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Diversity of bacteria and yeast in the naturally fermented cotton seed and rice beverage produced by Brazilian Amerindians

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ABSTRACT

Microorganisms associated with the fermentation of cotton seed and rice were studied using a combination of culture-dependent and -independent methods. Samples of the cotton seed and rice beverage were collected every 8 h during the fermentation process for analysis of the microbiota present over 48 h. The lactic acid bacteria (LAB) population reached values of approximately 8.0 log cfu/mL. A total of 162 bacteria and 81 yeast isolates were identified using polyphasic methods. LAB (*Lactobacillus plantarum*, *Lactobacillus vermiforme*, *Lactobacillus paracasei*) were the most frequently isolated bacteria. *Bacillus subtilis* was present from 16 h until the end of the fermentation process. A decrease in pH value from 6.92 (0 h) to 4.76 (48 h) was observed, and the concentration of lactic acid reached 24 g/L at the end of the fermentation process. DGGE (denaturing gradient gel electrophoresis) was performed to determine the dynamics of the communities of bacteria and yeast, and the analysis revealed a predominance of LAB throughout the fermentation process. No changes were observed in the yeast community. The yeast species detected were *Candida parapsilosis*, *Candida orthopsilosis*, *Clavispora lusitaniae* and *Rhodotorula mucilaginosa*. Our studies indicate that the DGGE technique combined with a culture-dependent method is required to discern the dynamics in the fermentation of cotton seed and rice.

1. Introduction

In several countries, conventional foods and beverages are produced largely from cereals. However, some of these products have not received sufficient scientific attention. They often ferment spontaneously, resulting in improved shelf-life and better nutritional properties compared with the raw material. Single or mixed cereals are used as a substrate in the production of fermented foods, and the final product can vary according to the microbial population and fermentation conditions. Fermentation procedures have been used to develop new foods with enhanced health properties (Blandino et al., 2003).

In Brazil, several Amerindian tribes (Araweté, Kayapó, Karajá, Java and Tapirapé) use small-scale fermentation to produce foods and beverages with high nutritional value that also have medical and religious significance. Several substrates, such as cotton seed, rice, cassava, corn, peanut, banana and pumpkin, are used as raw materials to ferment and produce beverages, which are staple foods for adults and children (Wagley, 1988).

Generally, spontaneous cereal-based fermentations are performed by proto-cooperation between yeast, lactic acid bacteria (LAB) and fungi (Blandino et al., 2003). Nout and Sarkar (1999) suggested that the growth of yeast in fermented foods is favored by acidification of the environment, which is created by bacteria. Furthermore, they suggested that the growth of bacteria is stimulated by the presence of yeast, which can provide growth factors, such as vitamins and soluble nitrogen compounds.

Studies of a Brazilian beverage produced from cassavas and peanuts showed the presence of both bacteria and yeast during fermentation. The *Lactobacillus* genus was the predominant bacteria found in beverages produced from rice, cassavas and peanuts (Almeida et al., 2007; Ramos et al., 2010). *Lactobacillus* has been found in many other fermented foods, such as the cereal-based Ogi and Doenjang and sourdough (Omemu et al., 2007; Kim et al., 2009; Iacumim et al., 2009). The yeast species, *Candida*, *Pichia* and *Saccharomyces*, have been described in the fermentation of cassava and peanut cauim (Schwan et al., 2007; Ramos et al., 2010).

In the past, there was no well-supported data for nutritional, technical and quality control of indigenous fermented food. However, in the last two decades, the number of books and articles focused on indigenous fermented beverages and foods from around

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the world has increased (Blandino et al., 2003). The microbiological and nutritional properties of these foods are important and should be studied.

In addition to the standard morphological, cultural and biochemical methods, various molecular techniques can be used to identify different microbial species. Molecular, culture-independent approaches have proven to be powerful tools for providing a more complete inventory of the microbial diversity in food samples. The ability to simultaneously analyze multiple samples without prior knowledge of the diversity of the ecosystem makes population fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), useful in monitoring the diversity and dynamics of complex microbial ecosystems. DGGE takes advantage of the sequence-dependent separation of PCR fragments amplified from the total DNA of a sample that are equivalent in size (Miletto et al., 2007). The ability to extract and sequence bands from DGGE gels offers an additional, valuable tool for identifying the predominant organisms in the ecosystem. Although initially developed to detect mutations in human genes (Myers et al., 1987), DGGE has also been widely used to study the population diversity and dynamics of various food ecosystems, such as fermented maize dough (Ben Omar and Ampe, 2000), cheese (Hoorde et al., 2008), meat (Audenaert et al., 2010) and cauim (Ramos et al., 2010).

