

## IDENTIFICATION OF THE BACTERIAL COMMUNITY RESPONSIBLE FOR TRADITIONAL FERMENTATION DURING SOUR CASSAVA STARCH, CACHAÇA AND MINAS CHEESE PRODUCTION USING CULTURE-INDEPENDENT 16S rRNA GENE SEQUENCE ANALYSIS

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### ABSTRACT

We used a cultivation-independent, clone library-based 16S rRNA gene sequence analysis to identify bacterial communities present during traditional fermentation in sour cassava starch, cachaça and cheese production in Brazil. Partial 16S rRNA gene clone sequences from sour cassava starch samples collected on day five of the fermentation process indicated that *Leuconostoc citreum* was the most prevalent species, representing 47.6% of the clones. After 27 days of fermentation, clones (GenBank accession numbers GQ999786 and GQ999788) related to unculturable bacteria were the most prevalent, representing 43.8% of the clones from the bacterial community analyzed. The clone represented by the sequence GQ999786 was the most prevalent at the end of the fermentation period. The majority of clones obtained from cachaça samples during the fermentation of sugar cane juice were from the genus *Lactobacillus*. *Lactobacillus nagelli* was the most prevalent at the beginning of the fermentation process, representing 76.9% of the clones analyzed. After 21 days, *Lactobacillus harbinensis* was the most prevalent species, representing 75% of the total clones. At the end of the fermentation period, *Lactobacillus buchneri* was the most prevalent species, representing 57.9% of the total clones. In the Minas cheese samples, *Lactococcus lactis* was the most prevalent species after seven days of ripening. After 60 days of ripening, *Streptococcus salivarius* was the most prevalent species. Our data show that these three fermentation processes are conducted by a succession of bacterial species, of which lactic acid bacteria are the most prevalent.

**Key words:** fermentation, cassava, cachaça, cheese, clone library

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## INTRODUCTION

Culture-based methods are not designed to detect unculturable cells and often fail to characterize less abundant microbial populations or those microorganisms that require selective enrichment (17). These limitations have led to the development of culture-independent techniques. The most commonly used techniques are PCR-based and nucleic acid-based detection methods (28). Compared to conventional methods, culture-independent methods are generally less time consuming and well suited for analyzing microbial communities over time. These methods may also help in the study of microbial populations and their diversity in food ecosystems (16).

Sour cassava starch (“polvilho azedo”) and Minas cheese are traditional fermented foods produced in Brazil. These products are mostly manufactured on a small scale using traditional recipes (5, 18). During sour cassava starch production, the starch from cassava root is fermented in a tank for a period of time that ranges between 20 and 70 days (7, 18). Using culture-based methods, *Lactobacillus plantarum* and other lactic acid bacteria have been reported to be the most prevalent microorganisms associated with spontaneous fermentation of cassava starch in Brazil (18). These microorganisms are responsible for generating organic acids and aromatic compounds in this food product (3, 4, 9, 10, 18). Cheese production in Brazil involves milk fermentation by lactic acid bacteria, which are either deliberately added as starter cultures or are adventitious microbial populations, the growth of which is the result of the selective pressures encountered under the conditions used in the fermentation process (5, 20). Lactic acid bacteria occur naturally as part of the indigenous microbiota of raw milk, and the microbial diversity contributes to the large differences in organoleptic characteristics found in raw-milk cheeses (1).

Cachaça is the most important Brazilian distilled beverage (13, 26). During traditional cachaça production, lactic acid

bacteria are considered contaminants in the fermentation process. These bacteria compete with yeasts for the sucrose found in the sugar cane juice, which then reduces the ethanol yield of fermentation (12). However, a precise correlation between bacterial contamination and reduced alcohol content during cachaça fermentation has not yet been shown (25).

The bacterial communities associated with sour cassava starch, cachaça and Minas cheese have been determined using cultivation techniques in combination with the molecular identification of isolates (14, 18, 20, 14). The aim of the present study was to examine the structure of the bacterial communities found in these traditional fermented products using direct DNA extraction, 16S rRNA gene amplification, cloning and sequencing.

