

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

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- 1 Impact of turning, pod storage and fermentation time on microbial ecology and volatile
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#### 23 Abstract

24 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 25 26 work, we studied the impact of cocoa post-harvest processing on both microbial communities 27 structure and volatile composition. Cocoa beans samples were fermented in wooden boxes in 28 Ivory Coast at different time intervals with turning and without turning, and derived from 29 pods stored for two different duration times. Cocoa beans were analyzed using a molecular 30 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 31 32 profiles. The results showed that the main parameter that influenced microbial communities 33 structure was fermentation time, followed by turning, whereas, pods storage duration had a 34 minor impact. Similar results were obtained for aromatic profile, except for pods storage 35 duration that significantly affected volatile compound production. Global statistical analysis 36 using Canonical Correspondence Analysis (CCA), showed the relationship between microbial communities and volatile composition. Furthermore, this study allowed the identification of 37 38 discriminating microbial and chemical markers of cocoa post-harvest processing.

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#### 46 **1. Introduction**

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

51 Cocoa has an intrinsic quality potential that depends on several parameters, such as cocoa 52 variety, age of the cocoa tree, soil chemical composition, post-harvest treatments, and 53 industrial process (Kongor et al., 2016). In particular, postharvest processing applied on beans 54 can enhance or degrade the sanitary and/or organoleptic qualities of cocoa (Saltini et al., 55 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 56 57 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 58 are the first type of microorganism involved followed by Lactic Acid Bacteria (LAB), and 59 60 then Acetic Acid Bacteria (AAB) (Schwan and Wheals, 2004). Several recent studies tried to 61 understand the role of these microorganism groups in cocoa fermentation and to control this 62 phenomenon by inoculating selected strains previously isolated from cocoa (Lefeber et al., 63 2012, Crafack et al., 2013). The major results of these works showed that yeast strains have a 64 greater impact on the improvement of cocoa aromatic quality when compared to bacterial 65 strains (Crafack et al., 2013; 2014; Ho et al., 2014; Koné et al., 2016; Hamdouche et al., 2017). 66

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).

72 Aroma profile significantly contributes to cocoa quality and thus is crucial for the 73 acceptability of chocolate. This profile varied mainly with fermentation and drying time 74 (Rodriguez-Campos et al., 2011; 2012). Aroma precursors previously formed during 75 fermentation and drying steps were highly developed during roasting. Roasting reduces 76 acidity by volatile acids evaporation and removes bitter, acidic and astringent flavors (Beckett 77 2000, Ramli et al., 2006). Maillard reactions generate a valuable source of aromatic 78 compounds as pyrazines, pyrroles, furans and thiazoles (Belitz et al., 2009). The 2-79 methylpyrazine and 2, 3, 5, 6 tetramethylpyrazine are the main volatile compounds present in 80 roasted cocoa. Both are responsible for the sweet and strong cocoa flavor respectively 81 (Lefeber et al., 2012).

The most used techniques for volatile compounds extraction are distillation, direct solvent 82 83 extraction, solid phase extraction (SPE), headspace methods and solid phase micro-extraction (SPME). After concentration, gas chromatography is the most suitable analytical method. 84 85 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 86 87 volatile compounds detection and identification: flame ionization, flame photometry, atomic 88 emission, and mass spectrometry. SPME-GC-MS for the headspace analysis of volatile 89 compounds allowed sensitive and representative analysis of cocoa products with high 90 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,

95 fermentation time and turning) during cocoa post-harvest processing on microbial and volatile 96 profiles; (ii) to identify discriminating microbial and/or aroma markers of each process; (iii) to 97 establish relationship between microbial communities associated with cocoa beans and 98 volatile compounds produced.

#### 99 2. Material and Methods

#### 100 **2. 1. Cocoa beans sampling**

101 Four hundred cocoa pods were harvested in November 2014 in Akoupé (Ivory Coast). The 102 number of harvested pods was divided in two batches (200 pods each), the first batch was 103 stored during 2 days and the other one during 8 days. After storage, all pods were opened 104 using a machete. For each pods opening delay, approximately 30 kg of pulp-bean mass were 105 extracted manually, recovered and distributed in two wooden boxes for fermentation during 7 106 days. Two types of processing were applied on these cocoa beans: fermentation with and 107 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, 108 5, 6 and 7 days of fermentation and sun dried during 7 days on wooden drying racks. During 109 drying step, no turning was done. The sampling was performed in sterile conditions by using 110 111 sterile gloves and plastic bags. Samples were sent by plane to Cirad laboratory, Montpellier, 112 France and stored at room temperature. The microbial and chemical analyses were carried out 113 immediately.

#### 114 2. 2. Microbial ecology analysis

#### 115 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<sub>2</sub>-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<sup>-1</sup>; 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).