In this study, conventional morphological, cultural and biochemical methods combined with rRNA gene sequencing and PCR–DGGE were used to assess the microbial communities involved in the fermentation of a beverage made with cotton seed and rice.

2. Materials and methods

2.1. Beverage preparation and sampling

The cotton seed and rice beverage was prepared by the local Amerindian Tapirapé–Tapi'itãwa tribe, which resides next to the Urubu Branco (Mato Grosso, Brazil) mountain located 10.8°S and 51.3°W in the extreme north of Mato Grosso State. The protocol for the manufacture of the rice beverage is described in Fig. 1. Briefly, the cotton seed and rice were soaked separately in water and then crushed. Five kilograms of rice and 2.5 kg of cotton were added to approximately 10 L of water. This viscous mixture was left to cook for 30 min until the substrate was uniformly distributed. The cooked mash was left to ferment in an open vessel for 48 h at room temperature (approximately 30 °C) to obtain the final beverage product. Four fermentations were performed under the conditions described above. The pH of the cotton seed and rice beverage varied from 6.9 (sample at 0 h) to 4.7 (sample at 48 h) during the fermentation process, and the amount of lactic acid reached 24 g/L by the 48th hour of fermentation.

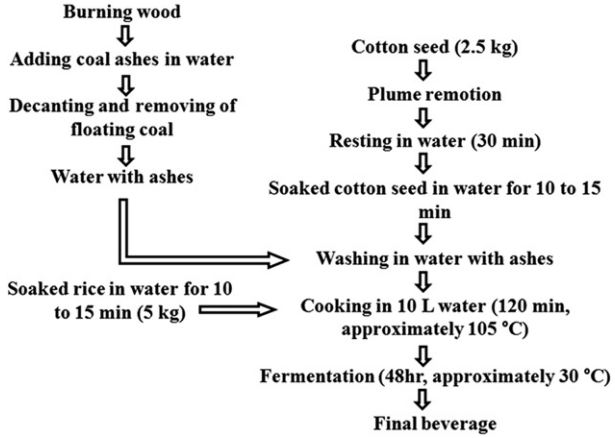

Fig. 1. Protocol for the manufacture of the beverage composed of cotton seed and rice.

Samples were aseptically collected in sterile bottles and maintained at 4–6 °C until analysis (approximately 4 h). For each sample collected at 8 h intervals, 20 mL of the cooked and fermenting substrate was added to a bottle containing 180 ml of saline peptone diluents (0.1% peptone, 0.5% NaCl, and 0.03% $Na_2H_2PO_4$).

2.2. Cell densities of bacteria, yeast and filamentous fungi

The samples were mixed in a stomacher at normal speed for 60 s, and 10-fold dilutions were prepared. Five different types of culture media were used to study the microbial communities. Plate count agar (PCA, Merck) was used as a general medium for the viable mesophilic bacteria population. Eosin Methylene Blue Agar (Oxoid) was used for Enterobacteriaceae, and MYGP agar [0.3% yeast extract (Merck), 0.3% malt extract (Merck), 0.5% peptone (Himedia), 1.0% glucose (Merck), 2.0% agar (Merck)], containing 100 mg of chloramphenicol (Sigma, St. Louis, USA) and 50 mg of chlortetracycline (Sigma) per 1000 ml was used to inhibit bacterial growth in the yeast counts. MRS (De Man Rogosa Sharpe, Merck) agar containing 0.1% cysteine–HCl was used for LAB growth under anaerobic conditions. MRS plates were incubated in acrylic anaerobic jars. After spreading, the plates were incubated at 28 °C for 48 h for bacteria and 5 days for filamentous fungi and yeast. All samples were plated in triplicate.

2.3. Identification of bacteria and yeast

The colonies of microorganisms were counted and selected randomly for 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 inducing spore formation (80 °C for 10 min). For subsequent identification, biochemical and motility tests were performed as recommended in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and The Prokaryotes (Hammes and Hertel, 2003), and the tests were confirmed using API 50 CHB galleries (BioMerieux). Presumptive lactobacilli were counted on MRS agar. Isolates were examined for colony and cell appearance, catalase activity, Gram stain, motility and the production of CO_2 from glucose in MRS broth with a Durham tube. Biochemical characterization of the strains was performed with an API ID 32 for lactococci and enterococci and an API 50 CHL (BioMerieux) for lactobacilli and *Leuconostoc*. *Lactobacilli* were identified as Gram-positive, catalase-negative, and oxidase-negative regular fermentative rods. They were classified as obligatory homofermentative, facultative heterofermentative and obligatory heterofermentative lactobacilli because of their ability to produce CO_2 from glucose and gluconate.