## MATERIALS AND METHODS

### Sample collection

Sour cassava starch samples were collected from a factory in the city of Conceição dos Ouros, Minas Gerais, Brazil. The samples were collected 5, 27 and 45 days after the fermentation process started, according to Lacerda *et al.* (18). Cachaça fermentation samples were collected from a traditional distillery in the city of Esmeraldas, Minas Gerais at 7, 21 and 35 days after fermentation process started. Preparation procedures and sample collection for sour cassava starch and cachaça are described in Lacerda *et al.* (18) and Gomes *et al.* (13), respectively. After 7 and 60 days of maturation, Minas cheese samples were collected from a farm in the city of São Roque de Minas, Minas Gerais. This semi-hard Minas cheese, also known as Canastra cheese, is produced with raw milk and has been made at the farmhouse production level for the last 200 years using traditional procedures. Milk coagulation is a result of employing natural whey cultures as starter cultures (indigenous lactic acid bacteria) and commercial rennet. The natural microbiota present in the milk and in the environment is responsible for ripening (6). All samples were aseptically

collected using sterile flasks and were stored at -20°C until the DNA was extracted.

#### DNA extraction, amplification and clone libraries

DNA was extracted from 1 g of each sour cassava starch and cachaça fermentation sample, which was diluted in 9 mL sterile distilled water. Each 1-gram cheese sample was homogenized in 9 mL of 0.1% buffered peptone water. These suspensions were centrifuged at 15,000 rpm for 10 min, and DNA was extracted from the pellet using the procedure described by Lacerda *et al.* (18).

16S rRNA gene sequences were PCR amplified using the bacterial domain specific primers 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1492r (5'GGTTACCTTGTTACGACTT3') (19). These primers amplify approximately 1,600 base pairs of the 16S rRNA gene. PCR reactions were performed in a final reaction volume of 50 µL, containing 0.1-0.5 µg DNA, dNTPs (200µM each), primers (0.8µM each), MgCl<sub>2</sub> (1.5 mM), 1 U *Taq* DNA polymerase (Invitrogen) and the supplied buffer. The amplification conditions were as described in Morlon-Guyot *et al.* (22). The amplified fragments were purified using the Wizard system, ligated into the pGEM<sup>®</sup>-T Vector System II (Promega, Madison, USA) and transformed into competent *E. coli* (XL1-Blue) cells according to the manufacturer's instructions (Invitrogen). Blue/white screening on Luria-Bertani (LB) agar containing ampicillin (Sigma), X-gal (Invitrogen) and isopropyl β-D-1-thiogalactopyranoside (IPTG, Promega) was used to randomly select approximately 30 white colonies for each sample. The clones were stored in freezing medium at -80°C.

#### Sequencing of cloned DNA and identification

Both strands of the cloned inserts were sequenced using the universal sequencing primers that correspond to the regions flanking the cloning site. Sequencing was performed using a DYEnamic Terminator Cycle Sequencing Kit in a MegaBACE<sup>™</sup> 1000 automated sequencing system (Amersham

Biosciences, USA). These partial sequences were searched against sequenced deposited in GenBank using the advanced BLAST similarity search option (2). Partial sequences were approximately 600 base pairs long (range 350–940 bp). The nucleotide sequences and other related sequences were aligned using MEGA version 4. The 16S rRNA gene clone sequences that had a greater than 97% similarity to sequences deposited in GenBank were designated as belonging to the corresponding species (see accession numbers in Table 1).

### RESULTS AND DISCUSSION

We sequenced 129 clones to identify the specific phlotypes of bacteria associated with the traditional processes used in sour cassava starch and Minas cheese fermentation and cachaça production. The overall structure of the bacterial communities found associated with these food products are summarized in Table 1.

Out of 45 clones examined from sour cassava starch fermentation, the most common species found after five days of fermentation were *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc citreum* and *Enterobacter* sp. *Leuconostoc citreum* was the most prevalent species at the start of the fermentation process, representing 47.6% of the total clones analyzed. After 27 days of fermentation, two and five clones (GenBank accession number GQ999786 and GQ999788, respectively) were similar to uncultured bacterium clones, representing 43.8% of the bacterial community analyzed at this stage of the fermentation process. *Enterobacter cloacae*, *Lactobacillus manihotivorans*, *Lc. lactis* subsp. *lactis*, *Lb. plantarum* and *Lactobacillus* sp. were found at lower frequencies. After 45 days of fermentation, an uncultured bacterium clone (GenBank accession number GQ999786) was the most prevalent. This uncultured bacterium clone was distantly related (90% identity) to some sequences deposited in GenBank, such as *Enterobacter* sp. (GenBank accession

numbers GQ478379 and FJ646658). At this fermentation time, *Lb. plantarum* and *Enterobacter* sp. were found at a low frequency.