126 2. 2. 2. PCR amplification of extracted DNA

127 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 128 129 GGG GCA CGG GGG GCA TCC TAC GGG AGG CAG CAG-3', Sigma) and 518r (5'-ATT 130 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 131 DNA fragment will remain partially double stranded and that the region screened is in the 132 133 lowest melting domain (Sheffield et al., 1989). Each mixture (final volume 50 µL) contained 134 about 100 ng of DNA template, DNA primers at 0.2 mM, deoxyribonucleotide triphosphate 135 (dNTPs) at 200 µM, 1.5 mM MgCl<sub>2</sub>, 5 µL of 10X of Taq reaction buffer MgCl<sub>2</sub> free and 1.25 136 unit of a-Taq polymerase (Promega). In order to increase the specificity of amplification and 137 to reduce the formation of spurious by-products, DNA was amplified by a "touchdown" PCR 138 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

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

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

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

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

Electrophoresis experiments were performed at 60°C using a denaturing gradient ranging from 30 to 60% (100% corresponded to 7 M urea and 40% (v/v) formamide, Promega). The migration was carried out at 20 V for 10 min and then at 80 V for 12 h. Then the gel was stained for 30 min with Gel Red solution and then photographed as described above in 2.2.1 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  $\mu$ L 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). The raw sequences data were edited using Bioedit Software and compared to the GenBank database using the BLAST program (Altschul et al., 1997) (http://www.ncbi.nlm.nih. gov/BLAST/). Sequences having a percentage of identity of 97% or greater were considered to belong to the same species (Palys et al., 1997).

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

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

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

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

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

190 group shows the explanatory variables that are the microbial communities (DNA bands191 identified by PCR-DGGE).

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194 **2. 3. Volatile compound analysis** 

- 195 2. 3. 1. Sample preparation and SPME-HS conditions
- 196 The shell and the bean were both analysed, because the objective of the work was to evaluate
- 197 the volatile composition of exported merchant cocoa (fermented and dried beans). In food
- 198 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.
- 201 Two hundred grams of unshelled dried cocoa beans from Ivory Coast were put in liquid 202 nitrogen and ground using a waring blender (SEB, France), to obtain cocoa flour, which was 203 stored at - 80°C until analysis. Three grams of cocoa powder were introduced into vial (10 204 mL), 30  $\mu$ L 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 205 206 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-207 208 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 209 210 to the sample headspace at 50°C for 45 min.
- 211 2. 3. 2. Gas Chromatography Analytical Conditions

212 The analysis of the volatile compounds extracted from cocoa powder was performed using an 213 Agilent 6890N gas chromatography-mass spectrometer (GC-MS) in automatic injection mode, on a polar capillary column DBWAX, 30 m length  $\times 0.25$  mm internal diameter  $\times 0.25$ 214 215 um film thickness (Agilent, Palo Alto, CA, USA). The GC-MS conditions have been 216 established in Cirad laboratory, the injection temperature was 250°C. The GC oven 217 temperature was initially set at 40°C for 5 min, increased to 140°C at a rate of 2°C/min and 218 then increased at a rate of 10°C/min to 250°C. The carrier gas was high purity Helium at 1 mL.min<sup>-1</sup>. Splitless injection mode was used at 250°C for 2 min. The selective mass detector 219 220 was a quadrupole (Hewlett Packard, Model 5973), with an electronic impact ionization 221 system at 70 eV and at the temperature source at 230°C.

222 2. 3. 3. Volatile compounds identification

For the identification of compounds, pure standards were not used, this is why the 223 identification was just tentative. The identification was done: (i) by comparing the mass 224 spectra with commercial database Wiley275.L, HP product no. G1035A); (ii) and by 225 226 comparing of the linear retention index calculated (RI) with those found in the literature data. 227 Retention index (RI) was calculated in the same chromatographic conditions using series of nalkane C8-C20 (Supelco, Bellefonte, USA). Peak areas were used for relative quantification 228 229 of compounds using the MSD Chemstation software (version E.02.02.1431, Agilent 230 Technologies).

231 **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 volatilecompounds produced (variables to be explained).

#### 237 **3.** Non-volatile organic acids analysis

#### 238 **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  $\mu$ m filter (Sigma-Aldrich), then transferred to an HPLC vial (1 mL) (Thomson ).

#### 245 **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<sup>-1</sup> 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.

#### 253 3. Results and discussion

**3. 1. Microbial community structures** 

#### 255 **3. 1. 1. Variation in bacterial community structures**

256 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 258 two fermentation days, a few DNA bands (Y, B V, W) were observed and identified by 259 sequencing as belonging to Enterobacteriaceae, Lactobacillus fermentum, and Acetobacter 260 261 pasteurianus taxa respectively (Fig. 1.a,b; Table 1). At days 4 and 5, additional DNA bands 262 (A, E, F, J, L, M, N) were detected and identified as belonging to Lactobacillus curvatus, 263 Bacillus megaterium, Bacillus sp, Acetobacter sp and Providencia sp species, respectively. 264 All eight bacterial taxa remained detected until the last day of fermentation. This is 265 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 266 267 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).