All of the yeast isolates were characterized by their morphology and spore formation as well as their assimilation and fermentation of different carbon sources following the protocol of Barnett et al. (2000). Bacterial and yeast DNA from the pure cultures were extracted according to Makimura et al. (1999), respectively. By sequencing portions of the 16S rRNA gene and ITS region, we identified representative bacteria and yeast isolates at the species level. For amplification of the 16S rRNA gene, we used primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1512r (5′-CGG CTA CCT TGT TAC GAC T-3′). For yeast identification, the ITS region was amplified using primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR was performed as described by Ramos et al. (2010). Searches in GenBank (http://

www.ncbi.nlm.nih.gov/BLAST/) were performed to determine the closest known relatives of the partial ribosomal DNA sequences that were obtained.

2.4. DNA extraction from beverage samples and PCR analysis

Total DNA was extracted from samples at different times during the fermentation process using the protocol described by Ramos et al. (2010).

The DNA from the bacterial community was amplified with primers 338fgc (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3′) (the GC clamp is underlined) and 518r (5′-ATT ACC GCG GCTGCT GG-3′), which span the V3 region of the 16S rRNA gene (Ovreas et al., 1997). The DNA from the yeast population was amplified using the primers NS3 (5′-GCA AGT CTG GTG CCA GCA GCC-3′) (positions 553–573 of *Saccharomyces cerevisiae*) and YM951r (5′-TTG GCA AAT GCT TTC GC-3′) (positions 951–935 of *S. cerevisiae*). The NS3 primer had a GC clamp sequence at the 5′ end (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Haruta et al., 2006)). The PCR mix (25 μl) contained the following: 0.625 U Taq DNA polymerase (Promega, Milan, Italy), 2.5 μl 10× buffer, 0.1 mM dNTP, 0.2 μM of each primer, 1.5 mM $MgCl_2$ and 1 μl of extracted DNA. The amplification was performed according to Ramos et al. (2010). Aliquots (2 μl) of the amplified products were analyzed by electrophoresis on 1% agarose gels before they were used in DGGE analysis.

2.5. DGGE analysis

The PCR products were analyzed by DGGE using a BioRad DCode Universal Mutation Detection System (BioRad, Richmond, CA, USA) according to Ramos et al. (2010). To determine the composition of the microbiota, individual bands observed in the DGGE profiles were excised from the acrylamide gel and re-amplified to provide a template for sequencing. The conditions for re-amplification were the same as those described for DGGE analysis, using the same primer without the GC clamp.

The composition of the microbial communities at different times during the fermentation process was determined based on the presence or absence of amplicons detected by DGGE. To determine the diversity of amplicons, the gels were analyzed using the Diversity Database program. Hierarchical clustering was performed using the Systat 8.0 program and was based on similarity matrices that were generated by the agreement method (simple matching) using Euclidean distance and the Ward algorithm.

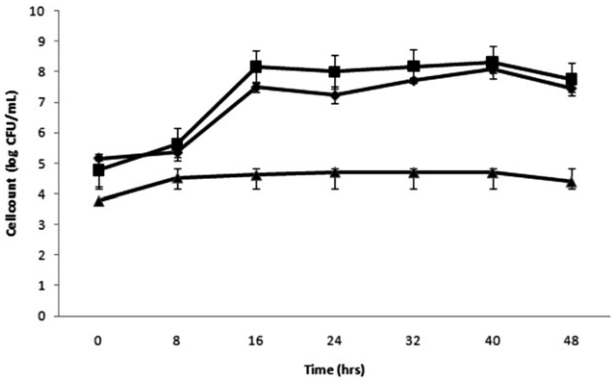

Fig. 2. Number (log cfu/mL) of total aerobic mesophilic bacteria (◆), LAB (■), and yeast (▲) during fermentation of the cotton seed and rice beverage.

3. Results

3.1. Bacteria and yeast identification

The counts from the different groups of microorganisms found in the cotton seed and rice beverage are shown in Fig. 2. The yeast population did not alter significantly during the fermentation process; the population ranged from 3.8 log cfu/mL to 4.7 log cfu/mL. LAB were detected throughout the fermentation process. At the beginning of fermentation, the LAB population was approximately 5.0 log cfu/mL; after 16 h of fermentation, it reached 8.1 log cfu/mL and maintained this level until the end of the process. These bacteria were quantitatively the dominant group. The total amount of aerobic mesophilic bacteria was detected at the beginning of fermentation (5.1 log cfu/mL), and after 16 h, this population increased to 7.5 log cfu/mL.

