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Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation

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ABSTRACT

Bacteria, yeasts and filamentous fungi were isolated during natural coffee processing. Bacteria were isolated in greater numbers at the beginning of the fermentation, when the moisture of the coffee beans was around 68%. Gram-positive bacteria represented 85.5% of all bacteria isolated, and *Bacillus* was the predominant genus (51%). Gram-negative species of the genera *Serratia*, *Enterobacter* and *Acinetobacter* were also found. Approximately 22% of 940 randomly chosen isolates of microorganisms were yeasts. *Debaryomyces* (27%), *Pichia* (18.9%) and *Candida* (8.0%) were the most commonly found genera, and these three genera tended to appear more often as the fruit was fermented and dried. *Aspergillus* was the most abundant genus besides *Penicillium, Fusarium* and *Cladosporium*, with 42.6% of the total fungi isolates. The genera and species identified included members known to have pectinase and cellulase activities. Of the 10 organic acids analyzed and quantified in coffee beans, acetic and lactic acids may have been generated by microbial activity. Butyric acid was not detected in any sample.

1. Introduction

Brazil is the largest producer of coffee (4.2 M tons), followed by Paraguay, Venezuela, Colombia, Indonesia, Ethiopia, India, Mexico and another 40 countries. Two coffee species dominate the world market: *Coffea arabica* (*arabica*) and *C. canephora* (*robusta*). Arabica and Robusta coffees account for 76.4% and 23.6% of world production, respectively (Coltro et al., 2006). Coffee cherries are processed by one of the two methods (Schwan and Wheals, 2003). In Colombia, Central America and Hawaii, the 'wet' method is used for Arabica coffee. In the wet method the hand-picking mature cherries are mechanical depulping and then fermented for approximately 24–48 h to remove the mucilage layer. The dry process, which results in so-called unwashed or natural coffee, is the oldest and simplest method of processing coffee. The dry process is often used in countries where rainfall is scarce and long periods of sunshine are available to dry the coffee properly. The dry method is used for about 95% of Arabica coffee produced in Brazil, most coffee produced in Ethiopia, Haiti, Indonesia and Paraguay, and some Arabica produced in India and Ecuador. This method involves fermentation of whole fruit and usually produces coffee that is heavy in body, sweet, smooth and complex. The coffee fruits are spread on the ground (earth, platforms, concrete or asphalt) in layers approximately 10 cm thick, heaped at night and respread each day (Schwan and Wheals, 2003). Over the course of 10–25 days of sun drying, natural microbial fermentation occurs that can influence in the final quality of the product (Schwan and Wheals, 2003; Silva et al., 2000). Fermentation of pectinaceous sugars produces ethanol and acetic, lactic, butyric and higher carboxylic acids. The formation of butyric and propionic acids from bacterial fermentation causes a loss of quality due to diffusion of the acids into the beans (Amorim and Amorim, 1977).

Bacteria, yeasts and filamentous fungi have already been reported during fermentation by the 'wet' method (Masoud and Kaltoft, 2006; Avallone et al., 2001), but only one comprehensive study of dry processing has been published (Silva et al., 2000). The microbiota involved in 'dry' processing are much more varied and complex than those found during wet fermentation, but the actual role of each group of microorganisms during coffee fermentation by natural processing is still unknown.

The microbial succession and consortium of bacteria, yeast and filamentous fungi and their metabolites during natural coffee fermentation remain to be studied. An understanding of microbial dynamics during natural fermentation should enable more rapid fermentation and better quality of the final product. Therefore, the objectives of this work were to investigate the natural microbial fermentation of coffee cherries, to isolate and

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characterize the microorganisms involved, and to evaluate the biochemical characteristics of coffee during fermentation, drying and storage.

2. Materials and methods

2.1. Sampling

One hundred and eight kg of coffee cherries from *C. arabica* var. Acaiá were hand-picked at the mature stage (red cherries) from a farm at 750–800 m above sea level situated in Lavras in the state of Minas Gerais, Brazil. The beans were fermented and dried by the natural method; they remained on a concrete platform for about 25 days until reaching a moisture level of 11–12%. The environmental temperature was 25–28 °C during the day and 18–20 °C during the night. The beans were then packed in either polystyrene bags or jute sacks and stored in a cold chamber at 3 °C with 59% relative humidity for 140 days.