272 Comparison of bacterial DNA profiles of fermented cocoa beans with and without turning 273 showed that the majority of revealed bands were common in the two types of fermentation 274 processing whatever the pod storage time. However, some bands (M, E, F) identified as 275 Bacillus genus were mainly revealed in DGGE profiles obtained from cocoa beans fermented 276 with turning (Fig. 1.a,b; Table 1). Furthermore, some bands (G, H) were detected only in 277 fermentation with turning and were identified as belonging to Acetobacter sp. These 278 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 279 280 consequence of temperature elevation after 2 days and at the turning time, which promoted 281 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 282

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, W) was not present during fermentation with turning, at 5, 6 and 7 days (Fig. 1.b-Table 1). 285 286 This result was unexpected because A. pasteurianus was the most prevalent AAB species involved in spontaneous cocoa from Ivory coast, Brazil and Malaysia (Hamdouche et al., 287 288 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 analysis. The disappearance of A. pasteurianus species was correlated with acetic acid content 292 293 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 investigated the pod storage effect on cocoa microbial ecology except the study of our 303 304 colleagues Kedjebo et al. (2016) who studied the effect of post-harvest treatment on the occurrence of ochratoxin A (OTA) in raw cocoa beans from Ivory Coast. They showed that 305 OTA content in raw cocoa beans increased with pods storage duration (0, 4 and 7 days) which 306 307 reflected the increase of ochratoxinogenic fungi activity (Aspergillus strains) during cocoa

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

310 Principal Component Analysis (PCA) was applied on bacterial DGGE profiles to represent 311 the dataset variance. We observed that fermented cocoa samples tended to group according to 312 fermentation time along the first component axis (F1 representing 53.44% of the total 313 variance). For 2 day storage samples, two main groups containing cocoa beans samples turned 314 fermented during 2 and 4 days on the left side of the axis and those fermented during 5, 6 and 315 7 days on the right side of the axis could be observed (Fig. 2). The picture is similar for 8 days 316 storage of non-turned samples. Eight-day storage of turned samples appeared as two main 317 groups: 2 day fermented on the left side of the axis and the rest of the samples on the right 318 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.

#### **326 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).

346 Canonical analysis of correspondence (CAC) was applied in order to describe the relation 347 between variations in microbial community structures and changes in cocoa post-harvest 348 parameters. CAC results showed that microbial communities associated with turning parameter consisted mainly of bacterial communities: Bacillus, LAB and Acetobacter species 349 350 (Fig. 4). This result was expected for AAB because they are aerobic microorganisms, which 351 are usually affected by the aeration. For *Bacillus*, it could be explained by the increasing of 352 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 353 354 duration was not related to the composition of microbiota.

#### 355 **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.

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

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#### **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, 2005; Rodriguez-Campos et al., 2012; Ho et al., 2014). In all fermentations, aldehydes content 368 369 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).

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

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#### 3. 2. 2. Variation of ketone compounds

385 For this family, the detected compounds were 2-heptanone, 2-pentanone, 2-nonanone, 386 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 387 388 general, acetoin quantity decreased with fermentation time. This compound is mainly 389 produced from pyruvate by alcoholic fermentation and from 2,3-butanediol by yeast 390 (Pretorius, 2000). Acetoin abundance in the first stage of fermentation could be correlated to 391 yeasts intervention in this stage. The quantity of others ketones varied slightly during 392 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 393 394 probably used as substrate by microorganisms during fermentation. Ketones were 395 significantly important (p < 0.05) in cocoa beans fermented without turning (Fig. 7.a,b). Some 396 ketones that were probably present naturally (Afoakwa et al., 2008, Rodriguez et al., 2012), 397 were degraded and catabolized in the case of the aerated fermentation process, where more 398 diversity and abundance of bacteria were observed by PCR-DGGE (Fig. 1.a). There was no 399 difference in ketones level for the two pod storage durations, except in the case of 400 fermentation with turning, whither the dominance of ketones was observed in pods stored at 401 the shorter time (2 days) (Fig. 7.a.b).

#### 402 **3. 2. 3. Variation of alcohol compounds**

403 The alcohols compounds found in cocoa beans samples were: ethanol, 2-pentanol, isopentanol, 2-heptanol, 2,3-butanediol, and 2-phenethylethanol. Among them, 2-404 phenethylethanol and 2,3 -butanediol were dominant during the four types of fermentations 405 406 (Fig. 5.c.i.o and Fig. 6.c.i); as already showed by Rodriguez-Campos et al. (2011). Ethanol was surely produced by the majority of yeasts (Ho et al., 2014, Koné et al., 2016). It was 407 408 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, subsequently it was oxidized in acetic acid (Schwan and Wheals, 2004) mainly by AAB 414 during the second (aerobic) stage. 415

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 oxidized to phenylethyl acetate with the presence of oxygen by LAB (Smit et al., 2005). 421 422 Furthermore, alcohol compounds could be evaporated with the aeration brought by turning process. This result agreed with those obtained by Camu et al. (2008) that showed the 423 424 evaporation of ethanol during the aeration of cocoa beans mass, as well as favoring the AAB growth that oxidized ethanol into acetic acid. 425

Total alcohols level was significantly higher in cocoa obtained from pods stored longer (Fig
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.