A total of 162 bacterial and 81 yeast isolates were identified using physiological and sequencing methods (Table 1 and Table 2). Isolates were classified as either Gram-positive or Gram-negative. There were 148 (91%) Gram-positive isolates and 14 Gram-negative (9%) isolates. The LAB (57%) were the predominant bacterial group, followed by the *Bacillus* genus (21%), *Corynebacterium* genus (14%) and Enterobacteriaceae family (9%). *Lactobacillus*

Table 1
Bacteria identified by 16S region and detected by DGGE analysis during 48 of fermentation. ND = no detected by platting; (+) present by PCR–DGGE analysis; (−) absence by PCR–DGGE analysis; letters in DGGE column correspond to bands in PCR–DGGE gel. Percentages calculated from total number of bacteria isolates.

Bacteria %	Accession number	DGGE	0	8	16	24	32	40	48
L. plantarum	HM163460	E	ND (−)	2.5 (−)	5.6 (+)	1.2 (+)	0.6 (+)	4.9 (+)	3.1 (+)
L. brevis	HM104306.1	H	ND (−)	ND (−)	ND (−)	ND (−)	1.8 (+)	ND (+)	ND (−)
L. vermiforme	M59295.1	B	0.6 (+)	1.2 (+)	2.5 (+)	1.2 (+)	0.6 (+)	ND (+)	3.1 (+)
L. paracasei	AB362762.1	F	ND (−)	ND (−)	5.6 (+)	1.8 (+)	1.2 (+)	0.6 (+)	0.6 (+)
L. sakei	EU081005.1	I	ND (−)	ND (−)	ND (−)	ND (−)	ND (+)	1.8 (+)	ND (+)
Leuconostoc lactis	HM058981.1	(−)	ND (−)	ND (−)	0.6 (−)	0.6 (−)	ND (−)	ND (−)	ND (−)
Leuconostoc mesenteroides sub. *mesenteroides*	HM059009.1	C	ND (+)	1.2 (+)	ND (+)	ND (+)	ND (−)	ND (−)	ND (−)
Lactococcus lactis	HM218820.1	M	ND (+)	ND (+)	ND (−)	ND (−)	ND (−)	ND (−)	ND (−)
Bacillus sp.	AY176766	A	2.5 (−)	4.9 (+)	1.8 (+)	ND (+)	ND (−)	ND (−)	ND (−)
Bacillus subtilis	AY219900.1	D	ND (−)	ND (−)	5.6 (+)	1.8 (+)	1.2 (+)	0.6 (+)	0.6 (+)
Bacillus cereus	GU321330.1	(−)	0.6 (−)	1.2 (−)	ND (−)	ND (−)	ND (−)	ND (−)	ND (−)
Carnobacterium maltaromaticum	AY543031.1	K	ND (−)	ND (−)	ND (−)	ND (−)	1.2 (+)	0.6 (+)	ND (+)
Enterococcus faecium	GU904671.1	G	0.6 (−)	1.2 (+)	8.6 (+)	1.2 (+)	ND (−)	ND (−)	ND (−)
Corynebacterium	–	(−)	4.3 (−)	2.5 (−)	3.7 (−)	1.2 (−)	1.8 (−)	ND (−)	ND (−)
Enterobacteriaceae *family*	–	(−)	3.7 (−)	1.2 (−)	1.8 (−)	ND (−)	ND (−)	ND (−)	ND (−)
E. coli	HM218820.1	L	1.2 (+)	0.6 (+)	ND (−)	ND (−)	ND (−)	ND (−)	ND (−)
Uncultured bacterium	DQ004294.1	J	ND (−)	ND (−)	ND (−)	ND (−)	ND (+)	ND (+)	ND (+)

Table 2
Yeast identified by ITS region and detected by DGGE analysis during 48 h of fermentation. ND = no detected by platting; (+) present by PCR–DGGE analysis; (−) absence by PCR–DGGE analysis; letters in DGGE column correspond to bands in PCR–DGGE gel. Percentages calculated from total number of isolates.

Yeast %	Accession number	DGGE	0	8	16	24	32	40	48
Rhodotorula mucilaginosa	AF515475.1	A	6.2 (+)	7.4 (+)	9.9 (+)	7.4 (+)	8.6 (+)	4.9 (+)	ND (+)
Candida parapsilosis	FJ662413.1	B	3.7 (+)	2.5 (+)	4.9 (+)	2.5 (+)	3.7 (+)	2.5 (+)	2.5 (+)
Clavispora lusitaniae	FJ183442.1	C	6.2 (+)	6.2 (+)	4.9 (+)	3.7 (+)	2.5 (+)	2.5 (+)	1.2 (+)
Candida orthopsilosis	FN812686.1	D	ND (−)	ND (+)	ND (+)	2.5 (+)	3.7 (+)	ND (+)	ND (−)

plantarum group (12%) was the most common species, as it was isolated at various times during fermentation of the beverage.

