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Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and citrate lyase production

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ABSTRACT

Microbial fermentation is an indispensable process for high quality chocolate from cocoa bean raw material. lactic acid bacteria (LAB) are among the major microorganisms responsible for cocoa fermentation but their exact role remains to be elucidated. In this study, we analyzed the diversity of LAB in six cocoa producing regions of Ivory Coast. Ribosomal 16S gene sequence analysis showed that *Lactobacillus plantarum* and *Leuconostoc mesenteroides* are the dominant LAB species in these six regions. In addition, other species were identified as the minor microbial population, namely *Lactobacillus curieae*, *Enterococcus faecium*, *Fructobacillus pseudoficulneus*, *Lactobacillus casei*, *Weissella paramesenteroides* and *Weissella cibaria*. However, in each region, the LAB microbial population was composed of a restricted number of species (maximum 5 species), which varied between the different regions. LAB implication in the breakdown of citric acid was investigated as a fundamental property for a successful cocoa fermentation process. High citrate lyase producer strains were characterized by rapid citric acid consumption, as revealed by a 4-fold decrease in citric acid concentration in the growth medium within 12 h, concomitant with an increase in acetic acid and lactic acid concentration. The production of citrate lyase was strongly dependent on environmental conditions, with optimum production at acidic pH (pH < 5), and moderate temperature (30–40 °C), which corresponds to conditions prevailing in the early stage of natural cocoa fermentation. This study reveals that one of the major roles of LAB in the cocoa fermentation process involves the breakdown of citric acid during the early stage of cocoa fermentation through the activity of citrate lyase.

1. Introduction

Fermentation of cocoa beans is the first stage in the chocolate production process. Microorganisms, namely yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and *Bacillus*, are indispensable in achieving a successful fermentation of cocoa and they constitute a key factor that strongly influences the quality of the end-product - the chocolate (De Vuyst and Weckx, 2016; Ouattara et al., 2008; Pereira et al., 2013; Schwan, 1998). Initially, in the cocoa fermentation time-course, the microbial activity generates metabolites and creates conditions that inactivates the embryo of the beans (De Vuyst and Weckx, 2016). Secondly, an array of essential biochemical reactions and chemical changes are triggered for the development of the complex (and much-appreciated) flavor of chocolate (Afoakwa et al., 2012; Pereira et al., 2012). The growth and development of the microbiota involved in natural cocoa bean time-dependent fermentation is closely related to environmental conditions (temperature, pH, and oxygen tension) and the composition of the pulp, which serves as a substrate.

Among these conditions, the pH is a relevant parameter that strongly affects the behavior and metabolism of microorganisms fermenting cocoa beans (Lefeber et al., 2010; Schwan and Wheals, 2004). Indeed, citric acid imparts an initial pH of around 3.5 to the fresh cocoa pulp at the onset of fermentation (Lefeber et al., 2010; Pettipher, 1986). This acidic pH is primarily more favorable to fungal growth, notably yeasts under anaerobic conditions. Consequently, increasing the pH of fermenting cocoa mass is indispensable for bacterial growth and many biochemical reactions influencing the production of high quality chocolate (Beckett, 2009). An effective and successful cocoa fermentation process is characterized by a progressive increase in the pH, which could reach between 5 and 8 at the end of the fermentation process (Ouattara et al., 2014; Pereira et al., 2012). The increase in pH is essentially due to the breakdown and reduction in the citric acid content of the cocoa pulp (Schwan and Wheals, 2004). Thus, citrate catabolism constitutes a biochemical pathway of considerable

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interest in achieving efficient cocoa fermentation (Lefeber et al., 2010; Ouattara et al., 2016). Yeasts were initially reported to be mainly responsible for the breakdown of citric acid during cocoa fermentation (Schwan and Wheals, 2004) but LAB were also considered as important players in this process, and these bacteria have attracted more interest during the last decade (Lefeber et al., 2011a). In LAB, citrate is metabolized into various compounds, such as succinic acid, lactic acid, acetoin or butane 2,3-diol, depending on the targeted metabolic pathways (Illeghems et al., 2015). Citrate catabolism involves a particular enzyme, citrate lyase (EC 4.1.3.6), which catalyzes the breakdown of citrate into oxaloacetate and acetate (Bekal et al., 1998). The production of citrate lyase is induced by citrate and it quickly disappears after citrate depletion in the medium (Bekal et al., 1998). Bacteria producing this enzyme are often used to provide flavor and texture to many fermented products (Bekal et al., 1998).

Citrate lyase, is a multifunctional complex including three subunits, α, β and γ, whose functions are coordinated. First, the γ subunit (CL ligase; EC 6.2.1.22) catalyzes the activation of citrate lyase by introducing an acetyl group at the thioester residue of the prosthetic group linked to its acyl carrier protein (ACP), to form an acetyl-S-ACP (Schmellenkamp and Eggerer, 1974). Then, the α subunit (citrate: acetyl-ACP transferase; EC 2.8.3.10) replaces the acyl group with a citryl group to form the citryl-S-ACP (Dimroth and Eggerer, 1975). Finally, the β subunit (citryl-S-ACP lyase; EC 4.1.3.34) splits citryl-S-ACP into oxaloacetate and regenerated acyl-S-ACP (Dimroth and Eggerer, 1975).

The type of LAB able to synthesis citrate lyase is undoubtedly of interest in cocoa fermentation. Thus, studying these bacteria will lead to the identification of strains with a high potential for citrate lyase production that could be used as starters for improving cocoa fermentation.

The aim of this study was to evaluate, by molecular typing, the diversity of LAB isolated from fermenting cocoa in six different regions of Ivory Coast and to assess the impact of environmental conditions encountered during cocoa fermentation on the activity of citrate lyase in these strains.