#### 434 **3. 2. 4. Variation of ester compounds**

435 The six detected esters were ethyl acetate, isobutyl acetate, isoamyl acetate, phenylethyl 436 acetate, methyl isopentanoate, and methyl isovalerate (Fig .5.d.j.p and Fig. 6.d.j). Ethyl 437 acetate was the dominant ester in all fermentations. According to Rodriguez-Campos et al. 438 (2012), esters concentration decreased with fermentation time, but in our case, no significant 439 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 440 441 ester seems to be produced only at the beginning of cocoa fermentation as reported previously 442 (Rodriguez-Campos et al., 2011), and also in wine fermentation (Rojas et al., 2003) mainly in 443 the first day of fermentation. This may be due to the abundance of glucose and ethanol during 444 this stage, because they are the main substrates for ethyl acetate synthesis (Yong et al., 1981). 445 Moreover, Ethyl acetate is a product of esterification from acetic acid and ethanol (Pretorious, 446 2000); this concept explains the correlation between the amount of this ester, acetic acid and 447 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 448 study. A large quantity of these compounds was observed at the beginning of fermentation 449 and decreased sharply after 2 days (Fig. 5.c.i.o.e.k.q and 6.c.i.e.k). The concentration of this 450 451 ester was significantly higher in cocoa fermented without turning (Fig 7.a,b). Esters were 452 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.

460 **3. 2. 5. Variation of acid compounds** 

461 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 462 significantly during fermentation realized with turning (Fig. 5.e.k.q and Fig. 6.e.k). In the case 463 464 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 465 evolution of acetic acid amount in this case, could be explained by the fact that there was no 466 beans turning, therefore no homogenization in the metabolites produced and beans 467 composition. Whatever the pods storage delay, a normal evolution of acetic acid amount 468 during fermentation with turning was observed. According to the literature, acetic acid 469 470 increased and reached its maximum around 72h of cocoa fermentation then decreased after 88 471 h (Shwan and Wheal, 2004; Lefeber and al., 2012). This data confirmed our result, which 472 showed that turning of cocoa beans allowed to realize fermentation with a normal evolution 473 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 474 475 (with AAB growth and/or activity) occurs in unmixed cocoa, and the rise in temperature plus 476 the aeration favors the volatilization nutrients depletion (Camu et al., 2008) during 477 fermentation with turning.

During fermentation, assimilation of citric acid by LAB generates a slight rise in pH to 4.5 478 and temperature to 37°C of cocoa pulp (Camu et al., 2007) which create a favorable 479 environment for the growth of AAB involved in the second phase of fermentation (acetic 480 fermentation). During this phase, AAB are responsible of the oxidation of ethanol to acetic 481 482 acid (Schwan and Wheals, 2004). Our results on acetic acid bacterial communities structure 483 was related to acetic acid production, because low acid acetic level was observed in parallel 484 with the disappearance of the main AAB, which was A. pasteurianus (band V and W) (Fig. 1) 485 in the absence of aeration.

486 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 487 488 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 489 490 the literature, acetic acid is produced by oxidation of the ethanol during the second 491 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, 492 493 which is volatile, could vary according to the ethanol content and time.

#### 494 **3. 2. 6. Variation of others volatile compounds**

*Pyrazines:* Tetramethylpyrazine was the unique compound belonging to pyrazine family that 495 496 was detected (Fig. 5.f.l.r and Fig. 6.f.l). In most cases, tetramethylpyrazine compound has 497 been reported to produce characteristics notes of roasted cocoa that is important for flavor 498 quality (Afoakwa et al., 2008; Serra-Bonvehi, 2005). Furthermore, various studies reported on 499 the microbial origin of pyrazines in fermented food as cocoa and soybeans (Selamat et al., 500 1994; Besson et al., 1997). In our case, tetramethylpyrazine was probably produced by 501 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 502

fermentation. Our results showed that tetramethylpyrazine level had no significant difference
during fermentation times, pods storage times and between fermentation processing. Pyrazine
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 detected at the end of cocoa fermentation (6 and 8 days) (Rodriguez-Campos et al., 2012). 509 The presence of this compound only in fermented cocoa beans obtained from long pods 510 storage when more microbial diversity was detected, could be explained by the increase of 511 nutrient degradation, including phenolic acids, thus 2-methoxyphenol elaboration. We also 512 513 connected the production of this compound to the natural heating during sun drying. In general, phenolic acids are degraded thermically or decomposed by microorganisms into 514 phenols, as 2-methoxyphenol that results from a heat degradation of ferulic acid during coffee 515 516 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. 8). This is consistent with the results obtained on volatile acids by Afoakwa et al. (2013a; 524 525 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.

# 530 3. 4. Correlations between microbial ecology and volatile compounds produced in cocoa 531 fermentation

532 In order to measure the impacts of turning during fermentation (fermentation time and pods 533 storage time), PCA was applied on all volatiles compounds detected in fermented cocoa beans 534 (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 535 in cocoa fermented without turning, while aldehydes were significantly abundant in the 536 537 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, 538 fermentation process and pod storage time. Principal axis F1 (38.86%) separated cocoa beans 539 540 samples fermented (5, 6 and 7 days) without turning from the beans fermented with turning 541 (Fig. 9). ACP analysis allowed identifying discriminant compounds that were affected by 542 studied parameters. Discriminant and significant compounds in fermentation without turning 543 were acetic acid, isobutyric acid, 2,3-butanediol, acetoin, phenethyl acetate, isoamyl acetate. 544 In the case of turning, all aldehydes compounds detected and some esters were identified as 545 discriminant compounds. The same axis F1 regrouped samples according to fermentation 546 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 547 548 other samples. F2 axis (18.45%), separated the two pods storage times starting from 5 days of 549 fermentation, by 2-methoxyphenol and acetophenone that were significant (abundant) in long storage (8 days). 550

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 could exist between microbial community structures and volatile composition of cocoa beans. 553 554 CAC was applied on all data obtained in order to determine these relationships (Fig. 10). Microbial taxa detection by DGGE and their correlation with volatile compounds had a 555 556 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 compounds (Ho et al., 2014; Koné et al., 2016). Moreover, the CAC described the relation 559 560 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. *pasteurianus* abundance. This hypothesis could be verified by the identification of volatile 562 563 profile for each microbial species detected during fermentation.

