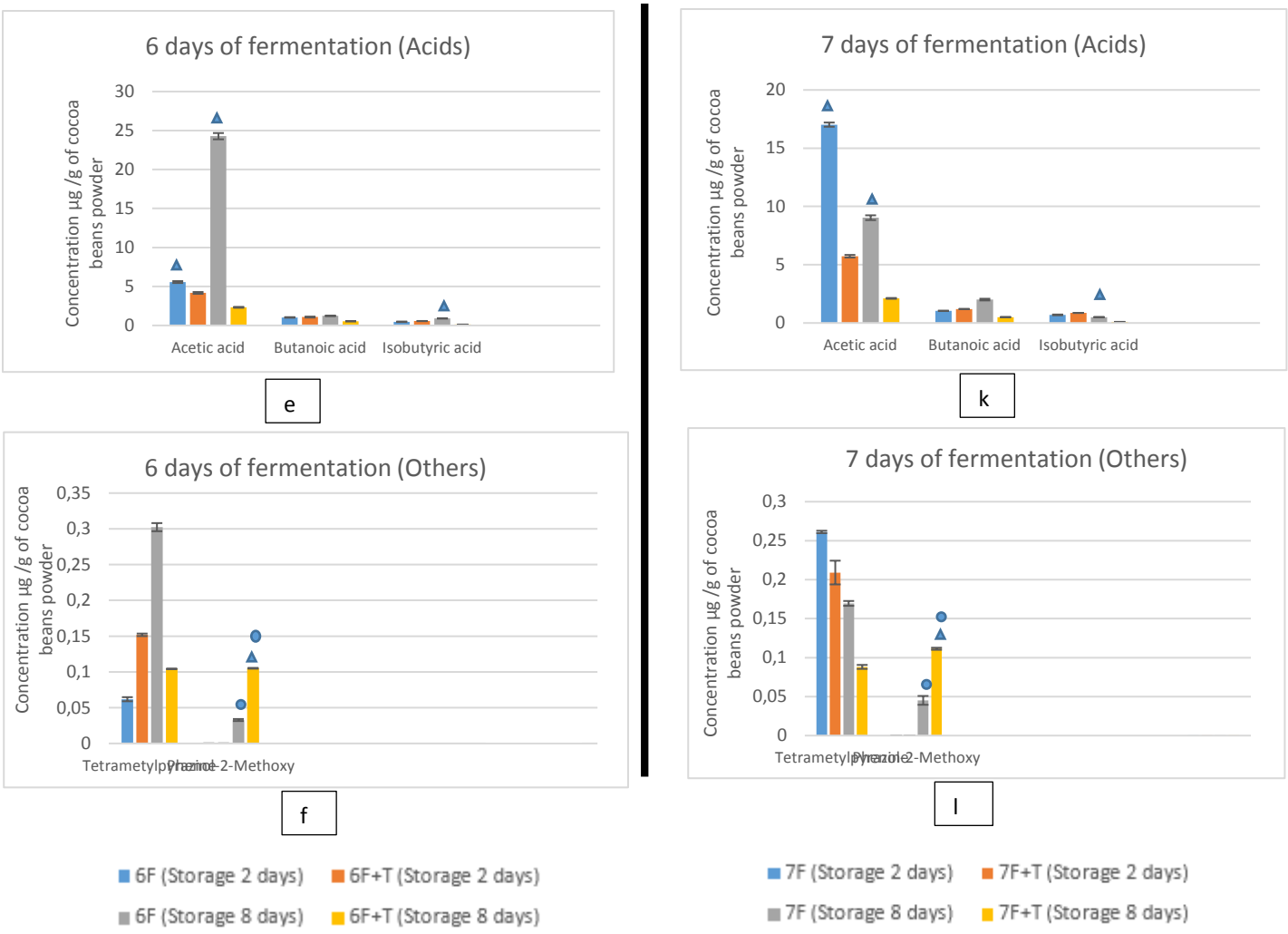