2. Materials and methods

2.1. Fermentation, isolation and growth conditions of LAB

Cocoa pods were harvested on farms from six cocoa producing regions of Ivory Coast (Agneby-Tiassa, Guemon, Indénié-Djuablin, Loh-Djiboua, Nawa and Sud-Comoé). Six spontaneous heap fermentations of 50 kg cocoa beans (one fermentation in each region) were conducted for six days, using banana leaves. For this purpose, beans were laid out on banana leaves and covered with other banana leaves. During fermentation, 100 g samples were regularly withdrawn and collected in a Stomacher bag, at 12 h intervals, for analysis. For each fermentation, a total of 13 samples were collected. Numeration and isolation of LAB were carried out using the decimal dilution method (Pereira et al., 2012). 25 g of fresh cocoa bean sample were added to 225 mL of 0.1% (w/v) buffered (pH 7.2) peptone water then allowed to rest for one hour at 30 °C (Oxoid, Basingstoke, United Kingdom) in a 500 mL sterile flask, and shaken for 2 to 5 min at room temperature to obtain an homogenous sample containing the bacteria (initial dilution). One milliliter (1.0 mL) of the bacteria-enriched peptone water was diluted in 9 mL of 0.1% tryptone salt solution (10-fold dilution) and then, from this solution, a serial dilution was performed up to 10^{-8}. In our experiment, this serial dilution was only plated onto the rich elements composition medium of Man-Rogosa-Sharpe (MRS) agar (Oxoid) supplemented with 50 $\mu g \cdot mL^{-1}$ of nystatin to inhibit fungal growth as commonly used for LAB diversity identification (Lefeber et al., 2011b; Pereira et al., 2012; Ouattara et al., 2016), albeit, the biodiversity both in term of species and strains was reported to be improved when using more than a culture medium (Camu et al., 2007). So in our conditions, plates were incubated at 30 °C for 48–72 h, under anaerobic conditions, for subsequent colony counts and bacterial enumeration. For this purpose, a maximum of 15 isolates were randomly selected from two successive dilutions medium and analyzed LAB were identified as being Gram-positive, oxidase and catalase negative, and unable for sporulation. For each 100 g sample, the total number of LAB isolates range from 3 to 13.

The isolates were stored at −80 °C in MRS buffer medium supplemented with 20% (v/v) glycerol, in Eppendorf tubes, for further investigation.

2.2. PCR amplification of 16S ribosomal RNA genes (16S rRNA gene)

A genotypic approach toward the 16S rRNA genes in all the isolates was performed to discriminate and identify LAB microorganisms isolated previously. This approach is now generally accepted as the best target for studying phylogenetic relationships (Vandamme et al., 1996; Rossello-Mora and Amann, 2001). For this purpose, a multiple alignment (https://npsaprabi.ibcp.fr/NPSA/npsa_clustalw.html) of 16S rRNA genes from various species of LAB enabled us to design the forward (5′-GGYRTGCCTAATACATGCAAGT-3′) and reverse (5′-CCCGGGAACGTATTCACCGCG-3′) primers. These specific primers anneal to the most highly conserved 5′ and 3′ regions of the 16S rRNA gene, respectively, and after PCR they generate an amplicon of approximately 1400 bp. To perform the PCR reactions, bacteria grown for 24 h on agar plates were suspended in 100 μL of sterile distilled water and the resulting suspensions were used as DNA templates. PCR amplification was carried out in a Sensoquest Labcycler, as described previously (Ouattara et al., 2011). Reactions were performed in a final volume of 50 μL containing 1 μL of bacterial suspension, 1.25 U of Taq DNA polymerase (Biolabs, Lyon, France), 5 μL of 10 × standard buffer; 1 μL deoxynucleoside triphosphate (10 mM), 2 μL of each primer (10 μM) (Eurofins Genomics, Allemagne) and 38.75 μL of water. After an initial denaturation at 95 °C for 4 min, reactions were run for 35 cycles, each cycle comprising: denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. Finally, a 10 min extension at 72 °C was carried out. The presence and yield of specific PCR products were monitored using agarose 0.8% (w/v) gel electrophoresis at 70 V, for 2 h, in 1 × Tris Borate EDTA buffer and visualized with ethidium bromide staining and UV transillumination.

2.3. 16S rRNA gene restriction and sequence analysis

The 1400 bp PCR products were directly digested with two restriction enzymes, *Hae*III and *Taq*I, in separate reactions. The digestions were carried out for one hour at 37 °C for restriction enzyme *Hae*III and at 65 °C for *Taq*I in a final volume of 20 μL containing 12 μL of PCR product, 2 μL of commercially supplied incubation buffer, 5 μL of water and 1 μL (10 U) of the restriction enzyme (Biolab, Lyon, France). Digestion products were run on a 2% agarose gel in Tris-Borate EDTA buffer at 35 V overnight. Gels were stained with ethidium bromide, visualized by transillumination, and digitalized with a gel print system. Strains were grouped according to their restriction profiles. For further identification, at least four representative strains from each restriction group were randomly chosen for amplification of the hyper-variable region of the 16S rRNA genes, about 500 bp, using the primers F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R520 (5′-ACCGCGGCTGCTGGC-3′) (Anzai et al., 2000). PCR reactions were performed in the same conditions as described previously. PCR products were purified using the PCR clean-up (Macherey Nagel, Germany) and then sequenced using the primer F27.

The basic local alignment search tool (BLAST, blastN) from the NCBI database site (blast.ncbi.nlm.nih.gov/) was used to find the closest sequences relative to the amplified 16S RNA genes in order to identify our LAB strains.

The phylogenetic tree was constructed from the partial sequences of