Ampe *et al.* (3) used denaturing gradient gel electrophoresis (DGGE) to show that the dominant bacteria

involved in the spontaneous fermentation of sour cassava starch in Colombia were all lactic acid bacteria, mainly those closely related to *Bifidobacterium minimum*, *Lc. lactis*, *Streptococcus* sp., *Enterococcus saccharolyticus* and *Lb. plantarum*.

**Table 1.** Most closely related bacterial species to the sour starch cassava, cachaça and cheese species based on BLASTn analysis.

| UFMGCB                     | Most closely related species/GenBank accession number               | Times <sup>a</sup> |    |     | Percent similarity | bp analyzed | Identification                                 | GenBank accession number |
|----------------------------|---|--------------------|----|-----|--------------------|-------------|--|--------------------------|
|                            |   | I                  | II | III |                    |             |  |                          |
| <b>Sour cassava starch</b> |   |                    |    |     |                    |             |  |                          |
| UFMG-PB10                  | <i>Enterobacter aerogenes</i> JCM1235 [NR_024643]                   | -                  | 2  | -   | 97.4%              | 735         | <i>Enterobacter aerogenes</i>                  | GQ918333                 |
| UFMG-PB11                  | <i>Enterobacter cloacae</i> ATCC13047T [AJ251469.1]                 | -                  | 1  | -   | 98.4%              | 680         | <i>Enterobacter cloacae</i>                    | GQ999778                 |
| UFMG-PB6                   | <i>Leuconostoc mesenteroides</i> LM2 [AY675249]                     | 3                  | -  | -   | 98.8%              | 827         | <i>Leuconostoc mesenteroides</i>               | GQ999779                 |
| UFMG-PB20                  | <i>Lactobacillus manihotivorans</i> LSAF000162 [AF000162.1]         | -                  | 1  | -   | 97%                | 694         | <i>Lactobacillus manihotivorans</i>            | GQ999780                 |
| UFMG-PB8                   | <i>Leuconostoc pseudomesenteroides</i> NRIC 1777 [AB023237.1]       | 1                  | -  | -   | 97%                | 837         | <i>Leuconostoc pseudomesenteroides</i>         | GQ999781                 |
| UFMG-PB9                   | <i>Lactobacillus plantarum</i> DSM 20205 [M58827]                   | 1                  | 3  | 1   | 99.6%              | 815         | <i>Lactobacillus plantarum</i>                 | GQ999782                 |
| UFMG-PB03                  | <i>Lactobacillus plantarum</i> DSM 20205 [M58827]                   | -                  | 1  | -   | 96%                | 556         | <i>Lactobacillus</i> sp.                       | GQ999783                 |
| UFMG-PB12                  | <i>Lactococcus lactis</i> subsp. <i>lactis</i> NCDO 604T [AB100803] | 5                  | 1  | -   | 99.6%              | 538         | <i>Lactococcus lactis</i> subsp. <i>lactis</i> | GQ999784                 |
| UFMG-PB07                  | <i>Leuconostoc citreum</i> ATCC49370 [AF111948]                     | 10                 | -  | -   | 99%                | 770         | <i>Leuconostoc citreum</i>                     | GQ999785                 |
| UFMG-PB02                  | <i>Enterobacter</i> sp. [GQ478379]                                  | -                  | 2  | 6   | 90%                | 933         | Uncultured bacterium clone                     | GQ999786                 |
| UFMG-PB01                  | <i>Enterobacter</i> sp. [GQ360072]                                  | 1                  | -  | -   | 99%                | 765         | <i>Enterobacter</i> sp.                        | GQ999787                 |
| UFMG PB30                  | Enterobacteriaceae bacterium [AB461659]                             | -                  | 5  | -   | 99%                | 589         | Uncultured bacterium clone                     | GQ999788                 |
| UFMG-PB44                  | <i>Enterobacter</i> sp. [GQ360072]                                  | -                  | -  | 1   | 99%                | 721         | <i>Enterobacter</i> sp.                        | GQ999789                 |