#### 564 4. Conclusion

This study measured the effect of three cocoa process parameters on both microbial 565 community structures and volatile composition of cocoa beans, and showed that fermentation 566 time has a major impact on the aroma, followed by turning. In our study, pod storage duration 567 568 (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 aeration could have a negative impact on the cocoa volatile composition, because of the high 572 573 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.

583 At this stage, the impact of post-harvest processing was measured on cocoa volatile 584 composition but not specifically on aroma quality. That aspect yet remains to be explored in 585 the future.

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#### 590 **References**

Afoakwa, E. O., Kongor, J. E., Takrama, J. F., & Budu, A. S. (2013a). Changes in
acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp preconditioned cocoa (Theobroma cacao) beans. *International Food Research Journal, 20*(3),
1215-1222

Afoakwa, E. O., Paterson, A., Fowler, M., & Ryan, A. (2008). Flavor formation and character
in cocoa and chocolate: a critical review. *Critical reviews in food science and nutrition, 48(9),*

**597** 840-857.

- Afoakwa, E. O., Quao, J., Takrama, J., Budu, A. S., & Saalia, F. K. (2013b). Chemical
  composition and physical quality characteristics of Ghanaian cocoa beans as affected by pulp
  pre-conditioning and fermentation. *Journal of Food Science and Technology*, *50(6)*, 10971105.
- 602 Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., & Lipman,
- 603 D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein databases search
- 604 programs. *Nucleic Acids Research*, 25, 3389-3402.
- Beckett, S. T. (2000). The Science of Chocolate. Royal Society of Chemistry Paperbacks.
- Belitz, H. D., Grosch, W., & Schieberle, P. (2009). Coffee, tea, cocoa. *Food chemistry*, 938-970.
- Besson, I., Creuly, C., Gros, J. B., & Larroche, C. (1997). Pyrazine production by Bacillus
  subtilis in solid-state fermentation on soybeans. *Applied microbiology and biotechnology*,
  47(5), 489-495.
- 611 Bonnarme, P., Amarita, F., Chambellon, E., Semon, E., Spinnler, H. E., & Yvon, M. (2004).
- Methylthioacetaldehyde, a possible intermediate metabolite for the production of volatile
  sulphur compounds from L-methionine by *Lactococcus lactis*. *FEMS microbiology letters*,
  236(1), 85-90.
- Camu, N., Gonzalez, A., De Winter, T., Van Schoor, A., De Bruyne, K., Vandamme, P.
- 616 (2008). Influence of turning and environmental contamination on the dynamics of populations
- of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation
- 618 in Ghana. *Applied and Environmental Microbiology*, 74, 86-98.
- Cocolin, L., Bisson, L. F., & Mills, D. A. (2000). Direct profiling of the yeast dynamics in
  wine fermentations. *FEMS Microbiology Letters*, *189*, 81-87.

- 621 Crafack, M., Keul, H., Eskildsen, C. E., Petersen, M. A., Saerens, S., Blennow, A., & Nielsen,
- 622 D. S. (2014). Impact of starter cultures and fermentation techniques on the volatile aroma and
- sensory profile of chocolate. *Food research international*, *63*, 306-316.
- 624 Crafack, M., Mikkelsen, M. B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., ... &
- 625 Nielsen, D. S. (2013). Influencing cocoa flavour using *Pichia kluyveri* and *Kluyveromyces*
- 626 *marxianus* in a defined mixed starter culture for cocoa fermentation. *International journal of*
- 627 *food microbiology*, *167(1)*, 103-116.
- de Melo Pereira, G. V., Magalhães, K. T., de Almeida, E. G., da Silva Coelho, I., & Schwan,
- 629 R. F. (2013). Spontaneous cocoa bean fermentation carried out in a novel-design stainless
- 630 steel tank: Influence on the dynamics of microbial populations and physical-chemical
- 631 properties. International journal of food microbiology, 161(2), 121-133.
- 632 Ducki, S., Miralles-Garcia, J., Zumbé, A., Tornero, A., & Storey, D. M. (2008). Evaluation of
- 633 solid-phase micro-extraction coupled to gas chromatography-mass spectrometry for the
- headspace analysis of volatile compounds in cocoa products. *Talanta*, 74(5), 1166-1174.
- 635 Guehi, S. T., Dabonne, S., Ban-Koffi, L., Kedjebo, D. K., & Zahouli, G. I. B. (2010). Effect
- of turning beans and fermentation method on the acidity and physical quality of raw cocoa
- 637 beans. Advance Journal of Food Science and Technology, 2(3), 163-171.
- Hamdouche, Y., Guehi, T., Durand, N., Kedjebo, K. B. D., Montet, D., & Meile, J. C. (2015).
- 639 Dynamics of microbial ecology during cocoa fermentation and drying: Towards the
- 640 identification of molecular markers. *Food Control*, 48, 117-122.
- Hamdouche, Y., Nganou D. N., Meile, J.C., Durand, N., Teyssier, C., Montet, D. (2016).
- 642 Discrimination of post-harvest coffee processing methods by microbial ecology analyses.
- 643 *Food Control*, 65, 112-120.

