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# Microbiological diversity associated with the spontaneous wet method of coffee fermentation

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ABSTRACT

The evaluation of the microbiota present during coffee wet fermentation was done in two distinct regions of Minas Gerais, Brazil: one farm in the South of Minas Gerais (Lavras = L) and another farm in the savannah region (Monte Carmelo = MC). The yeast population ranged from 2.48 to 4.92 log CFU/g and from 2 to 4.81 log CFU/g, the mesophilic bacteria population ranged from 3.83 to 8.47 log CFU/g and from 5.37 to 7.36 log CFU/g, and the LAB population ranged from 2.57 to 5.66 log CFU/g and from 3.40 to 4.49 log CFU/g in the L and MC farms, respectively. *Meyerozyma caribbica* and *Hanseniaspora uvarum* were the dominant yeasts in coffee wet fermentation at L farm, and *Torulaspora delbrueckii* was the dominant yeast at MC farm. The species *Staphylococcus warneri* and *Erwinia persicina* were the predominant bacteria at L farm, and *Enterobacter asburiae* and *Leuconostoc mesenteroides* were the dominant species at MC farm. Lactic acid was the principal acid detected, reaching 2.33 g/kg at L farm and 1.40 g/kg at MC farm by the end of the process. The volatiles composition was similar for roasted coffee from the two different regions and furans, acids, and alcohol were the main groups detected. Temporal Dominance Sensation (TDS) analyses showed that the coffee beverage from L farm was dominated by citrus and herbaceous sensory characteristics, while the coffee from MC farm was dominated by citrus, herbaceous, and nuts sensory characteristics. Evaluating the microbiota in these two regions was important in improving the knowledge of the microbial species present during coffee wet fermentation in Brazil.



## 1. Introduction

The coffee fruit usually consists of an outer layer of skin (peel), called the exocarp under which there is a layer of pulp, followed by mucilaginous mesocarp (mucilage) firmly adhered to the rigid layer called parchment (endocarp). The parchment protects two seeds surrounded by a thin membrane known as silver skin. The coffee fruit are subject to a fermentation process in order to remove the mucilage and pulp before drying.

Three methods might be used to process coffee: the dry, semi-dry, and wet processes (Brando and Brando, 2014; Silva, 2014). In the dry method, the newly harvested whole fruit is fermented and dried on platforms after which the coffee beans are removed by hulling and polishing (removed the husk layer that cover the dry coffee beans). In the semi-dry method the coffee peel, pulp and part or all mucilage are removed mechanically and then the coffee is fermented and dried. The amount of mucilage removed depends on the characteristic of the machine used. In the wet method the peel and pulp are removed mechanically, leaving the mucilage adhered to the beans. These pulped coffees are then transferred to water tanks where they are allowed to ferment for 6 to 72 h (depending on the environmental temperature), during which the remaining mucilage are degraded and solubilized. The beans are then removed from the tanks and sun dried.

Brazil is the largest producer and exporter of coffee. The State of Minas Gerais, in the Southeast of Brazil, produces about 53% of all the coffee in Brazil, of which 85% is processed by dry method. In the last decade, coffee producers in the southern and central areas of Minas Gerais began to process coffee via wet fermentation (Sindcafé-MG, 2014). This processing produces a coffee with a different composition and sensory characteristics than coffee processed by the semi-dry or dry methods, which are alternatives used by the producer to suit different markets.

Microorganisms are naturally present during coffee processing and use the various compounds in the pulp and mucilage as nutrients during the fermentation stages. They secrete organic acids and other metabolites that may affect the final sensory characteristics of the beverage (Silva, 2014). This microbiota may vary according to several factors: regional characteristics, coffee fruit composition and the method of fermentation. It is important to know the microbiota present during the processing of coffee, especially when selecting starter cultures that can

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be used in producing differentiated final products and inhibiting the growth of mycotoxigenic fungi (Masoud and Jespersen, 2006; Massawe and Lifa, 2010; Silva et al., 2012).

Studies have been performed to identify the microbiota present in wet processing of coffee fruits in India, Hawaii, and Mexico (Agate and Bhat, 1966; Avallone et al., 2001; Frank et al., 1965). Classical morphological and biochemical characterization has been used to identify the microbiota, and species of *Erwinia*, *Klebsiella*, and *Leuconostoc* and the yeasts *Kloeckera*, *Candida*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae* var. *ellipsoideus*, and *Cryptococcus* have been identified as contributing to these fermentations. Using a molecular approach, PCR–DGGE, Masoud et al. (2004) identified *Pichia kluyveri*, *P. anomala*, *Hanseniaspora uvarum*, *Kluyveromyces marxianus*, *Candida pseudointermedia* and *Torulaspora delbrueckii*, during wet coffee fermentation in Arusha, Tanzania (East Africa).

Recently, yeasts species found in coffee processed by the wet method in Brazil were reported by Pereira et al. (2014). *Pichia fermentans* (YC5.2) and *Saccharomyces* sp. (YC9.15) were studied as having a potential for use as starter cultures for coffee wet fermentation. The bacteria population was not mentioned in their work (Pereira et al., 2014).

Studies still are needed in Brazil to improve the knowledge of the microbiota present in coffee processing for each producing region due to the variation in climate and altitude. Our aim was to study the microbial diversity involved in wet coffee fermentation in two main producing regions of Brazil having distinct environmental characteristics using culture-dependent and culture-independent methods. The target metabolites present during the fermentation process were also analyzed using headspace solid-phase microextraction/gas chromatography (HS–SPME/GC) and high-performance liquid chromatography (HPLC). The sensory profile of the final coffee beverage was also evaluated.

