Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization

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1. Introduction

Cassava is a very important staple crop for the diet of many tropical countries. Small-scale fermentation is widely used for root preservation and the elaboration of well-appreciated cassava fermented dishes and foods. Sour cassava starch is typically processed in Southern America (Colombia, Brazil) and traditionally used for the preparation of cheese-breads. The natural lactic fermentation of wet-extracted cassava starch, along with sun-drying, were proved to be the key steps for conferring sour cassava starch some specific functional properties, such...
as expansion during dough baking (Westby and Cereda, 1994; Zakhia et al., 1996).

Previous studies have shown that the natural sour cassava starch fermentation was mainly due to the action of lactic acid bacteria (LAB), and that fermentation temperature and duration as well as the composition of the microflora influenced the expansion properties of the final cassava sour starch (Brabet, 1994; Figueroa et al., 1995). During these works, some LAB strains involved in the natural sour cassava starch fermentation were isolated, identified and characterized using classical microbiological techniques (Figueroa et al., 1995; Morlon-Guyot et al., 1998; ben Omar et al., 2000).

However, recent studies on the fermentation of pozol, a traditional maize-fermented food, demonstrated that classical microbiological approach failed to adequately describe the microflora of fermented foods and recommended that a polyphasic approach should be used for a better description of the microbial communities of these environments (Ampe et al., 1999b). Therefore, this paper aims at describing the dynamics of the microbial community during traditional cassava starch fermentation using culture-independent methods.

2. Materials and methods

2.1. Sour cassava starch

The cassava Mbra 383 variety usually cultivated in the Cauca Department, Colombia (around 1100 m above sea level), was processed into sour starch according to the traditional small-scale processing in this region. Twelve kilograms of the wet-extracted starch (45% to 50% of water content) was put in a 1-m-height PVC tube and covered with a 1- to 2-cm layer of running water. The PVC tubes were introduced in the traditional fermentation tank and the starch was allowed to naturally ferment for 30 days at ambient temperature (around 25°C) in liters tanks. After fermentation, the starch was laid on black polyethylene sheets and sun-dried until a final 10% water content was reached. Sour cassava starch was sampled at $t = 1, 6, 16$ and 30 days of fermentation for further analysis. Five fermentations were performed in the same conditions. Numerical results are the means of the data obtained for these five fermentations.

2.2. Scanning electron microscopy

Sour cassava starch samples were fixed with glutaraldehyde and dehydrated with an ethanol graded series as described by Giraud et al. (1994). Ethanol was then removed by application of CO$_2$ at the critical point. The samples were coated with platinum and SEM observation was performed with a JEOL JSM-6300F microscope (University of Montpellier UMI, France).

2.3. Analysis of pH, sugars and fermentation products

Ten grams of starch was diluted fivefold in sterile water. 0.2 ml of 2 N H$_2$SO$_4$ was added to 1.3 ml of this suspension and the mixture was centrifuged 10 min at 10,000 $\times g$. The concentrations of soluble starch, sugars, ethanol and organic acids in the supernatants were assayed by high-pressure liquid chromatography using an Aminex HPX87H column (BioRad, Richmond CA). Running conditions were: mobile phase, H$_2$SO$_4$ 6 mmol l$^{-1}$; flow rate, 0.8 ml min$^{-1}$; temperature, 65°C. Detection was performed using a refractometer (PU 4026 Philips, Heindoven). The pH was measured directly in the suspension. All results were the means of five determinations.