- Hamdouche, Y., Teyssier, C., & Didier, M. (2017). Impact of Acetic Bacteria on Cocoa
- 645 Fermentation. In Acetic Acid Bacteria: Fundamentals and Food Applications. Editor: Ilkin
- 646 Yucel Sengun. CRC Press.
- 647 Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K., & De Vos, P. (1996).
- 648 Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns
- 649 in bacterial phylogeny and taxonomy. *Journal of Microbiological Methods*, 26, 247-259.
- Ho, V. T., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation.
- 651 *International journal of food microbiology, 174, 72-87.*
- Holm, C. S., Aston, J. W., & Douglas, K. (1993). The effects of the organic acids in cocoa on
- the flavour of chocolate. *Journal of the Science of Food and Agriculture*, 61(1), 65-71.
- 654 ICCO 2017 : International Cocoa Organization.
- Jinap, S., Thien, J., & Yap, T. N. (1994). Effect of drying on acidity and volatile fatty acids
- 656 content of cocoa beans. *Journal of the Science of Food and Agriculture*, 65(1), 67-75.
- 657 Kedjebo, K. B. D., Guehi, T. S., Kouakou, B., Durand, N., Aguilar, P., Fontana, A., &
- Montet, D. (2016). Effect of post-harvest treatments on the occurrence of ochratoxin A in raw
- 659 cocoa beans. Food Additives & Contaminants: Part A, 33(1), 157-166.
- 660 Koné, M. K., Guéhi, S. T., Durand, N., Ban-Koffi, L., Berthiot, L., Tachon, A. F., & Montet,
- D. (2016). Contribution of predominant yeasts to the occurrence of aroma compounds during
- 662 cocoa bean fermentation. *Food Research International*, 89, 910-917.
- Kongor, J. E., Hinneh, M., Van de Walle, D., Afoakwa, E. O., Boeckx, P., & Dewettinck, K.
- 664 (2016). Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour
- 665 profile—A review. *Food Research International*, 82, 44-52.

Kowalchuk, G.A., Stephen, J.R., de Boer, W., Prosser, J.I., Embley, T.M., & Woldendorp,
J.W. (1997). Analysis of ammonia-oxydizing bacteria of the beta subdivision of the class
Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and
sequencing of PCR amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology*, *63*, 1489-1497.

- 671 Leesing, R. (2005). Identification and validation of specific markers for traceability of
  672 aquaculture fish for import/export (PhD thesis). France: University of Montpellier 2.
- Lefeber, T., Gobert, W., Vrancken, G., Camu, N., & De Vuyst, L. (2011). Dynamics and
  species diversity of communities of lactic acid bacteria and acetic acid bacteria during
  spontaneous cocoa bean fermentation in vessels. *Food Microbiology*, 28(3), 457-464.
- Lefeber, T., Janssens, M., Camu, N., & De Vuyst, L. (2010). Kinetic analysis of strains of
  lactic acid bacteria and acetic acid bacteria in cocoa pulp simulation media toward
  development of a starter culture for cocoa bean fermentation. *Applied and environmental microbiology*, *76*(23), 7708-7716.
- Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm
  implementation of a starter culture for improved cocoa bean fermentation and its influence on
  the flavour of chocolates produced thereof. *Food microbiology*, *30*(2), 379-392.
- Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., & De Vuyst, L. (2012). On-farm
  implementation of a starter culture for improved cocoa bean fermentation and its influence on
  the flavour of chocolates produced thereof. *Food microbiology*, *30*(2), 379-392.
- Lima, L. J., Kamphuis, H. J., Nout, M. R., & Zwietering, M. H. (2011). Microbiota of cocoa
  powder with particular reference to aerobic thermoresistant spore-formers. *Food microbiology*, 28(3), 573-582.