2.2. Microbiological analyses

Samples were aseptically taken every 24 h throughout coffee processing. Each sample included 200 fruits that were added to a bottle containing 1800 ml of saline-peptone water (0.1% bacteriological peptone, Himedia, Mumbai, India and 0.8% NaCl, Merck, Darmstadt, Germany). After mixing for 15 min at 150 rpm in an orbital shaker, ten-fold dilutions were prepared. Enumeration of microorganisms was carried out using five different culture media. Plate count agar (PCA, Merck) and WL differential medium (Cat. no. 242510 Difco) were used as general media, Eosyn Methylene Blue Agar (Oxoid, Basingtstoke, UK) was used to enumerate Enterobacteriaceae, MRS medium (Oxoid) with 0.25% (v/v) of nystatin (Sigma, St. Louis, USA) was used for the enumeration of lactic acid bacteria (Almeida et al., 2007) and DG18 (Dicloran Glycerol 18%) agar (Oxoid) containing 100 mg chloramphenicol (Sigma) and 50 mg chlortetracycline (Sigma) per liter was used to enumerate yeasts and filamentous fungi (Hocking and Pitt, 1980). After spreading, plates were incubated at 28 °C for 48 h for bacteria and 5 days for filamentous fungi and yeast. For each type of medium containing isolated colonies, the square root of the number of colonies was taken at random for identification (Holt et al., 1994). Isolates were purified and stored at −80 °C in 20% glycerol.

2.2.1. Identification of bacteria

The size, shape, color, height and edge of each colony were noted and Gram staining, presence of catalase and motility were assayed before replicating the bacteria onto slants of PCA and preserving them at −80 °C in 20% glycerol for subsequent identification.

Gram-negative bacteria were identified using Bac-Tray Kits I, II and III (Difco), following the manufacturer's instructions.

Gram-positive bacteria were subdivided into spore-formers and non-spore-formers by heating at 80 °C for 10 min to kills the vegetative cells (Almeida et al., 2007). Subsequent identification using biochemical and motility tests proceeded as recommended in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and The Prokaryotes (Hammes and Hertel, 2003) and confirmation was made using API 50 CHB galleries (BioMerieux). Presumptive lactobacilli were counted on MRS agar. Biochemical characterization of strains was performed with API ID 32 for lactococci and enterococci, and API 50 CHL (BioMerieux) for lactobacilli and leuconostocci.

2.2.2. Identification of yeasts

All yeast isolates were characterized by determining their morphology, spore formation, assimilation and fermentation of different carbon sources according to Kurztman and Fell (1998) and Barnett et al. (2000).

2.2.3. Identification of filamentous fungi

Filamentous fungi were initially cultured on PDA medium (Merck) and observed with an optical microscope for preliminary identification. This was done by morphotypic analysis of the colony, especially color and appearance, using the method of Pitt and Hocking (1997). Initial identification of the genera *Penicillium*, *Fusarium*, *Cladosporium* and *Aspergillus* was made with microscopic slide examination of spores and mycelium. Further support for fungi identification was found in Christensen (1982), Nelson et al. (1983) and Pitt and Hocking (1997).

2.3. Chemical analysis

Samples of coffee fruits and beans were taken and characterized with respect to total soluble solids, starch, total sugars, reducing sugars, total titratable acidity, phenolics, pH and moisture (A.O.A.C., 2000), pectins (Bitter and Muir, 1962), polyphenol oxidase (PPO) (Mazzafera and Robinson, 2000), peroxidase (Matsuno and Uritani, 1972), lignin and cellulose (Van Soest and Wine, 1968).

2.4. Organic acids

Organic acids were analyzed using a high-performance liquid chromatography system (Shimadzu, model LC-10A*i* (Shimadzu Corp., Japan) with a UV detector at 210 nm. A Shimadzu ion exclusion column (Shim-pack SCR-101H) operated at a temperature of 40 °C was used to achieve chromatographic separation. Two gram of sample was mixed with 18 ml of water for 30 min. Water-soluble acids were eluted with 0.01 M potassium dihydrogen phosphate buffer solution at a flow rate of 0.6 ml/min. Samples were microfiltered through a 0.2 μm cellulose acetate filter and directly injected (20 μl) onto the chromatographic column. Standard solutions (malic acid, lactic acid, acetic acid, butyric acid, propionic acid, citric acid, oxalic acid, succinic acid, tartaric acid) were prepared by dilution of the individual compounds in ultra pure water. All samples were examined in triplicate. The coefficient of variation was less than 5% in each case.

3. Results

3.1. Microbiological analyses

A total of 940 microbial colonies isolated from the experimental samples were analyzed. Eighty five percent of the isolates could be identified to species level. The incidence of bacteria, yeast and filamentous fungi and the variation in water activity (a_w) during coffee cherry and bean fermentation and drying are shown in Fig. 1. Ripe coffee cherries presented 69% moisture at the beginning of fermentation (time 0) that represented 0.85 a_w (Fig. 1). From the 12th to the 16th day of fermentation the decrease in the moisture values was not $>0.23\%$. The moisture reached 15%, 13% and 11%, on the 18th, 20th and 22nd fermentation/drying days, respectively. On the 22nd day, the coffee beans had 11% moisture and 0.52 a_w. This a_w value was considered safe for bean storage because it does not favor microorganism development (Bytof et al., 2005). After fermentation and