The LAB that were isolated included isolates from *L. plantarum* group (17.9% of total bacteria), *Lactobacillus paracasei* (9.9%), *Lactobacillus vermiforme* (9.3%), *Lactobacillus sakei* (1.8%), *Lactobacillus brevis* (1.8%), *Carnobacterium maltaromaticum* (1.8%), *Leuconostoc lactis* (1.2%) and *Leuconostoc mesenteroides* (1.2%). The isolates belonging to the *L. plantarum* group were detected after 8 h of fermentation and remained in the samples to the end of the fermentation process. With the exception of the sample taken at the 40 h time point, the *L. vermiforme* species was isolated throughout the fermentation process. The *C. maltaromaticum* isolates were only found in fermentation samples taken at the 32 and 40 h time points. The profile of different bacteria species isolated from cotton seed and rice after fermentation is shown in Table 3.

The *Bacillus* genus was detected throughout the fermentation process, and except for the beginning of the process, the *Bacillus subtilis* species was found at all time points during fermentation. Some physiological characteristics of bacilli are shown in Table 4. Isolates of *Corynebacterium* were not detected after 32 h of fermentation, and no representatives of the Enterobacteriaceae family, including *Escherichia coli*, were isolated after 16 h of fermentation.

The species of yeast isolated from the cotton seed and rice beverage included *Rhodotorula mucilagionasa*, *Candida parapsilosis*, *Clavispora lusitaniae* and *Candida orthopsilosis*. Some of the carbohydrates assimilated by yeasts are shown in Table 5. Representatives of the yeast species were detected throughout the fermentation process, except for *C. orthopsilosis*, which was only found after 8 h of fermentation.

3.2. DGGE analysis of the bacterial community

Traditionally, many plating procedures are only partially selective and might exclude members of the microbial community (Magalhães et al., 2010). Thus, to determine the composition of bacteria in the cotton seed and rice beverage, PCR–DGGE analysis was employed. The V3 region in the 16S rRNA gene was amplified, and the DGGE fingerprint is shown in Fig. 3. The results for the highlighted bands are shown in Table 1.

The bacterial DGGE profile indicated the presence of LAB throughout the fermentation process, consistent with what we found by plating. The *L. vermiforme* species was detected during the entire fermentation process. According to the results of DGGE, LAB were the most prevalent microbes found at the end of the fermentation process. *L. plantarum* group and *L. paracasei* were observed at the 16 h time point, whereas *L. brevis*, *L. sakei* and *C. maltaromaticum* were detected at the 32 h time point. *L. mesenteroides* sub. *mesenteroides*, *L. lactis* and *Enterococcus faecium* were present at the beginning and middle of the fermentation process.

Species from the *Bacillus* genus were detected at the 8 h time point, and *B. subtilis* in particular remained until the 48th hour of fermentation. *E. coli* was detected only in the early hours of the fermentation process. *Lactococcus lactis*, observed at the beginning of fermentation, was detected only by DGGE.

Bacterial taxonomic structure comparisons were performed by cluster analysis of the DGGE data according to the Pearson correlation, and dendograms were constructed using UPGMA methods (Fig. 4). The hierarchical cluster divided the fermentation process into two groups, the first containing samples from 0 to 24 h and the second containing samples from the 32 and 48 h time points.

3.3. DGGE analysis of yeast

To study the yeast diversity in the cotton seed and rice beverage, the NS3 region of the 18S rRNA gene from yeast was amplified, and DGGE analysis was performed. The DGGE profiles are shown in Fig. 5. The results of the highlighted bands are shown in Table 2. No differences were found in the community structure of yeast during fermentation, and all of the species identified were found throughout the fermentation process, except for *C. orthopsilosis*, which was detected from 8 to 40 h.

The hierarchical cluster divided the fermentation into two groups; the first group contained samples from 16 to 40 h and showed no difference in microbial composition (Fig. 6). The second group comprised the samples in the first hours (0 and 8 h) and the final time point (48 h) of fermentation. We observed that the results of the PCR–DGGE correlated well with the results of the culture-dependent method.

4. Discussion

Traditional and rudimentary fermented foods and beverages, especially those prepared from substrates rich in fermentable

Table 3
Percentage of bacteria fermenting different carbohydrates. Number in parenthesis indicates number of isolates.

