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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

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ABSTRACT

Yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) populations, isolated from cocoa bean heap and box fermentations in West Africa, have been investigated. The fermentation dynamics were determined by viable counts, and 106 yeasts, 105 LAB and 82 AAB isolates were identified by means of rep-PCR grouping and sequencing of the rRNA genes. During the box fermentations, the most abundant species were *Saccharomyces cerevisiae*, *Candida ethanolica*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Acetobacter pasteurianus* and *Acetobacter syzygii*, while *S. cerevisiae*, *Schizosaccharomyces pombe*, *Hanseniaspora guilliermondii*, *Pichia manshurica*, *C. ethanolica*, *Hanseniaspora uvarum*, *Lb. fermentum*, *Lb. plantarum*, *A. pasteurianus* and *Acetobacter lovaniensis* were identified in the heap fermentations. Furthermore, the most abundant species were molecularly characterized by analyzing the rep-PCR profiles. Strains grouped according to the type of fermentations and their progression during the transformation process were also highlighted. The yeast, LAB and AAB isolates were physiologically characterized to determine their ability to grow at different temperatures, as well as at different pH, and ethanol concentrations, tolerance to osmotic stress, and lactic acid and acetic acid inhibition. Temperatures of 45 °C, a pH of 2.5 to 3.5, 12% (*v*/*v*) ethanol and high concentrations of lactic and acetic acid have a significant influence on the growth of yeasts, LAB and AAB. Finally, the yeasts were screened for enzymatic activity, and the *S. cerevisiae*, *H. guilliermondii*, *H. uvarum* and *C. ethanolica* species were shown to possess several enzymes that may impact the quality of the final product.

1. Introduction

Different aspects can affect cocoa beans fermentations and therefore the quality of the final products. Fruit pods of the *Theobroma cacao* tree are opened manually and the beans, with the surrounding pulp, are fermented using traditional procedures, mainly heap and box fermentations, for four to seven days and thereafter dried.

Local practices, related to the management of cocoa bean fermentation, such as good harvesting practices, careful selection and correct handling of the beans, can influence cocoa quality (Lima et al., 2011; Papalexandratou et al., 2011a,c; Trognitz et al., 2013). Another important aspect to consider is the fermentation process and, more specifically, its microbial ecology. Most of the microorganisms that naturally contaminate the beans come from outer surface of the pods, the hands of the workers, from the machetes used to open the pods, from the baskets used to transport the beans, plants materials and, above all, from boxes with residues of previous fermentations and plantain leaves (Schwan and Wheals, 2004).

Fermentation process involved complex microbial activities and biochemical changes that have been recently deeply reviewed by Schwan and Fleet (2014). The microorganisms responsible for the fermentation are yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB); moreover species of *Bacillus* sp., other bacteria and filamentous fungi could also grow with consequent influence on quality of the process. First main event regards microbial activities on the mucilaginous pulp that results in the production of alcohol and acids, and an increasing of temperature. Secondly, fermentation is essential for the death of the embryo of the seed, which takes place through the production of heat and acetic acid. This is also important to eliminate bitterness and astringency and, lastly, for the formation of aroma precursors. High sugar concentration, high temperatures, pH changes, ethanol production, metabolism of organic acids and as a result their concentrations, turn cocoa beans fermentation into a stressful environment for microorganism growth. They exert a selection and inhibition on the natural occurring microbiota, while promoting those populations, which are better adaptable to these conditions. Another aspect that influences the quality of the process involves enzymatic activities. Particularly, yeast pectinolytic enzymes are considered to have an important role in degradation and solubilization of the pulp, that allows penetration of oxygen into the

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fermenting mass enabling aerobic acetic acid bacteria to grow as reported by Schwan and Fleet (2014).

In this study, the dynamics of yeasts, LAB and AAB, obtained from two different types of cocoa bean fermentation, that is, heap and box fermentations, have been investigated using a culture-dependent microbiological method and molecular techniques. Furthermore, the ability of isolates to tolerate different stress conditions has been tested in order to provide useful information on their ability to initiate and carry out the fermentation. Moreover, a panel of yeast's enzymatic activities potentially impacting the course of the fermentation and the quality of the final product were determined.

2. Materials and methods

2.1. Cocoa bean fermentations

Cocoa pods, of Forastero hybrid, were harvested in Ivory Coast (West Africa) during main crop (October to December) by means of traditional procedures, and opened manually with a machete. Two different spontaneous cocoa beans fermentation were carried out, that is, heap (H) and box (B) fermentations. Both were performed in triplicate using three different batches of fresh beans and located in the plantation area, under a roof. The heap fermentations involved 50 kg of fresh cocoa pulp-bean mass being piled into a heap on top of plantain leaves placed on the ground, and the heap being covered with other leaves. The box fermentations instead involved 1600 kg of fresh cocoa pulp-bean mass being placed in a wooden box, resulting in 100 cm of depth, arranged in tiers, with one slightly raised above the other, in order to facilitate the turning phase. In both cases, the mass was turned after 48 and again after 96 h using a shovel, until the beans were visually homogenous. After the fermentation processes, the cocoa beans were dried under the sun for 5 to 10 days, depending on the weather. The beans were turned until the humidity level was lower than 8% and this was performed weighing 10 g of grounded beans and by using a moisture analyzer (Mettler Toledo, Milan, Italy).