- 689 Menezes, A. G. T., Batista, N. N., Ramos, C. L., Silva, A. R. D. A., Efraim, P., Pinheiro, A.
- 690 C. M., & Schwan, R. F. (2016). Investigation of chocolate produced from four different
- 691 Brazilian varieties of cocoa (Theobroma cacao L.) inoculated with *Saccharomyces cerevisiae*.
- 692 *Food Research International*, 81, 83-90.
- 693 Meyer, B., Biehl, B., Said, M. B., & Samarakoddy, R. J. (1989). Post-harvest pod storage: A
- 694 method for pulp preconditioning to impair strong nib acidification during cocoa fermentation
- 695 in Malaysia. *Journal of the Science of Food and Agriculture*, 48(3), 285-304.
- 696 Moens, F., Lefeber, T., & De Vuyst, L. (2014). Oxidation of metabolites highlights the
- 697 microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation.
- 698 *Applied and environmental microbiology*, 80(6), 1848-1857.
- 699 Muyzer, G., De Waal, E. C., & Uitterlinden, A. G. (1993). Profiling of complex microbial
- 700 populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-
- amplified genes coding for 16S rRNA. *Applied Environmental Microbiology*, *59*, 695-700.
- 702 Nazaruddin, R., Seng, L. K., Hassan, O., & Said, M. (2006). Effect of pulp preconditioning on
- the content of polyphenols in cocoa beans (Theobroma cacao) during fermentation. *Industrial*
- 704 *Crops and Products, 24(1), 87-94.*
- 705 Palys, T., Nakamura, L. K., & Coha, F. M. (1997). Discovery and classification of ecological
- 706 diversity in the bacterial world: the role of DNA sequence data. International Journal of
- 707 *Systematic Bacteriology*, *47*, 1145-1156.
- Papalexandratou, Z., Camu, N., Falony, G., & De Vuyst, L. (2011). Comparison of the
- bacterial species diversity of spontaneous cocoa bean fermentations carried out at selected
- farms in Ivory Coast and Brazil. *Food microbiology*, 28(5), 964-973.

Papalexandratou, Z., Lefeber, T., Bahrim, B., Lee, O. S., Daniel, H. M., & De Vuyst, L.
(2013). *Hanseniaspora opuntiae, Saccharomyces cerevisiae, Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box
fermentations, underlining the importance of these microbial species for a successful cocoa
bean fermentation process. *Food microbiology*, *35*(2), 73-85.

- Papalexandratou, Z., Vrancken, G., De Bruyne, K., Vandamme, P., & De Vuyst, L. (2011).
- Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted
  species diversity of lactic acid bacteria and acetic acid bacteria. *Food microbiology*, 28(7),
  1326-1338.
- Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: novel approaches to theancient art of winemaking. *Yeast*, *16*(*8*), 675-729.
- Ramli, N., Hassan, O., Said, M., Samsudin, W., & Idris, N. A. (2006). Influence of roasting
  conditions on volatile flavor of roasted Malaysian cocoa beans. *Journal of Food Processing*
- *and Preservation*, *30*(*3*), 280-298.
- 725 Rodriguez-Campos, J., Escalona-Buendía, H. B., Contreras-Ramos, S. M., Orozco-Avila, I.,
- Jaramillo-Flores, E., & Lugo-Cervantes, E. (2012). Effect of fermentation time and drying
- temperature on volatile compounds in cocoa. *Food Chemistry*, 132(1), 277-288.
- 728 Rodriguez-Campos, J., Escalona-Buendía, H. B., Orozco-Avila, I., Lugo-Cervantes, E., &
- Jaramillo-Flores, M. E. (2011). Dynamics of volatile and non-volatile compounds in cocoa
- 730 (Theobroma cacao L.) during fermentation and drying processes using principal components
- analysis. *Food Research International*, 44(1), 250-258.
- 732 Rojas, V., Gil, J. V., Manzanares, P., Gavara, R., Piñaga, F., & Flors, A. (2002). Measurement
- 733 of alcohol acetyltransferase and ester hydrolase activities in yeast extracts. Enzyme and
- 734 *Microbial Technology*, *30*(2), 224-230.

- Rojas, V., Gil, J. V., Piñaga, F., & Manzanares, P. (2003). Acetate ester formation in wine by
  mixed cultures in laboratory fermentations. *International Journal of Food Microbiology*, *86(1)*, 181-188.
- Saltini, R., Akkerman, R., & Frosch, S. (2013). Optimizing chocolate production through
  traceability: A review of the influence of farming practices on cocoa bean quality. *Food control*, 29(1), 167-187.
- 741 Schwan, R. F., & Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role
- in chocolate quality. *Critical reviews in food science and nutrition, 44(4), 205-221.*
- 743 Selamat, J. I. N. A. P., & Mordingah Harun, S. (1994). Formation of methyl pyrazine during
- cocoa bean fermentation. *Pertanika*, *17*, 27-27.
- Serra-Bonvehí, J. (2005). Investigation of aromatic compounds in roasted cocoa powder. *European Food Research and Technology*, 221(1-2), 19-29.
- 747 Sheffield, V. C., Beck, J. S., Stone, E. M., & Myers, R. M. (1989). Attachment of a 40 bp
- 748 G+C rich sequence (GC-clamp) to genomic DNA fragments by polymerase chain reaction
- results in improved detection of single-base changes. *Proceeding of the National Academy of*
- 750 *Sciences of the United States of American, 86, 232-236.*
- Smit, G., Smit, B. A., & Engels, W. J. (2005). Flavour formation by lactic acid bacteria and
  biochemical flavour profiling of cheese products. *FEMS microbiology reviews, 29(3),* 591610.
- Smit, G., Smit, B. A., & Engels, W. J. (2005). Flavour formation by lactic acid bacteria and
  biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29(3),* 591610.

