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Detailed analyses of the bacterial populations in processed cocoa beans of different geographic origin, subject to varied fermentation conditions

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ABSTRACT

The quality of chocolate is influenced by several parameters, one of which is bacterial diversity during fermentation and drying; a crucial factor for the generation of the optimal cocoa flavor precursors. Our understanding of the bacterial populations involved in chocolate fermentation can be improved by the use of high-throughput sequencing technologies (HTS), combined with PCR amplification of the 16S rRNA subunit. Here, we have conducted a high-throughput assessment of bacterial diversity in four processed samples of cocoa beans from different geographic origins. As part of this study, we also assessed whether different DNA extraction methods could affect the quality of our data. The dynamics of microbial populations were analyzed postharvest (fermentation and sun drying) and shipment, before entry to the industrial process. A total of 691,867 high quality sequences were obtained by Illumina MiSeq sequencing of the two bacterial 16S rRNA hypervariable regions, V3 and V4, following paired-read assembly of the raw reads. Manual curation of the 16S database allowed us to assign the correct taxonomic classifications, at species level, for 83.8% of those reads. This approach revealed a limited biodiversity and population dynamics for both the lactic acid bacteria (LAB) and acetic acid bacteria (AAB), both of which are key players during the acetification and lactic acid fermentation phases. Among the LAB, the most abundant species were *Lactobacillus fermentum*, *Enterococcus casseliflavus*, *Weissella paramesenteroides,* and *Lactobacillus plantarum/paraplantarum*. Among the AAB, *Acetobacter syzygii*, was most abundant, then *Acetobacter senegalensis* and *Acetobacter pasteriuanus*. Our results indicate that HTS approach has the ability to provide a comprehensive view of the cocoa bean microbiota at the species level.

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1. Introduction

Cocoa beans are seeds enclosed within the mucilaginous pulp of cocoa pods, which are the fruits of cocoa trees (*Theobroma cacao* L.). Once harvested, these beans are widely used as the main raw material in the chocolate industry. The quality of chocolate is influenced by several parameters including good agricultural and post-harvest processing practices. The former includes the appropriate maintenance of plant population and phytosanitary state, soil and climate conditions, fruit maturation and harvest. Good post-harvest procedure includes optimized pod opening, fermentation, drying, and storage conditions (Lima et al., 2011). In particular, fermentation and drying are crucial stages for the correct formation of cocoa flavor precursors amino acids and short-chain peptides (Lima et al., 2011; Schwan and Wheals, 2004). After the pod has been opened, the cocoa beans undergo a natural fermentation of about 6–7 days, which is important not only for the removal of the mucilaginous pulp surrounding the seeds, but also to facilitate cocoa drying and to initiate the appropriate biochemical changes within the beans (Guehi et al., 2010). Cocoa fermentation consists of a succession of different microbial populations (including yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB)), that colonize the mucilaginous pulp as a substrate for growth, and in doing so, produce several compounds such as ethanol, lactic acid, acetic acid, and aldehydes (Meersman et al., 2013). Following fermentation, the beans may be naturally or artificially dried (in rainy regions) in order to reduce their moisture content from about 60% to 6–7%. This step is a crucial one in determining the quality of cocoa beans; if drying is too rapid, some of the chemical reactions started during fermentation will not run to completion, with the consequent development of acidity and bitterness. On the other hand, if drying is too slow, contamination with molds can occur with the possible production of mycotoxins and off-flavors, which also incurs substantial production losses (Hamdouche et al., 2014; Lima et al., 2011).

Several studies have addressed the diversity of the microbial flora during the spontaneous fermentation of cocoa beans, but most have relied on classical culture-dependent methods that are intrinsically biased because of their inability to cultivate all species. In recent years, culture-independent molecular techniques, coupled with classical methods, have enabled a more detailed assessment of the microbial population

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because of their high throughput advantages and their ability to detect species that may be difficult or even impossible to cultivate (Camu et al., 2007; Cocolin and Ercolini, 2008; Meersman et al., 2013; Nielsen et al., 2007; Papalexandratou et al., 2011a, 2011b, 2013). Until now, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) was the most popular molecular technique, despite its low-throughput limitations (De Melo Pereira et al., 2013). More recently, high-throughput sequencing (HTS) has permitted to achieve a greater resolution and detection sensitivity than the one obtained with the analyses of DGGE gel banding patterns (Illeghems et al., 2012; Parente et al., 2015; Połka et al., 2015). This type of analysis has proven to be a very powerful instrument for the assessment of microbial populations (Illeghems et al., 2012). However, HTS methodologies have some disadvantages, which include a dependence on DNA extraction methodologies and primer sensitivity, both of which can affect the richness and diversity of the detected microbial populations (Delmont et al., 2011; Meersman et al., 2013).

The issue of quantity and quality of DNA available for PCR-based analyses is particularly relevant for complex matrixes such as fermented cocoa beans that are rich in polysaccharides and flavonols (polyphenols) that naturally inhibit PCR (Porebski et al., 1997; Spaniolas et al., 2008; Tortajada et al., 2009). A number of studies comparing different methods of DNA extraction from cocoa or chocolate have been carried out in the past, but they were primarily focused on the detection of genetically modified soybean DNA in chocolate (Gryson et al., 2004; Haymes et al., 2004); relatively few were performed for analyzing the microbial composition of bacteria and filamentous fungi (Fredricks et al., 2005; Tortajada et al., 2009). Here, we have carried out a high-throughput assessment of bacterial diversity for samples of processed cocoa beans derived from Cameroun, Ghana, and Ivory Coast. Our aim was to evaluate whether geographic origin of the sample, and fermentation conditions, could impact bacterial composition, and whether the DNA extraction method used could further bias the accuracy of these analyses.