Bacteria (%)	Maltose	Lactose	Glucose	Sucrose	Raffinose	Cellobiose	Trehalose
L. plantarum	100 (29)	100 (29)	100 (29)	100 (29)	52 (15)	100 (29)	100 (29)
L. brevis	100 (3)	100 (3)	100 (3)	67 (2)	0	100 (3)	100 (3)
L. vermiforme	33 (5)	0	100 (15)	47 (7)	0	0	0
L. paracasei	100 (16)	100 (16)	100 (16)	100 (16)	0	56 (9)	100 (16)
L. sakei	33 (1)	33 (1)	100 (3)	100 (3)	0	100 (3)	100 (3)
Leuconostoc lactis	100 (2)	100 (2)	100 (2)	100 (2)	50 (1)	100 (2)	100 (2)
Leuconostoc mesenteroides sub. mesenteroides	100 (2)	100 (2)	100 (2)	100 (2)	100 (2)	0	100 (2)
Carnobacterium maltaromaticum	100 (3)	100 (3)	100 (3)	100 (3)	0	100 (3)	100 (3)
Enterococcus faecium	100 (19)	100 (19)	100 (19)	100 (19)	21 (4)	100 (19)	100 (19)

Table 4
Percentage of *Bacillus* isolates producing acid from different carbohydrates and amylase action. Number in parenthesis indicates number of isolates.

Bacteria (%)	Arabinose	Lactose	Glucose	Sucrose	Xylose	Mannitol	Amylase activity
Bacillus sp.	80 (12)	0	100 (15)	100 (15)	33 (5)	53 (8)	93 (14)
Bacillus subtilis	100 (16)	0	100 (16)	100 (16)	100 (16)	100 (16)	100 (16)
Bacillus cereus	0	0	100 (3)	100 (3)	0	0	100 (3)

carbohydrates, such as cassava, peanuts and cereals, contain a large number of microorganisms during the fermentation process (Almeida et al., 2007; Schwan et al., 2007; Ramos et al., 2010). These microorganisms are responsible for the physical and chemical changes of these products. In the case of the indigenous fermented beverage made with cotton seed and rice, a large number of microorganisms were isolated, and most were bacteria. We used culture-dependent and -independent methods to determine the diversity of the bacteria and yeast found in the beverage naturally fermented with cotton seed and rice. Using these methods, we found that the *Lactobacillus* and *Bacillus* species were the dominant bacterial groups. Representatives of the *Corynebacterium* and Enterobacteriaceae groups were found, but not until the end of the fermentation process. DGGE was not able to detect the *Corynebacterium* species, which may be due to the primer pair used in the PCR or the conditions for DGGE.

The predominance of LAB in fermented foods is common due to their ability to tolerate low pH levels (Van der Meulen et al., 2007). This tolerance along with the propensity of lactic acid bacteria to produce a variety of antimicrobial substances creates unfavorable conditions for the growth of pathogens as well as toxigenic and spoilage organisms. The presence of LAB in the cotton seed and rice beverage studied here might play a role in the reduced survival of bacterial pathogens despite the rudimentary preparation process.

There was a predominance of the *Lactobacillus* species at the end of the fermentation process, whereas *Lactococcus*, *Leuconostoc* and *Enterococcus* species were observed at the beginning of fermentation. These genera are typically associated with cereal kernels and flour, but they do not survive long-term acidification processes (Van der Meulen et al., 2007). Isolates belonging to the *L. plantarum* group are more acid-tolerant and often dominate the fermentation processes of vegetables and cereals, mainly because of their ability to transport and metabolize different plant carbohydrates (McDonald et al., 1990).

The LAB found in this study were also present in other fermented foods, such as Ogi (Omemu et al., 2007) and cauim (Almeida et al., 2007; Ramos et al., 2010). *E. faecium* has previously been identified as the predominant LAB in *doenjang*, a Korean fermented soybean paste (Kim et al., 2009). Our results indicate that LAB may play an important role during the fermentation of the cotton seed and rice beverage. LAB are able to ferment important carbohydrates, such as glucose, maltose and sucrose. *L. mesenteroides* sub. *mesenteroides*, *L. plantarum* group, *L. lactis* and *E. faecium* probably have an important role in fermenting raffinose, which is present in cotton seed, thereby releasing sucrose that can be used by other microorganisms. However, more studies are required to establish and characterize the role of LAB in the fermentation of cotton seed and rice.

In this study, representative organisms from *B. subtilis*, *Bacillus cereus* group and other *Bacillus* sp. were isolated during fermentation. They likely originated from the raw material, as these species have been isolated from a variety of animal and plant products (Odunfa, 1981). *Bacillus* species have been found in cauim from cassava (Almeida et al., 2007) and have been associated with Maari, a fermented product from Baobab seed (Parkouda et al., 2010). Strains of *B. subtilis* have been used as starter cultures in the controlled fermentation of Natto and Kinema (Owens et al., 1997). In studies with Soumbala, Ouoba et al. (2007) showed the ability of some *B. subtilis* and *Bacillus pumilus* species to degrade African locust bean oil, proteins and carbohydrates as well as to produce a pleasant aroma and antimicrobial substances to fight pathogens. Specific strains from the above studies were proposed to be suitable starter cultures for the controlled production of Soumbala. The *Bacillus* group of microorganisms is typically found in soils, and members secrete a wide range of degradative enzymes, including amylases and proteases, which may be important in the fermentation process. Almeida et al. (2007) have shown that *Bacillus licheniformis* has strong protease activity. In our studies, the majority of *Bacillus* species showed amylolytic activity (Table 4).