## 2. Materials and methods

### 2.1. Processing of coffee fermentation

The coffee fruits were fermented by the wet method. The experiments were performed in two geographically different regions of Minas Gerais, at a farm located in Monte Carmelo (MC), 870 m above sea level in the savannah (Cerrado) region, and at a farm located in Lavras (L), 919 m above sea level in the Atlantic Forest region. The experiments were done during two consecutive harvesting seasons (2012 and 2013).

The fruit (60 kg) of *Coffea arabica* L. var. Acaiá was harvested at the mature stage (cherries) and was mechanically depulped in a horizontal machine (model BDSV-04; Pinhalense, São Paulo, Brazil), followed by for 48 h fermentation in a tank with 60 l of water to remove the mucilage. At the L farm, the temperature of fermentation was between 14 and 23 °C, and at the MC farm it was between 20 and 28 °C. Fermentations were done in duplicate at each farm. After fermentation, the coffees were placed on suspended platforms for sun drying until they reached approximately 11% moisture (224 h at L farm and 336 h at MC farm), measured using Moisture Meter G600i (GEHAKA, São Paulo, Brazil). The following samples were collected: cherries before fermentation, along the fermentation (0, 6, 12, 24, 36, and 48 h), and during drying (60, 112, 224, and 336 h). Samples (500 g) were placed aseptically in sterile plastic bags, in triplicate, and immediately transferred to the laboratory in iceboxes for microbiological analyses. For physicochemical analyses, coffee samples were frozen at −20 °C until analyzed. Sensory analysis and volatile compounds were evaluated in dried coffee beans.

### 2.2. Characterization and identification of microbiota

#### 2.2.1. Quantification, isolation, and phenotypic characterization

Coffee cherry sample (10 g) was added to 90 mL of sterile peptone water (in g/L: 1 bacteriological peptone [Himedia, Mumbai, India]), homogenized for 2 min in a Stomacher (Mayo Homogenius HG 400, São Paulo, Brazil), and used for decimal serial dilution. Bacteria and yeasts were enumerated by spread plating on MRS agar (Merck) for lactic acid bacteria, on plate count agar (PCA) (in g/L: 5 tryptone [Himedia], 2.5 yeast extract [Merck], 1 glucose [Merck], 15 agar Merck]), for total bacteria and on YPD agar (in g/L: 10 yeast extract [Merck], 10 peptone [Himedia], 20 dextrose [Merck], 20 agar [Merck]) for yeasts. MRS plates were incubated in an anaerobic jars at 30 °C for 3–4 days and PCA and YPD plates were incubated at 30 °C for 3–7 days and the morphological properties of the colonies (cell size, cell shape, edge, color, and brightness) were recorded and the square root of the number of colonies counted for each morphotype was purified by streaking on new agar plates (same culture media used for plating) (Senguna et al., 2009). The pure cultures were stored in an ultra freezer at −80 °C in the same broth culture media used for plating, containing 20% glycerol (w/w).

The phenotypic characterization of the bacterial colonies was performed using Gram staining, catalase and oxidase activities, motility tests, growth in culture media with 1% and 5% (w/v) NaCl (salt tolerance), protease production, spore formation, and the ability to ferment glucose, sucrose, and xylose (Sigma, St. Louis, USA), as recommended in *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). Yeast colonies were characterized for morphology and biochemical assessments as described by Kurtzman et al. (2011).

A total of 435 isolates was obtained; 251 were from samples from L farm and 184 isolates were obtained from MC farm.

The microbiota identified was the same in both years analysed. However, variation of less than 5% was found in the counting.

#### 2.2.2. Genotypic identification

The isolates (435) were grouped by phenotypic characteristics, as mentioned above, and representatives of each phenotypic group were subjected to molecular analyses (236 isolates). The genotypic characterization of the selected isolates was first performed by rep-PCR and subsequently by DNA sequencing.

The bacterial and yeast cultures were grown under appropriate conditions, collected from agar plates with a sterile pipette tip, and resuspended in 50 μL of sterile Milli-Q water. The suspension was heated for 10 min at 95 °C, and three μL was used as a DNA template in the PCR experiments.

The fingerprints of the genomic DNA were obtained via the PCR amplification of repetitive bacterial and yeast DNA elements (rep-PCR) using the $(GTG)_5$ primer, as described by Nielsen et al. (2007). Amplified PCR products were separated via 2% (w/v) agarose gel electrophoresis at 70 V for 3 h, and the images were visualized and photographed using a transilluminator LPixImage (LTB 20 × 20 HE, LPix®, Brazil).

The bacteria and yeasts representative of each group were subjected to 16S rRNA gene and internal transcribed spacer (ITS) region gene sequencing, respectively. The amplification of the 16S rRNA gene used the primers 27 F and 1512R (Devereux and Willis, 1995). The ITS region was amplified using the primers ITS1 and ITS4 (Nielsen et al., 2007). The amplified PCR products were sent for sequencing at the Advanced Genetics Technologies Center — AGTC (Kentucky, USA); the ABI3730 XL automatic DNA sequencer was utilized.

The sequences were aligned using the BioEdit 7.7 sequence alignment editor and were compared to the GenBank database using the Basic Local Alignment Tool (BLAST) program (National Center for Biotechnology Information, Bethesda, MD) for the identification of isolates.

### 2.3. PCR–DGGE analysis

Samples of coffee cherries (3 g) were mixed with 5 mL of Milli-Q water for 10 min then the coffee cherries were removed and the liquid phase was centrifuged at 100 ×*g* for 10 min at 4 °C. The pellet was used for DNA extraction. The total DNA was extracted from samples using the "DNA Purification from Tissues" protocol (QIAamp DNA Mini Kit [Qiagen, Hilden, Germany]) in accordance with the manufacturer's instructions. The DNA from the bacterial community was amplified with