2. Materials and methods

2.1. Sample collection

Four samples of processed-dried cocoa beans were collected from different production sites in Africa: Ghana (S1), Ivory Coast (S2, S4), and Cameroon (S3) (Table 1). One sample from the Ivory Coast had been fermented with a starter culture, while the other three had undergone spontaneous fermentation processes. Samples originating from Ghana (S1) and Ivory Coast (S2–S4) were fermented in wooden boxes during the main cropping period (October–November 2014), while the sample from Cameroon (S3) was fermented during the mid-crop (June to July 2014), with the traditional heap method using banana leaves

2.2. DNA extraction

Processed cocoa beans (100 g) were homogenized using a grinder at maximum speed for 60 s and the resultant mixture collected in sterile tubes. DNA was harvested in triplicate using the 3 different DNA extraction methods, thus resulting in a total of 9 replicates per sample. DNA extraction was carried out according to the manufacturer's protocol for three different kits; the Maxwell® 16 FFS Nucleic Acid Extraction Kit (REF custom Cat. ×9431 Promega, USA), the GMO Extraction Kit (REF 4466336, Life Technologies, USA), and the Fast DNA Spin Kit for Soil (REF 116560000 MPbio, USA). For each assay, DNA was eluted in 100 μL of sterile TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0and stored at −20 °C until use. DNA was quantified with the Quant-iT™ HS ds-DNA assay kit (Invitrogen, Paisley, UK) using a QuBitTM fluorometer. Two μL samples of each extract were also resolved by electrophoresis on a 0.8% agarose gel to verify DNA quality.

2.3. DNA amplification

To analyze bacterial diversity, the V3–V4 region of the 16S rRNA gene was amplified by PCR using the universal primers 343f (5′-TACGGRAGGCAGCAG-3′), and 802r (5′-TACNVGGGTWTCTAATCC-3′). PCR amplification was carried out with the Phusion Flash High-Fidelity Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction mix comprised 12.5 μl of Phusion Flash High-Fidelity Master Mix, 1.25 μl of each primer (10 μM), 0.2 ng of DNA template, and PCR ultra-pure water. In order to perform simultaneous analyses of all samples in a single sequencing run, each sample was amplified using a forward primer with a 9-base extension at the 5′ end, which acts as a tag (Vasileiadis et al., 2015). The possibility of generating anomalous data, due to non-specific primer annealing, was reduced by incorporation of a two-step PCR, as described in Berry et al. (2011). PCR products generated from the second step were multiplexed as a single pool using equivalent molecular weights (20 ng). This pool was purified using the solid phase reversible immobilization (SPRI) method (Agencourt AMPure XP kit (REF A63880, Beckman Coulter, Italy, Milano)), then sequenced by Fasteris S.A. (Geneva Switzerland), using the TruSeq DNA sample preparation kit (REF 15026486, Illumina Inc, San Diego, CA) for amplicon library preparation. Sequencing was performed with the MiSeq Illumina instrument (Illumina Inc., San Diego, CA) with V3 chemistry generating 300 bp paired-end reads.

2.4. Sequence data preparation and analyses

The MiSeq Control software version 2.3.0.3, RTA v1.18.42.0, and CASAVA v1.8.2 were used for base calling, and the Illumina barcode demultiplexing process. As the V3-V4 16S rRNA gene amplicons are shorter than 500 bp, so 300 bp paired-end reads per amplicon were sufficient for re-constructing full-length V3–V4 regions. This task was performed using the “pandaseq” script (Bartram et al., 2011), which imposes a minimum 30 bp overlap between read pairs, with a maximum of 2 allowed mismatches. Sequences were further demultiplexed according to sample indexes and primers using the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Large homopolymers (≥10), sequences that aligned outside of the targeted V3–V4 region, chimeric amplicons, and sequences that were classified by the Bayesian classifier (80% bootstrap cutoff) as non-targeted taxa (versus the Mothur version of the ribosomal database project (RDP)) were purged following their identification using Mothur version 1.32.0 (Claesson et al., 2009; Edgar et al., 2011; Schloss et al., 2009; Wang et al., 2007). Two approaches were then followed for sequence data analyses, the operational taxonomic unit (OTU), and taxonomy-based approaches. Mothur

Table 1
Description and labels used to identify the four processed beans samples analyzed, together with details about geographical origin and fermentation conditions. For each sample, different DNA extraction methods were compared.

Sample label	Origin	Starter	Fermentation method	Crops	Fermentation time	Drying time	DNA extraction method
S1	Ghana	no	Wooden box	Main	6 days	4 days	MP-Bio/R-Bio/LifeTech
S2	Ivory Coast	no	Wooden box	Main	6 days	4 days	MP-Bio/R-Bio/LifeTech
S3	Cameroon	no	Ground/banana leaves	Mid	7 days	5 days	MP-Bio/R-Bio/LifeTech
S4	Ivory Coast	yes	Wooden box	Main	6 days	4 days	MP-Bio/R-Bio/LifeTech

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