Isolates belonging to the *B. cereus* group were found only during the first hours of cotton seed and rice fermentation. The inhibition of *B. cereus* growth and sporulation could be attributed to the suboptimal pH, the presence of organic acids and competitive constraints (Rossland et al., 2005). Assimilation of arabinose, xylose and mannitol are important carbohydrates to discriminate between *B. cereus* and *Bacillus subtilis* according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Some *Bacillus* species could not be differentiated from the complex mixture of microorganisms in the cotton seed and rice beverage. The analysis of the V3 region of the 16S rRNA and the size (approximately 200 bp) of the DGGE amplicons did not provide a sufficiently high resolution to identify some *Bacillus* at the species level. When corresponding bands in different samples were similar to a different species of *Bacillus*, they were identified as *Bacillus* sp. (Band A).

No differences were found in yeast community structure during the fermentation of cotton seed and rice, except for *C. orthopsilosis*, which was isolated only in the 24 and 32 h samples and detected by DGGE after 8–40 h of fermentation.

Candida species have an impact on human health and have been reported to be present in fermented foods. *C. parapsilosis* has been shown to be present in chorote, a traditional Mexican fermented product (Castillo-Morales et al., 2005), and in cassava and peanuts

Table 5
Percentage of yeast assimilating different carbohydrates and amylase action. Number in parenthesis indicates number of isolates.

Yeast (%)	Maltose	Lactose	Glucose	Sucrose	Raffinose	Cellobiose	Trehalose	Amylase activity
Rhodotorula mucilaginosa	94 (34)	0	100 (36)	100 (36)	100 (36)	50 (18)	100 (36)	0
Candida parapsilosis	100 (18)	0	100 (18)	100 (18)	0	0	100 (18)	0
Clavispora lusitaniae	100 (22)	0	100 (22)	100 (22)	0	23 (5)	23 (5)	0
Candida orthopsilosis	100 (5)	0	100 (5)	100 (5)	0	0	100 (5)	0

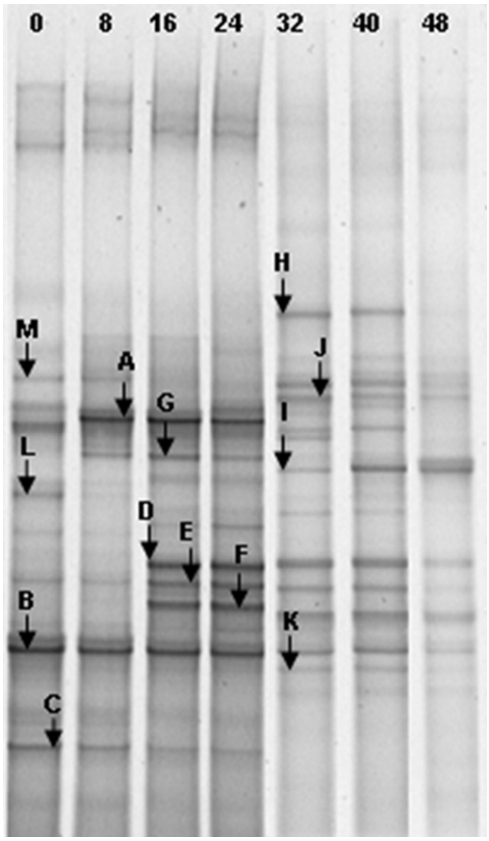

Fig. 3. Denaturing Gradient Gel Electrophoresis profiles of bacterial V3 regions of the 16S rRNA gene amplified from the cotton seed and rice beverage throughout the 48 h of fermentation. A = *Bacillus* sp., B = *L. vermiforme*, C = *Leuconostoc mesenteroides* sub *mesenteroides*, D = *B. subtilis*, E = *L. plantarum*, F = *L. paracasei*, G = *Enterococcus faecium*, H = *L. brevis*, I = *L. sakei*, J = Uncultured bacterium, K = *Carnobacterium maltoromanticum*, L = *E. coli*, M = *Lactococcus lactis*.