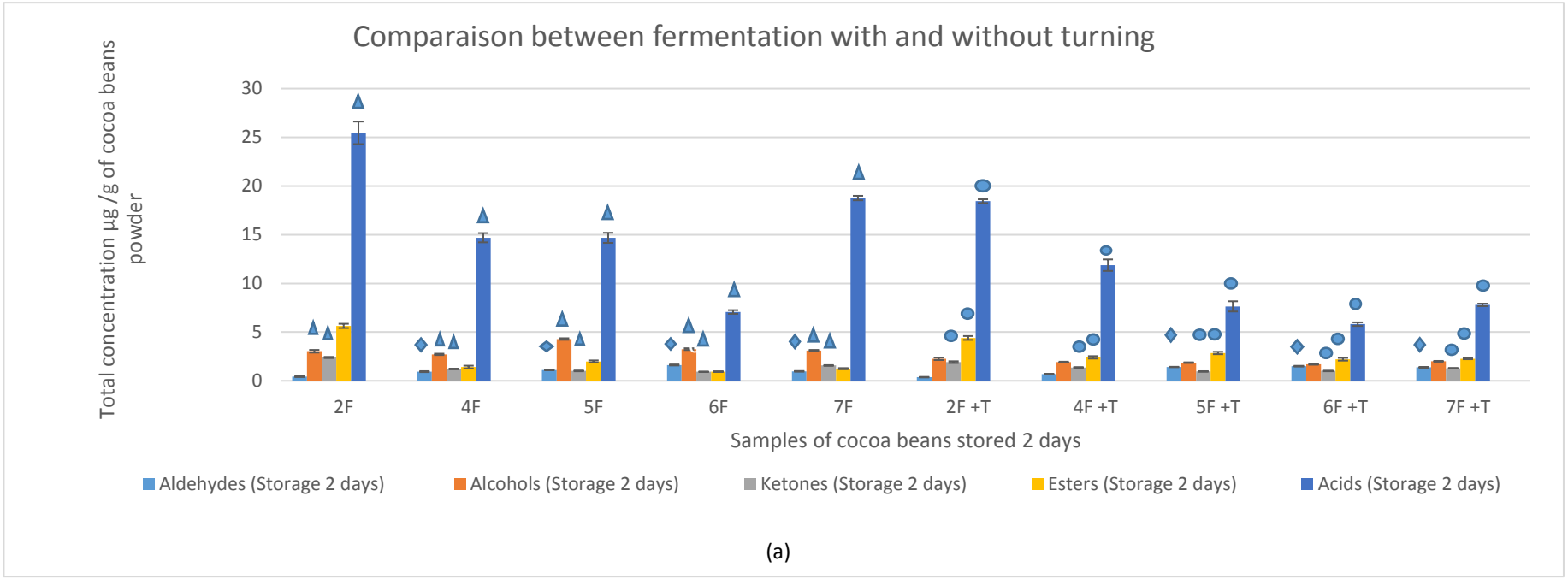