2.4. DNA isolation

Total DNA was extracted from fermented starch at different fermentation times by a method previously tested for high starch-containing foods (Ampe et al., 1999b). One gram of starch was resuspended in 10 ml 0.9% NaCl and homogenized for 30 s at maximal speed with an UltraTurrax T25 (Janke and Kunkel, IKA® Labortechnik). Two tubes (1.5 ml each) of this suspension were then centrifuged at 7000 $\times g$ for 10 min. Five hundred microliters of lysosome (20 $\mu$g l$^{-1}$) in TES buffer (50 mM Tris pH 8—1 mM EDTA—8.56% (w/v) saccharose) and 10 $\mu$l of mutanolysin (1 U $\mu$l$^{-1}$) were added to
each pellet. Samples were vortexed for 1 min and incubated for 1 h at 37°C. Fifty microliters of proteinase K (10 mg ml⁻¹) was added and the tubes were incubated for 50 min at 50°C then for 10 min at 65°C. Three hundred microliters of warm (65°C) buffer (0.2 M NaCl, 0.1 M Tris–HCl pH8, 2% SDS) was added and the tubes were incubated for 10 min at 65°C. Three hundred microliters of 5 M NaCl was added and the tubes were gently mixed for 30 s, incubated at 4°C for 10 min, and centrifuged at 7000 × g, 4°C for 10 min. The supernatant was divided into two tubes, and precipitated with 780 μl of isopropanol by incubation at −20°C for 30 min. The pellets were recovered by centrifugation at 12,000 × g, 4°C for 10 min, washed with 1 ml 70% ethanol, vacuum-dried and resuspended in 100 μl of water. The tubes corresponding to the same sample were then mixed and 700 μl of water was added. Eight hundred microliters of phenol pH 8 was added. Tubes were mixed for 3 min and centrifuged at 12,000 × g at room temperature for 10 min. The aqueous phase was extracted once more with phenol and two to three times with phenol:chloroform:isoamyl alcohol (25:24:1) pH 8 before a final extraction with chloroform:isoamyl alcohol (24:1). The aqueous phase was then precipitated with isopropanol, and the pellets were washed with 70% ethanol, vacuum-dried and resuspended in 200 μl water. The extract quality was routinely checked using 1% agarose-TBE 0.5 X gels.

2.5. PCR-DGGE analysis

The purified DNA was amplified with primers specific to the bacteria or eukarya domains. The bacterial community DNA was amplified using primers gc338f (5’CGCCGCGCGCGCGCGGC CGGGCGGGCGGGCGGGCGGGGCTCC TACGCGAGCGCAGCAG) and 518r (5’ATTACCGCGGCTGCTGG) spanning the V3 region of the 16S rDNA (Øvreas et al., 1997) as previously described (Ampe et al., 1999b). The eukaryotic community DNA was amplified using primers gc-Euk1427f (5’TCTGTGATGCCTAGATGTT TCTGGG) and Euk1616r (5’GCCTGTGATGTTACAA GGGCAGG) spanning the 1427–1637 region of the 18S rDNA, as described by van Hannen et al. (1999).

The PCR products were then analyzed by DGGE using the Bio-Rad DCode apparatus following the procedure first described by Muyzer et al. (1993). Samples were applied onto 8% (w/v) polyacrylamide gels in 1X TAE with a denaturing gradient ranging from 25% to 50% UF (100% corresponded to 7 M urea and 40% [v/v] formamide). The gels were run for 10 min at 20 V and 3 h at 200 V, stained with ethidium bromide for 10 to 15 min, then rinsed for 20 to 30 min with distilled water and documented with the GelDoc system (BioRad, Richmond, CA).

A DGGE standard was prepared as follows: (1) total DNA was extracted from exponentially grown Lactobacillus plantarum LMG18053, Lb. cellobiosus ATCC9846, Lb. paracasei I 2030, Leuconostoc mesenteroides ATCC10832, Ln. dextranicum INRA18G, Pediococcus pentosaceus ATCC43200, Weissella paramesenteroides ATCC333313, Lactococcus lactis ATCC11454 and Streptococcus salivarius spp. thermophilus CNCM10303. These were chosen among 40 strains as best spanning the whole gradient of the DGGE gels prepared here (data not shown). (2) PCR products were generated from each of these DNA preparations. (3) A mixture of these PCR products was made with final concentrations of: 10 ng μl⁻¹ of PCR products from Lb. plantarum, Lc. lactis and L. paracasei, and 2 ng μl⁻¹ of PCR products of DNA from the other strains. Ten microliters of this standard was routinely used in all DGGE gels.

2.6. Sequences of DGGE fragments

DGGE fragments were cut out with a sterile scalpel. The DNA of each fragment was eluted in 20-μl sterile water overnight at 4°C. One microliter of the eluted DNA of each DGGE band was reamplified using the same conditions as above. The success of this operation was checked by running 3 μl of the PCR products in DGGE gels as described above with sour starch-amplified DNA as control. The PCR products which yielded a single band co-migrating with the original band were then purified and sequenced.