- 757 Ter Braak, C. J. (1986). Canonical correspondence analysis: a new eigenvector technique for
- multivariate direct gradient analysis. *Ecology*, 67(5), 1167-1179.
- 759 Tomlins, K. I., Baker, D. M., Daplyn, P., & Adomako, D. (1993). Effect of fermentation and
- 760 drying practices on the chemical and physical profiles of Ghana cocoa. *Food Chemistry*,
- 761 *46(3)*, 257-263.
- Visintin, S., Alessandria, V., Valente, A., Dolci, P., & Cocolin, L. (2016). Molecular
- identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid
- bacteria isolated from heap and box cocoa bean fermentations in West Africa. *International*
- *journal of food microbiology, 216, 69-78.*
- Yong, F. M., Lee, K. H., & Wong, H. A. (1981). The production of ethyl acetate by soy yeast
- 767 Saccharomyces rouxii NRRL Y-1096. International Journal of Food Science & Technology,
- 768 *16(2)*, 177-184.



**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.

![](_page_35_Figure_0.jpeg)

**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, 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, 8S4F T, 8S4F T, 8S4F T, 8S5F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F , 8S2F , 8S4F , 8S4F , 8S4F , 8S5F , 8S6F , 8S6F , 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.

![](_page_36_Figure_0.jpeg)

**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.

![](_page_37_Figure_0.jpeg)

**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, 2S4F T, 2S4F T, 2S5F T, 2S5F T, 2S6F T, 2S6F T, 2S7F T and 2S7F T) and without turning (2S2F , 2S2F , 2S4F , 2S4F , 2S4F , 2S5F , 2S6F , 2S6F , 2S6F , 2S6F and 2S7F) during 2, 4, 5, 6 and 7 days fermentation and from cocoa beans stored during 8 days, fermented with turning (8S2F T, 8S4F T, 8S4F T, 8S4F T, 8S5F T, 8S6F T, 8S6F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F , 8S2F , 8S4F , 8S4F , 8S5F , 8S5F , 8S6F , 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.

![](_page_38_Figure_0.jpeg)

![](_page_39_Figure_0.jpeg)

**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.  $\diamond$  indicates significant differences between fermentation times.

![](_page_41_Figure_0.jpeg)

![](_page_41_Figure_1.jpeg)

![](_page_42_Figure_0.jpeg)

**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.  $\blacktriangle$  indicates significant differences between fermentation with and without turning.  $\bigcirc$  indicates significant differences between the two pods storage duration.  $\diamondsuit$  indicates significant differences between fermentation times.

![](_page_43_Figure_0.jpeg)

![](_page_43_Figure_1.jpeg)

**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).  $\blacktriangle$  indicates significant differences between fermentation with and without turning. • indicates significant differences between the two pods storage duration. • indicates significant differences between fermentation times.

![](_page_44_Figure_0.jpeg)

(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.

![](_page_45_Figure_0.jpeg)

**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, 2S5F T, 2S5F T, 2S6F T, 2S6F T, 2S7F T and 2S7F T) and without turning (2S2F, 2S2F, 2S4F, 2S4F, 2S5F, 2S5F, 2S6F, 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, 8S4F T, 8S5F T, 8S6F T, 8S6F T, 8S7F T and 8S7F T) and without turning (8S2F, 8S4F, 8S4F, 8S5F, 8S5F, 8S6F, 8S6F, 8S7F and 8S7F) during 2, 4, 5, 6 and 7 days fermentation.

![](_page_46_Figure_0.jpeg)

**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.

Band	Family/ Genus/species	Percent identity	Query cover	E-value	References
Α	Lactobacillus curvatus	93%	99%	5e-74	FJ609221
Y	Enterobacteriaceae	100%	100%	2e-97	KM021355
В	Lactobacillus fermentum	99%	100%	1e-95	LC042465
С	Enterococcus casseliflavus	99%	100%	1e-78	KJ571214
E	Bacillus megaterium	94%	100%	2e-77	KJ004421
F	Bacillus sp	99%	100%	8e-97	HG794259
G		98%	100%	1e-89	
Н		98%	100%	6e-88	
J	Acetobacter sp	99%	100%	2e-92	AB853266
Т		90%	99%	1e-53	
X	Acinetobacter sp	92%	100%	2e-51	KJ814994
K	Bacillus sp	100%	100%	2e-97	KM983003
L	Providencia sp	90%	97%	5e-64	AB920789
Μ	Bacillus sp	90%	100%	5e-43	FJ235680
Ν	Providencia sp	97%	100%	2e-67	KM059194
0	Enterobacter aerogenes	99%	100%	1e-95	LN623623
Р	Bacillus sp	91%	97%	1e-53	KC236480
Q	Enterobacter sp	99%	100%	8e-97	GU944492
S	Klebsiella sp	92%	99%	7e-57	GQ416571
V	A actabactor nastourianus	1000/	1000/	1e-83	VM082001
W	Aceiobacier pasteurianus	100%	100%	5e-83	MN1903001
R	Klebsiella pneumoniae	100%	100%	2e-97	KP761422
D, I	NI				

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

NI: not identified band

Table 2: Identification of DGGE bands for yeast spe	cies
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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

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

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

\*: 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

![](_page_49_Picture_0.jpeg)