Figure 6: Concentration of aldehydes (a,g,), ketones (b,h), alcohols (c,i), esters (d,j), acids (e,k) and other volatile compounds (f,l) produced in cocoa beans previously stored during 2 and 8 days, fermented 6 days (6F) and 7 days (7F) with turning (+T) and without turning. ▲ indicates significant differences between fermentation with and without turning. ● indicates significant differences between the two pods storage duration. ◆ indicates significant differences between fermentation times.



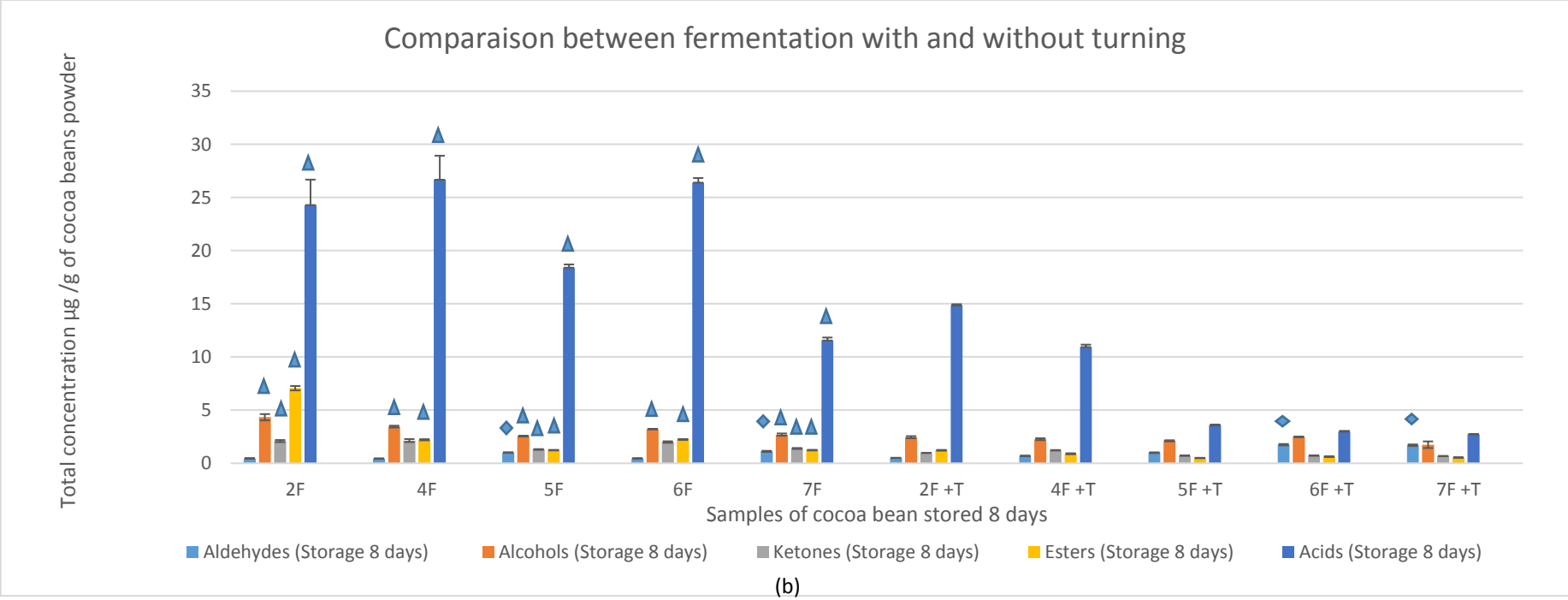
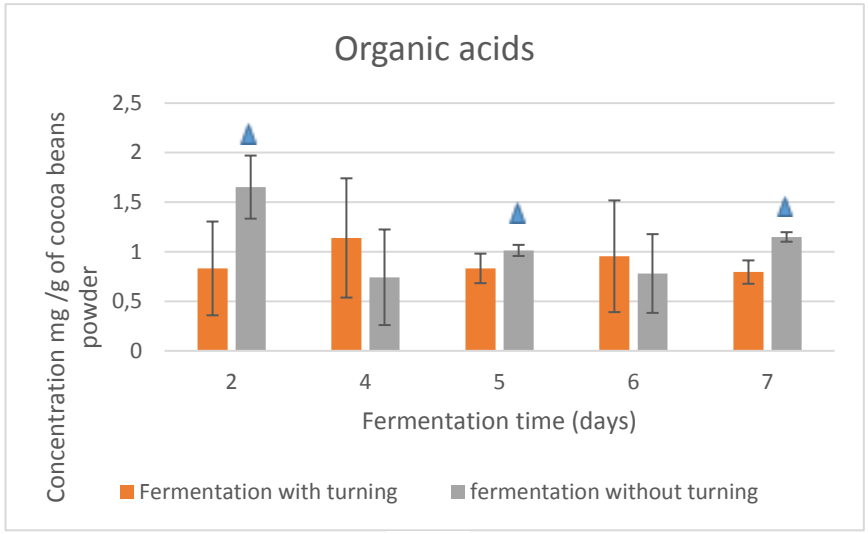
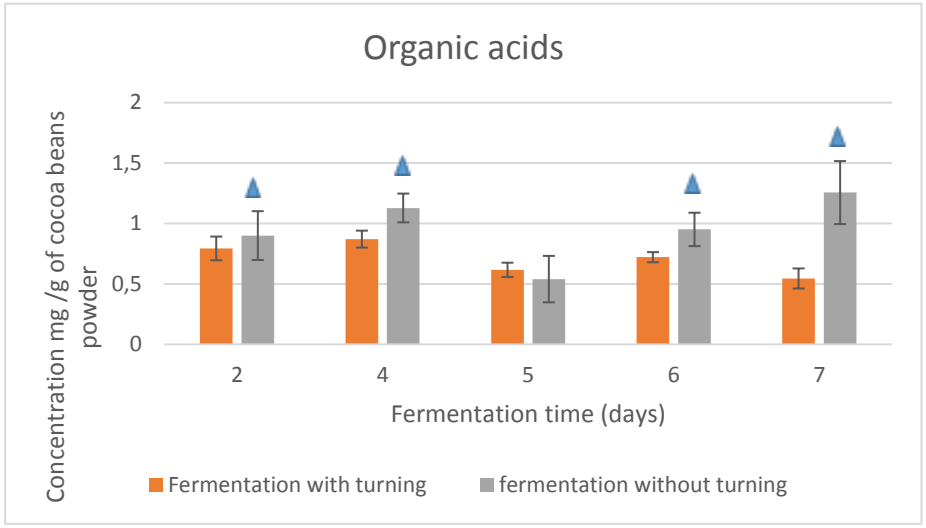