Sequences of these gene fragments were determined by the dideoxy chain-termination method with the ABI PRISM dye terminator kit (Perkin Elmer).
The sequencing products were loaded and analyzed on a 373 DNA sequencer (Applied Biosystems). DGGE fragments were sequenced using primer gc338f.

To determine the closest known relatives of the partial 16S rDNA sequences obtained, searches were performed in public data libraries (RDP and Genbank) with the FASTA, BLAST and RDP programs (Maidak et al., 1999). The CHECK_CHIMERA command of the RDP facilities was used to try to detect chimeric sequences.

The Genbank accession numbers for the 16S rDNA partial sequences of DGGE bands were AF192510 through AF192519.

2.7. RNA extraction and quantitative hybridization with phylogenetic probes

The total RNA was extracted from sour cassava starch and pure strains using a method previously optimized for high starch-containing food samples (Ampe et al., 1998). The recovered RNAs were used for quantification with probes S-S-Lbma-0207-a-A-20 (Ampe, 2000) and S-*=Lab-0722-a-A-25 (Sghir et al., 1998) specifically targeting Lb. manihotivorans and all LAB species, respectively. Synthetic HPLC purified oligonucleotides (Eurogentec, Belgium) were 3'-end labeled with digoxigenin following the instructions of the manufacturer (Boehringer Mannheim). RNA blotting and hybridization was performed as described before (Stahl et al., 1988; Ampe et al., 1999a). The bound probe was quantitated by densitometry in relation to reference standards after autoradiography. The control RNA content was estimated by hybridization with the universal probe S-*=Univ-1390-a-A-18 (Zheng et al., 1996) prior to use as internal standards, with E. coli RNA (Boehringer Mannheim) as absolute reference. Abundances of Lb. manihotivorans were expressed as the fraction of the total LAB rRNA in the sample (namely RNA indexes). The lower limit for detecting a unique SSU rRNA in the 1 μg of nucleic acid spotted on the membrane was approximately 3 ng of SSU-like rRNAs. Results were given as the mean of three determinations.

2.8. Chemicals and reagents

HPLC-pure primers were from Eurogentec (Seraing, Belgium), Taq polymerase and dNTPs from Promega (Charbonnières, France), E. coli RNA, DIG-labelling kit from Roche Diagnostics (Meylan, France). Other molecular biology products were from Sigma (Saint-Quentin Fallavier, France). All other chemicals were from Prolabo (Lyon, France).

3. Results

3.1. Scanning electron microscopy

Phase contrast and scanning electron microscopy (Fig. 1) indicated that (i) several different morphotypes were involved in the traditional sour cassava starch fermentation including long and short rods, coccoid cells, as well as some typical bifidobacteria (Fig. 1a–d); (ii) there was a large increase in cell number in the time course of fermentation; (iii) a small fraction of starch was degraded during the fermentation, as suggested from some alveoles detected on the surface of starch granules after 6 days of fermentation (Fig. 1b). Fig. 1f shows the layered raw starch structure on some degraded granules as the starch degradation increases; (iv) a network of fibers, most probably corresponding to bacterial polysaccharides, appeared to be synthesized during the second half of the fermentation process (Fig. 1e).

3.2. pH, sugars and fermentation metabolites

As seen from Table 1, the pH rapidly decreased during the fermentation time course and reached a final value equal to the pK₄ of lactic acid (3.5). Lactate was the most important fermentation metabolite found in the samples, although significant amounts of acetate were also measured. Other fermentation metabolites such as butyrate or propionate, as well as free sugars, were not detected.

3.3. DGGE fingerprinting of bacterial and eukaryotic communities

Two separate extractions of total DNA from each sour cassava starch sample were performed. One microliter of 10⁻¹ dilutions and undiluted total DNAs were subjected to amplification, and the equal-size
16S rDNA PCR products were analyzed by DGGE. Repeated extractions as well as dilutions of a given sample gave similar fingerprints (data not shown).