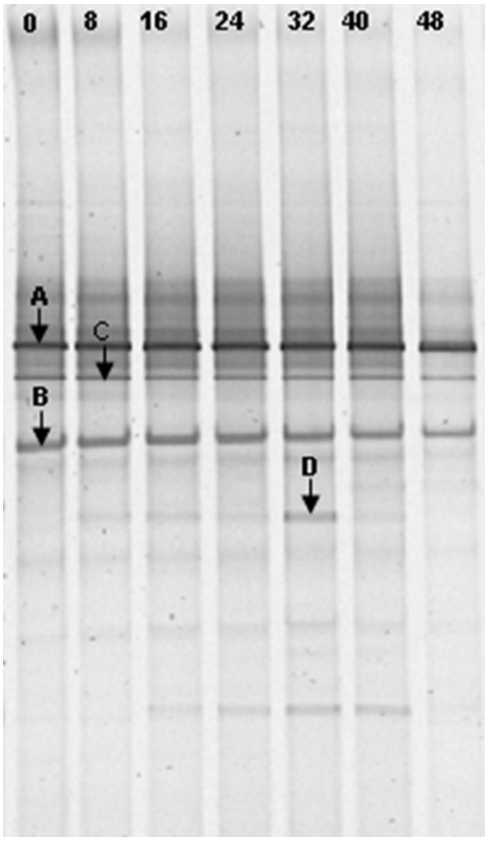

Fig. 5. Denaturing Gradient Gel Electrophoresis profiles of the yeast NS3 regions of the 18S rRNA gene amplified from the cotton seed and rice beverage throughout the 48 h of fermentation. A = *Rhodotorula mucilaginosa*, B = *Candida parapsilosis*, C = *Clavispora lusitaniae*, D = *Candida orthopsilosis*.

with rice cauim (Almeida et al., 2007; Ramos et al., 2010). *C. orthopsilosis* can be distinguished from *C. parapsilosis* by its ITS region as reported by Tavanti et al. (2005). None of the yeasts isolates in our study were able to secrete amylase.

Of the yeast present in the cotton seed and rice beverage, *C. lusitaniae* is a cosmopolitan species that can be found in a large variety of substrates, including fruits, soil, water and humans (Lachance and Phaff, 1998). The other species detected in this study was *Rhodotorula mucilaginosa*. This yeast may be found in soils and vegetation, and it is a common species found in food. *R. mucilaginosa* produces pink or red pigments and causes discoloration of foods, such as fresh poultry, fish and beef. This species has also been reported to cause spoilage in dairy products, such as yogurt, cheese, butter and cream (Frazier and Whesthoff, 2001). Only *R. mucilaginosa* can use raffinose as a carbon source. Carbohydrates, such as glucose, maltose, trehalose and sucrose, can be used by representatives of all species isolated in this work. The function of the yeast species in the cotton seed and rice beverage is not known. The yeast may cause spoilage or contribute to the development of flavor. More studies on yeast metabolism are needed to understand the role of this microorganism during fermentation, which would aid in the development of a starter culture. In this way, it may be that yeast species participate in gossypol detoxification in cotton seed extracts, as previous work with *Candida tropicalis* (Weng and Sun,

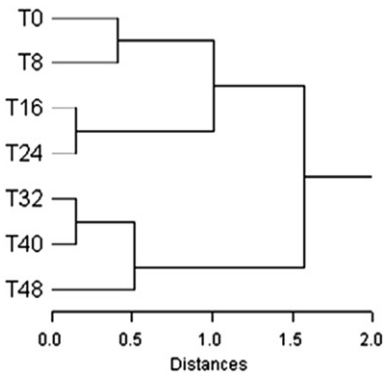

Fig. 4. Cluster analysis of rRNA 16S amplicons present throughout the 48 h of fermentation of the cotton seed and rice beverage.

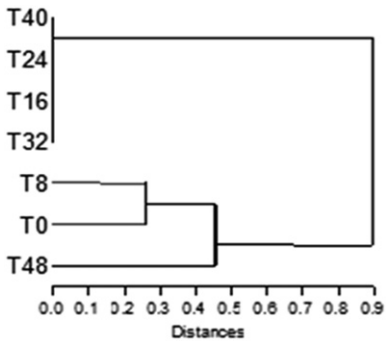

Fig. 6. Cluster analysis of rRNA 18S amplicons present throughout the 48 h of fermentation of the cotton seed and rice beverage.

2006) has shown. In addition, it is known that gossypol is inactivated by high temperatures (Kenar, 2006); thus, it would not be expected to be found here due to the cooking process prior to preparation of the beverage.

5. Conclusion

This report is the first on the microbial population involved in the fermentation of cotton seed and rice by Amerindians in Brazil. Our results showed that LAB were the predominant microorganisms present in the cotton seed and rice beverage, and no changes in the yeast community were detected during fermentation. This study indicated that conventional culturing and PCR–DGGE need to be combined to optimally describe the microbiota associated with the cotton seed and rice beverage. More studies should be performed to understand the role of these different microorganisms in the fermentation process to develop a controlled process using a selected culture.

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