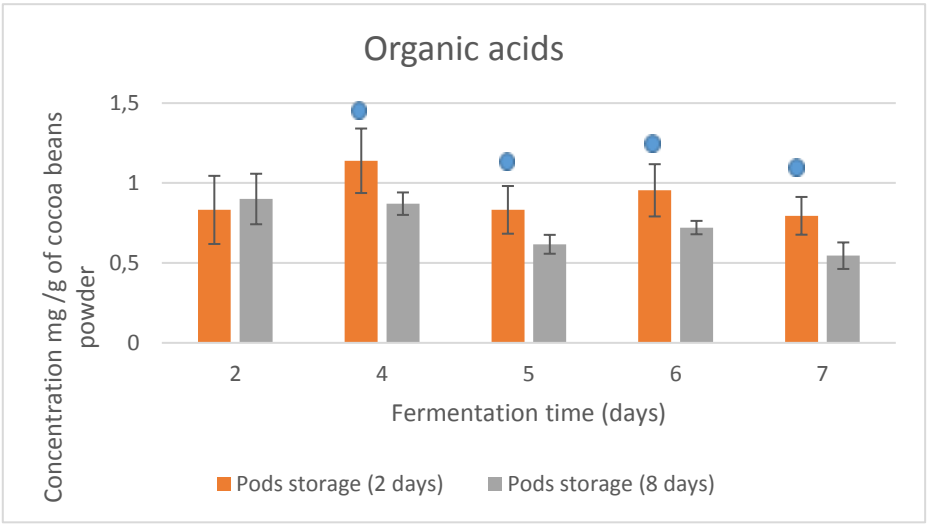
Figure 7: Total Concentration of volatile compounds produced in cocoa beans previously stored during 2 days (a) and 8 days (b), fermented without turning at 2, 4, 5, 6, 7 days (2F, 4F, 5F, 6F, 7F) and with turning (2F+T, 4F+T, 5F+T, 6F+T, 7F+T). ▲ indicates significant differences between fermentation with and without turning. ● indicates significant differences between the two pods storage duration. ◆ indicates significant differences between fermentation times.



(a)



(b)



(c)

Figure 8: Concentration of organic acids present in cocoa beans previously stored during 2 days (a) and 8 days (b), fermented with turning and without turning. In (c) the two storage durations (2 and 8 days) were compared. ▲ indicates significant differences between fermentation with and without turning. ● indicates significant differences between the two pods storage duration.