The fingerprints obtained for the sour cassava starch sampled during the fermentation time course showed 11 visible bands (Fig. 2). Bands #1, 4, 6, 8 and 10 were the most intense ones. The same bands were observed during the time course of fermentation, but the relative intensity of several bands (e.g. band #6) varied significantly reflecting the changes in the bacterial assemblage.

Individual bands observed in the DGGE profiles were then excised from acrylamide gels (Fig. 2) and reamplified with primers gc338f and 518r. Before sequencing, each PCR-reamplified DGGE band was run on a denaturing gradient gel to confirm that the expected product was obtained. All sequences re-

![Fig. 1. Scanning electron micrographs of sour cassava starch samples taken after 1 (a), 6 (b and c), 15 (d and e) and 30 (f) days of fermentation. The 30-day-fermented starch was sampled after sun-drying and represents the end product.](image-url)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>pH</th>
<th>Lactate (μmol g⁻¹)</th>
<th>Acetate (μmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.75 ± 0.11</td>
<td>6.57 ± 1.40</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>3.80 ± 0.05</td>
<td>15.5 ± 6.06</td>
<td>5.63 ± 7.39</td>
</tr>
<tr>
<td>15</td>
<td>3.78 ± 0.11</td>
<td>14.7 ± 13.4</td>
<td>4.53 ± 5.06</td>
</tr>
<tr>
<td>30</td>
<td>3.47 ± 0.05</td>
<td>67.7 ± 17.0</td>
<td>4.97 ± 2.34</td>
</tr>
</tbody>
</table>
Fig. 2. DGGE analysis of PCR-amplified 16S ribosomal DNA fragments from sour cassava starch bacterial communities. DNA was derived from the fermentation time course of a single experiment, but repeated experiments gave similar profiles. The positions and numbering of the bands sequenced (Table 2) and discussed in the text are indicated. A DGGE standard (Std) derived from PCR products of LAB pure strains was included in all the gels run and the corresponding identity of the bands is indicated on the right side of the standard.

A similar work was performed to study the eukaryotic community of sour cassava starch. The DGGE fingerprints revealed identical profiles for all tested samples. Two main bands were visible, but the profiles of fermented cassava were identical to that of unfermented cassava (data not shown); therefore, the observed bands obviously corresponded to cassava nuclear DNA, and no DNA from yeast or fungi was therefore found in the sour cassava starch samples.

3.4. Quantification of Lb. manihotivorans rRNA

Previous works report on the isolation of LAB strains from different traditional cassava starch fer-
mentations in Colombia, the majority of which belonged to the species *Lb. plantarum*, *Lb. manihotivorans* and *Ln. mesenteroides* (Figueroa et al., 1995; ben Omar et al., 2000). The sour cassava starch samples used in our study showed bands with sequences corresponding to *Lb. plantarum* and *Ln. mesenteroides*, but none of the sequences retrieved corresponded to *Lb. manihotivorans*. At the same time, the PCR-DGGE product obtained with DNA from a pure culture of *Lb. manihotivorans* co-migrated with that of *Lc. lactis* in our experimental conditions. As band #4—identified by sequencing to be from *Lc. lactis*—was very intense, the presence of *Lb. manihotivorans* in the analysed samples could not be dismissed by this experiment only. Therefore, the total RNA was extracted from the sour cassava starch samples and the relative importance of *Lb. manihotivorans* was quantified using a phylogenetic probe targeting the 16S rRNA of this species (Ampe, 2000). As (i) all identified organisms were lactic acid bacteria and (ii) a bias might be introduced by the presence of cassava RNA, the

### Table 2

Identity of bands retrieved from DGGE analysis of the sour cassava starch bacterial community