2.2. Sampling procedure

Samples were taken at the beginning of the fermentation (time zero) and after 1, 2, 4 and 6 days, and microbiological analysis was performed immediately after sampling. Cocoa beans coming from different points of the box/heap were mixed and collected in sterile bags. Twenty-five grams of cocoa beans and the adhering pulp were added to 100 mL of Ringer's solution (Oxoid, Milan, Italy) and homogenized manually. Serial dilutions in Ringer were prepared and analyzed on several microbiological media. The yeasts were enumerated by spreading on WL Nutrient agar (WLN, Oxoid) containing 1 μg/mL tetracycline (Sigma-Aldrich, Milan, Italy); the LAB were counted by means of pour plate inoculation on de Man Rogosa Sharp agar (MRS, Oxoid) supplemented with 2 μg/mL of natamycin (DSM Food Specialities, The Netherlands); the AAB were determined by spreading on Acid Acetic Medium agar (AAM, 1% glucose, 0.8% yeast extract, 1.5% bacteriological peptone, 15 g/L agar, Oxoid) containing 2 μg/mL of natamycin (DSM Food Specialities). Plates were incubated at 37 °C for 2 days for MRS and at 30 °C for 3 to 5 days for both AAM and WLN media. After the incubation period, the colonies were counted (the mean and standard deviation were calculated). Five colonies randomly selected for each microbial group investigated were streaked for purification. The purified isolates were stored at −20 °C in YEPD broth (2% glucose, 1% yeast extract, 1% bacteriological peptone, Oxoid) for yeasts, and in MRS broth and AAM broth for LAB and AAB, respectively. The broths all contained 25% of glycerol (Sigma). The temperatures and pH values were measured directly on the fermentation mass immediately before taking the samples, using a digital pH meter provided with a temperature probe (Mettler Toledo).

2.3. Statistical analysis

The data obtained from the pH, temperatures and yeasts, as well as the LAB and AAB counts of the H and B fermentations were analyzed using one-way analysis of variance (ANOVA). ANOVA analysis was performed using the Statistica software package (version 7.1, StatSoft Inc., Tulsa, OK, USA).

2.4. DNA extraction from pure cultures

DNA was extracted from 1 mL of an overnight culture of yeasts, LAB and AAB, as previously described in Cocolin et al. (2000, 2004). The DNA was quantified using a Nanodrop Instrument (Spectrophotometer ND-1000, Thermo Fisher Scientific, Milan, Italy) and then standardized at 100 ng/L.

2.5. Identification and characterization of the isolates

The isolates were identified by means of rep-PCR grouping and rRNA gene sequencing. Rep-PCR was performed using a single oligonucleotide primer $(GTG)_5$ (Versalovic et al., 1994), as previously described by Dal Bello et al. (2010). Generated dendrograms were analyzed in order to identify clusters with a coefficient of similarity higher than 85%. Then, representatives of each group and isolates with unique rep-PCR profiles were identified by 16S and 26S rRNA gene sequencing for bacteria and yeasts, respectively. RNA genes were amplified with the P1V1-P4V3 and NL1-NL4 primers for bacteria and yeasts, respectively (Klijn et al., 1991; Kurtzman and Robnett, 1997). The PCR products were purified by means of a PCR Extract Mini Kit (5PRIME, Milan, Italy) and sent to a commercial sequencing facility (MWG Biotech, Ebersberg, Germany). The obtained sequences were compared with those present in GenBank, using the Blast search program (Altschul et al., 1997).

The rep-PCR profiles of the most abundant species, namely *Saccharomyces cerevisiae* (35 isolates), *Lactobacillus fermentum* (51 isolates) and *Acetobacter pasteurianus* (51 isolates), were further analyzed to study the intraspecific biodiversity. Arbitrary selected coefficients of similarity of 80% for the yeast and the LAB, and of 75% for the AAB were used.

2.6. Screening for tolerance to stress conditions

An analysis of tolerance to stress conditions was carried out by monitoring the growth of all the isolates at different temperatures, pH values and concentrations of ethanol, glucose and fructose, as already suggested (Daniel et al., 2009; Lefeber et al., 2010; Pereira et al., 2012). Briefly, after centrifugation of the overnight cultures, the pellets were washed in Ringer's solution and then suspended in 200 μL of the same solution, and this was followed by dilution in order to normalize the inoculum concentration at the same absorbance value of 0.02 OD. The cultures were inoculated directly in 96-well plates in yeast nitrogen base (YNB, Oxoid), MRS and mannitol yeast extract peptone (2.5% D-mannitol, 0.5% yeast extract, 0.3% bacteriological peptone, Oxoid) media for yeasts, LAB and AAB, respectively. Eight, 10, or 12% (vol/vol) of ethanol; 5, 15, or 30% (wt/vol) of glucose and 5, 15 or 30% (wt/vol) of fructose were added to the media. Growth was measured at 30, 37, and 45 °C, for temperature tolerance, and the media were adjusted to pH 2.5, 3.5 and 4.5 for the pH tolerance. Furthermore, the LAB and AAB cultures were screened for lactic acid and acetic acid resistance: 1, 2, 4 or 5% of lactic acid and acetic acid were added to the media separately (Pereira et al., 2012). The yeast, LAB and AAB cultures inoculated in the media without any modification were used as the reference. Growth was evaluated by measuring the absorbance value at 630 nm (Biotek ELx808, Milan, Italy) at 0, 24 and 48 h for the yeasts, at 0, 12 and 24 for the LAB and at 0, 24 and 36 h for the AAB. All the experiments were performed in triplicate; the mean OD value of the three biological replicates for each stress condition was compared with the OD value of the reference condition in order to define a growth percentage.