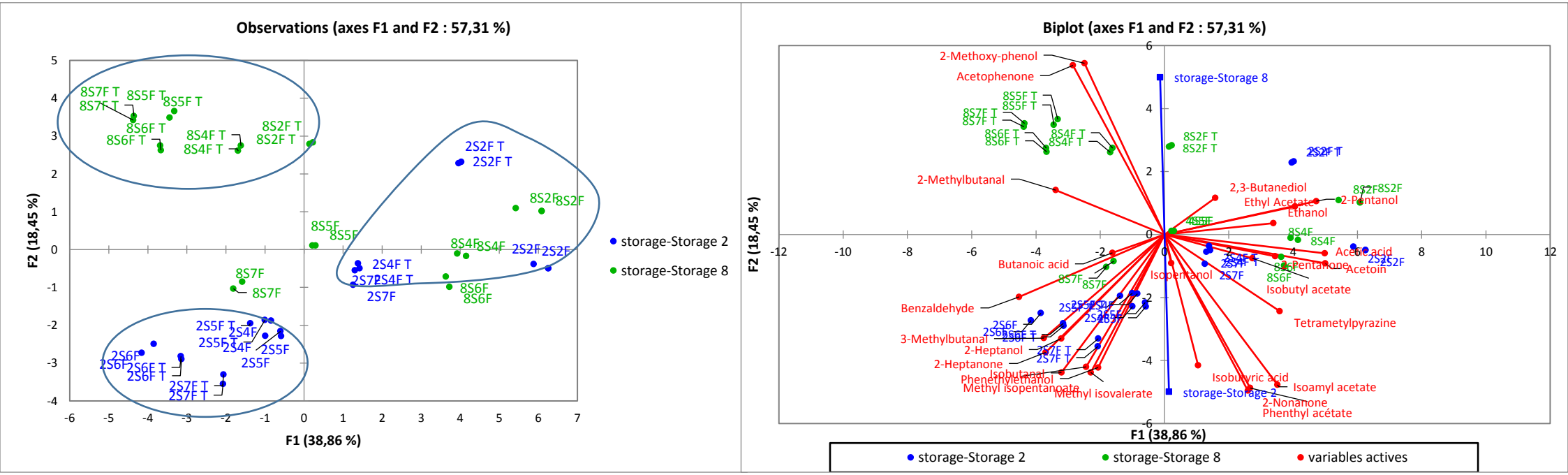


Figure 9 : Principal component analysis (PCA) of peak areas of volatiles compounds obtained from cocoa beans stored during 2 days, fermented with turning (2S2F T, 2S2F T, 2S4F T, 2S4F T, 2S5F T, 2S5F T, 2S6F T, 2S6F T, 2S7F T and 2S7F T) and without turning (2S2F , 2S2F , 2S4F , 2S4F , 2S5F , 2S5F , 2S6F , 2S6F , 2S7F and 2S7F) during 2, 4, 5, 6 and 7 days fermentation and from cocoa beans stored during 8 days, fermented with turning (8S2F T, 8S2F T, 8S4F T, 8S4F T, 8S5F T, 8S5F T, 8S6F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F , 8S2F , 8S4F , 8S4F , 8S5F , 8S5F , 8S6F , 8S6F , 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.