<table>
<thead>
<tr>
<th>Band #</th>
<th>Closest relative</th>
<th>% of identity (^{b})</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bf. minimum</em></td>
<td>98%</td>
<td>AF192510</td>
</tr>
<tr>
<td>2</td>
<td>chloroplast from Euphorbiaceae(^c)</td>
<td>94%</td>
<td>AF192511</td>
</tr>
<tr>
<td>4</td>
<td><em>Lc. lactis</em></td>
<td>93%</td>
<td>AF192512</td>
</tr>
<tr>
<td>5</td>
<td><em>Lb. plantarum</em></td>
<td>94%</td>
<td>AF192513</td>
</tr>
<tr>
<td>6</td>
<td><em>Streptococcus</em> sp.</td>
<td>92%</td>
<td>AF192514</td>
</tr>
<tr>
<td>7</td>
<td><em>Ln. mesenteroides</em></td>
<td>97%</td>
<td>AF192515</td>
</tr>
<tr>
<td>8</td>
<td><em>Ec. saccharolyticus</em></td>
<td>97%</td>
<td>AF192516</td>
</tr>
<tr>
<td>9</td>
<td><em>Ln. citreum</em></td>
<td>95%</td>
<td>AF192517</td>
</tr>
<tr>
<td>10</td>
<td><em>Lb. plantarum</em></td>
<td>93%</td>
<td>AF192518</td>
</tr>
<tr>
<td>11</td>
<td><em>Lb. plantarum</em></td>
<td>96%</td>
<td>AF192519</td>
</tr>
</tbody>
</table>

*\(^{a}\)Bands were extracted from the DGGE gel shown in Fig. 2.*

*\(^{b}\)Percentage of identical nucleotides between the sequences retrieved from the DGGE gel and the closest relative found in GenBank or RDP. Comparison was made using partial 16S rDNA sequences only (around 180 bases, corresponding to the region sequenced).*

*\(^{c}\)The public banks do not have the sequence for cassava chloroplast 16S rDNA (last verification: August 2000). The comparison could only be done with other members of the Euphorbiaceae.*

### Table 3

Importance of the species *Lb. manihotivorans* in sour cassava starch as determined by quantitative dot blot measurements of 16S rRNA

<table>
<thead>
<tr>
<th>Fermentation time (days)</th>
<th>% <em>Lb. manihotivorans</em> rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6±1.4</td>
</tr>
<tr>
<td>6</td>
<td>13.3±4.6</td>
</tr>
<tr>
<td>15</td>
<td>11.8±8.6</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
</tr>
</tbody>
</table>

The probes used (S-S-Lbma-0207-a-A-20 and S-*Lab-0722-a-A-25) were DIG-labelled for hybridization and the RNA controls used for standardization were from the *Lb. manihotivorans* LMG 18010\(^T\) and *Lb. plantarum* LMG 18053 strains. Results are presented as the relative percentage in comparison with the total LAB and are the means of data obtained from five separate fermentations ± standard deviation.

ND: not detected.

The molecular tools used in this study for following the microbial dynamics of traditional sour cassava starch fermentation confirmed that this process was ruled by lactic acid bacteria as previously suggested by many authors (Brabet, 1994; Figueroa et al., 1995; ben Omar et al., 2000). Besides, the sequences of several partial 16S rDNA genes retrieved from DGGE fingerprints corresponded to the dominant LAB species identified in traditional fer-
mentations of several starchy foods all over the world, such as *Lb. pontis* and *Lb. panis* for the sour dough in Europe (Vogel et al., 1994), *Ln. mesenteroides*, *Lb. plantarum*, *Lc. lactis* for cassava retting in the Congo (Brauman et al., 1996) or *Bifidobacterium minimum*, *Ln. mesenteroides*, *Enterococcus saccharolyticus*, *Lb. plantarum* for the pozol in Mexico (Ampe et al., 1999b). This suggests that some species might be typically associated with the fermentation of starchy foods. Surprisingly, *Bifidobacterium* and *Enterococcus* were until recently known to be mainly associated with the gastrointestinal tracts but not to participate in the spontaneous lactic acid fermentations of foods. The present study along with other observations on the Mexican pozol (Ampe et al., 1999b) open new orientations for studying the ecology of these organisms. On the other hand, as several sequences varied significantly from the closest reference 16S rDNA sequence, the sour cassava starch might be a reservoir for unknown bacterial species as already illustrated by the isolation of the new species *Lb. manihotivorans* so far not found in other natural environments (Morlon-Guyot et al., 1998).