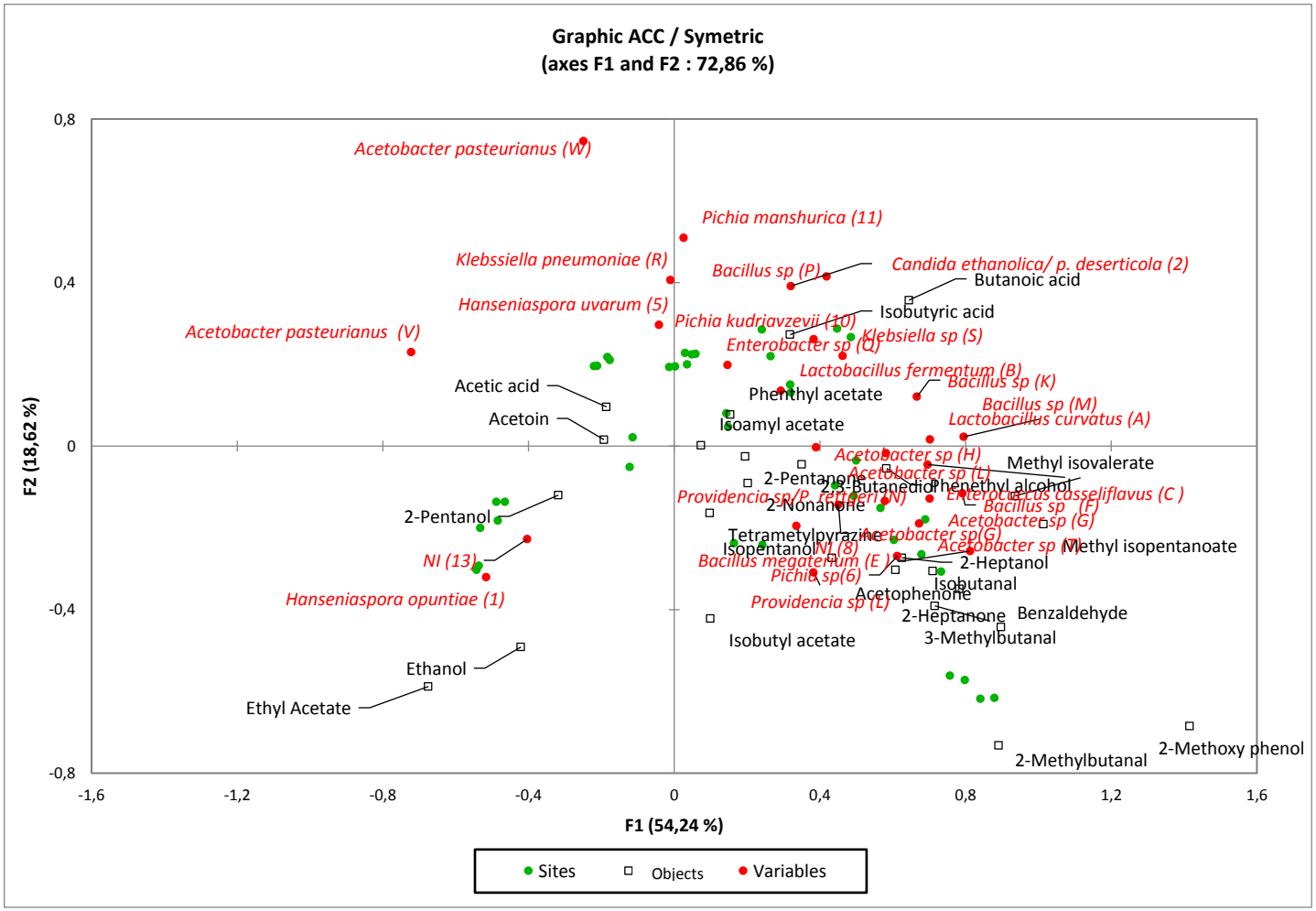


Figure 10: Canonical analysis of correspondence (CAC) applied on microbial DGGE band profiles and peak areas obtained from cocoa beans stored during 2 and 8 days and fermented with and without turning during 2, 4, 5, 6 and 7 days.

Table 1: Identification of bacterial DNA bands isolated from DGGE experiment

Band	Family/ Genus/species	Percent identity	Query cover	E-value	References
A	*Lactobacillus curvatus*	93%	99%	5e-74	FJ609221
Y	*Enterobacteriaceae*	100%	100%	2e-97	KM021355
B	*Lactobacillus fermentum*	99%	100%	1e-95	LC042465
C	*Enterococcus casseliflavus*	99%	100%	1e-78	KJ571214
E	*Bacillus megaterium*	94%	100%	2e-77	KJ004421
F	*Bacillus sp*	99%	100%	8e-97	HG794259
G **H** **J** **T**	*Acetobacter sp*	98% 98% 99% 90%	100% 100% 100% 99%	1e-89 6e-88 2e-92 1e-53	AB853266
X	*Acinetobacter sp*	92%	100%	2e-51	KJ814994
K	*Bacillus sp*	100%	100%	2e-97	KM983003
L	*Providencia sp*	90%	97%	5e-64	AB920789
M	*Bacillus sp*	90%	100%	5e-43	FJ235680
N	*Providencia sp*	97%	100%	2e-67	KM059194
O	*Enterobacter aerogenes*	99%	100%	1e-95	LN623623
P	*Bacillus sp*	91%	97%	1e-53	KC236480
Q	*Enterobacter sp*	99%	100%	8e-97	GU944492
S	*Klebsiella sp*	92%	99%	7e-57	GQ416571
V **W**	*Acetobacter pasteurianus*	100%	100%	1e-83 5e-83	KM983001
R	*Klebsiella pneumoniae*	100%	100%	2e-97	KP761422
D, I	*NI*				