Figueroa et al. (1995) have suggested that the traditional sour cassava starch fermentation was ruled by the succession of two microbial groups: heterofermentative LAB, mainly *Leuconostoc* species, would start the acidification followed by the development of homofermentative LAB dominated by *Lb. plantarum*. However, this hypothesis was based on a limited number of isolated strains and unavailable numerical data. In addition, studying the ecology of microorganisms by cultivation-dependent methods was shown to be strongly biased, even for fermented foods (Ampe et al., 1999b). The molecular tools used in the present study confirmed the importance of the species *Lb. plantarum* in the traditional fermentation of sour cassava starch, but also demonstrated that this species was present throughout the process and not only in the later stages as previously thought. By contrast, the very low intensities of the bands corresponding to the two *Leuconostoc* species suggests that this genus does not represent a dominant group during the fermentation. In addition, the molecular tools helped in identifying a number of organisms previously not mentioned among the sour cassava sour starch flora, such as *Bifidobacterium* and *Enterococcus* species, with intense corresponding bands in the DGGE profiles. Interestingly, the intensity of band #1 corresponding to *Bifidobacterium* increased during the fermentation, likely due to the progressive setting of anaerobic conditions as first suggested by Cereda (1973). On the contrary, the amount of *Lc. lactis* increased during the first 6 days of fermentation and then decreased probably due to the pH decrease and to the sensitivity of this species to low pH values. The same was observed for *Lb. manihotivorans*, another acid-sensitive species (Guyot et al., 2000), which represented 13% of the total flora after 6 days of fermentation but was not detected anymore at the end of the fermentation. Therefore, even though the traditional fermentation involves a succession of microbial populations as suggested by Figueroa et al. (1995), this succession seems to be ruled by the resistance vs. sensitivity of microorganisms towards the very acidic conditions (pH down to 3.5) reached within 2 weeks of fermentation and not by the type (homo vs. hetero) of fermentative metabolism (both *Lc. lactis* and *Lb. manihotivorans* are strictly homofermentative LAB). This is in agreement with Brabet (1994) who reported that 74–85% of the isolated microorganisms throughout three different sour cassava starch fermentations in Colombia were homofermentative LAB.

Cereda (1973) suggested that nonlactic acid bacteria—in particular clostridia—may also participate in the fermentation of cassava starch. In our study, neither the DGGE profiles nor the HPLC measurements evidenced the presence of these microorganisms, strongly suggesting that they play no significant role in this process.

In addition, the presence of yeasts and fungi was not detected using either SEM and PCR-DGGE with eukaryotic primers. This result is in agreement with Martínez and Zapata (1981) who found neither yeasts nor fungi using classical culture-dependent methods after 8 days of fermentation, and with Brabet (1994), who only found very low counts of yeasts and fungi using culture media. The absence of fungi is most probably due to the anaerobiosis during fermentation. However, the absence of yeasts remains to be explained as these were found at the end of cassava retting, another fermentation type performed in the Congo, with similar pH and anaerobiosis conditions.
to those prevailing in sour cassava starch fermentation (Brauman et al., 1996).

4.2. Combination of DGGE and RNA quantification with phylogenetic probes

This work represents the first attempt to study cassava starch fermentation with molecular tools. It clearly demonstrated that DGGE fingerprinting followed by 16S rDNA sequencing can successfully help in the faster identification of the bacterial species involved in cassava fermentation. This advantage would thus allow to avoid biased and time-consuming culture-dependent methods and open new perspectives for studying other cassava fermentation types, such as those performed in Western and Central Africa. However, it also pointed out one of the limits of DGGE analysis already reported by Murray et al. (1996): several DNA fragments sharing different sequences—and potentially corresponding to different species—can exhibit the same melting behaviour on a DGGE gel. In our study, the PCR products obtained from the amplification of DNA from \textit{Lc. lactis} and \textit{Lb. manihotivorans} could not be distinguished by the sole DGGE analysis. The importance of the latter species could only be demonstrated through the use of rRNA quantification with S-S-Lbma-0207-a-A-20 probe. This result emphasizes the need for combining several molecular methods to avoid the biases inherent to each one. Besides, it confirms the importance of pursuing efforts for isolating microbial species from their natural environments.

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References