NI: not identified band

Table 2: Identification of DGGE bands for yeast species

Band	Family/ Genus/ species	Percent identity	Query cover	E-value	References
1	*Hanseniaspora opuntiae*	98%	100%	6e-93	KC111446
2	*Candida ethanolica/ Pichia deserticola*	93%	100%	8e-56	KM234475
3	*Pichia sp*	99%	100%	1e-79	JX408867
4	*Pichia galeiformis*	93%	100%	3e-76	HM212622
5	*Hanseniaspora uvarum*	93%	100%	4e-65	KM816746
9	*Pichia sp*	95%	100%	4e-65	EU884437
10	*Pichia kudriavzevii*	99%	100%	2e-47	KC494718
11	*Pichia manshurica*	99%	100%	7e-11	JQ419868
12	*Candida ethanolica/ Pichia deserticola*	93%	99%	2e-64	KM005182
6 ,7, 13	*NI*				

NI: not identified band

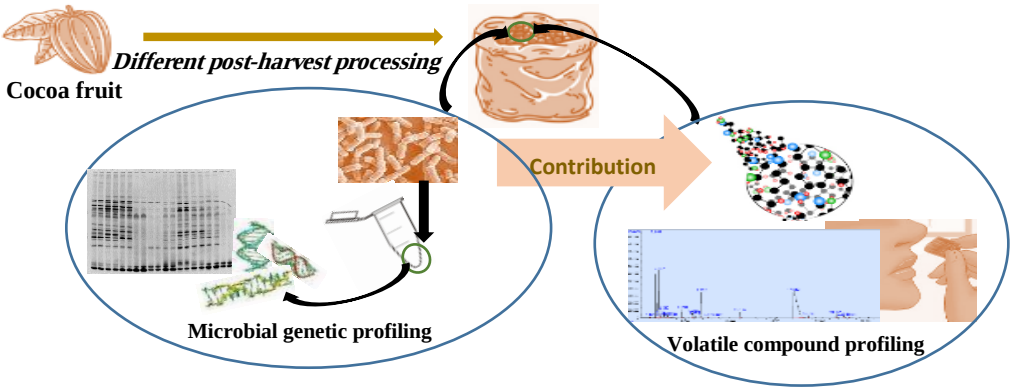
Table 3: Volatiles compounds identified in cocoa beans fermented with and without turning

Group	RT (min)	RI	RI*	Compound
Aldehydes	1.50	811	821	Isobutanal
	2.15	910	912	2-Methylbutanal
	2.19	913	910	3-Methylbutanal
	28.06	1497	1495	Benzaldehyde
Ketones	2.94	971	984	2-Pentanone
	9.51	1153	1170	2-Heptanone
	21.06	1367	1388	2-Nonanone
	35.21	1629	1645	Acetophenone
	14.83	1252	1272	Acetoin
Esters	1.94	886	885	Ethyl acetate
	2.28	920	916	Methyl isovalerate
	3.60	1011	1005	Isobutyl acetate
	3.72	1016	1015	Methyl isopentanoate
	6.92	1105	1117	Isoamyl acetate
	44.55	1802	1803	Phenylethyl acetate
Alcohols	2.46	934	929	Ethanol
	7.42	1115	1118	2-Pentanol
	11.44	1189	1206	Isopentanol
	17.96	1310	1332	2-Heptanol
	33.03	1588	1582	2,3-Butanediol
	49.58	1895	1925	2-Phenylethanol
Acids	24.26	1426	1449	Acetic acid
	32.01	1570	1588	Isobutyric acid
	37.58	1680	1691	Isovaleric acid
Others	26.06	1460	1468	Tetramethylpyrazine
	46.73	1842	1848	2-Methoxy-phenol

***:** Odour description and Retention Index (RI) obtained from literature: (Serra-Bonvehí, 2005; Rodriguez-Campos et al., 2011; 2012).

RI: Retention Index calculated

RT: Retention time


Different post-harvest processing
Cocoa fruit
Contribution
Microbial genetic profiling
Volatile compound profiling