| Cachaça fermentation |  |   |    |    |       |     |  |          |  |
|----------------------|--|---|----|----|-------|-----|--|----------|--|
| UFMG-CAB11           | <i>Lactobacillus nagelii</i><br>ATCC 700692 [AB162131]                           | 1 | -  | -  | 90%   | 541 | <i>Lactobacillus</i> sp.                               | GQ999790 |  |
| UFMG-CAB16           | <i>Lactobacillus paracasei</i><br>subsp. <i>paracasei</i> ATCC<br>25302 [D79212] | 3 | 1  | 1  | 100   | 812 | <i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> | GQ999791 |  |
| UFMG-CAB5            | <i>Lactobacillus nagelii</i><br>ATCC 700692 [AB162131]                           | 9 | 1  | -  | 99%   | 800 | <i>Lactobacillus nagelii</i>                           | GQ999792 |  |
| UFMG-CAB6            | <i>Lactobacillus satsumensis</i><br>NRIC 0604 [AB154519]                         | - | 1  | -  | 96%   | 533 | <i>Lactobacillus</i> sp.                               | GQ999793 |  |
| UFMG-CAB12           | <i>Lactobacillus harbinensis</i><br>SBT10908 [AB196123.1]                        | - | 9  | 4  | 100%  | 868 | <i>Lactobacillus harbinensis</i>                       | GQ999794 |  |
| UFMG-CAB9            | <i>Streptococcus salivarius</i><br>ATCC 25975(FJ154797.1)                        | - | -  | 1  | 100%  | 449 | <i>Streptococcus salivarius</i>                        | GQ999795 |  |
| UFMG-CAB10           | <i>Lactobacillus buchneri</i><br>ATCC 4005 [AB205055]                            | - | -  | 11 | 99%   | 781 | <i>Lactobacillus buchneri</i>                          | GQ999796 |  |
| UFMG-CAB14           | <i>Lactobacillus acetolerans</i><br>JCM 9904 [AB289008]                          | - | -  | 1  | 99%   | 763 | <i>Lactobacillus acetolerans</i>                       | GQ999797 |  |
| UFMG-CAB17           | <i>Lactobacillus plantarum</i><br>DSM 20205 [M58827]                             | - | -  | 1  | 98.8% | 421 | <i>Lactobacillus plantarum</i>                         | GQ999798 |  |
| Minas cheese         |  |   |    |    |       |     |  |          |  |
| UFMG-QB5             | <i>Lactobacillus plantarum</i><br>DSM 20205 [M58827]                             | 1 | 4  |    | 99.5% | 390 | <i>Lactobacillus plantarum</i>                         | GQ999799 |  |
| UFMG-QB6             | <i>Lactococcus lactis</i> NCDO<br>604T [AB100803]                                | 9 | 4  |    | 100%  | 499 | <i>Lactococcus lactis</i>                              | GQ999800 |  |
| UFMG-QB3             | <i>Lactobacillus brevis</i> ATCC<br>14869 [M58810.1]                             | - | 1  |    | 99%   | 700 | <i>Lactobacillus brevis</i>                            | GQ999801 |  |
| UFMG-QB16            | <i>Sphingomonas</i> sp. Clone<br>FI012 [AY349411]                                | - | 2  |    | 99%   | 614 | <i>Sphingomonas</i> sp.                                | GQ999802 |  |
| UFMG-QB55            | <i>Lactobacillus arizonensis</i><br>NRRLB14768 [AJ965482]                        | - | 7  |    | 99.8% | 496 | <i>Lactobacillus arizonensis</i>                       | GQ999803 |  |
| UFMG-QB26            | <i>Streptococcus salivarius</i><br>ATCC 7073_(AY188352.1)                        | 1 | 10 |    | 99.6% | 720 | <i>Streptococcus salivarius</i>                        | GQ999804 |  |
| UFMG-QB20            | Uncultured <i>Streptococcus</i><br>sp. clone SC002B48<br>[AY807774.1]            | - | 1  |    | 99.6% | 721 | Uncultured clone<br><i>Streptococcus</i> sp.<br>clone  | GQ999805 |  |

<sup>a</sup>Sour starch cassava: I-5 days, II-27 days, III-45 days. Cachaça fermentation: I-7 days, II-21 days, III-35 days; Minas cheese: I-7 days, II-60 days.

The presence of *Lb. manihotivorans* was only detected when 16S rRNA genes were hybridized with phylogenetic probes. Using culture-dependent methods, Lacerda *et al.* (18) showed that *Lb. plantarum* and *Lactobacillus fermentum* were the species most frequently associated with sour cassava starch fermentation at two factories in the state of Minas Gerais. However, the results obtained using clone library sequence analysis showed that the prevalence of lactic acid bacteria was limited to the first five days of fermentation, and a lower prevalence of these bacteria was detected at later time points. *Lb. plantarum* was detected in the three fermentation samples assayed but at low frequencies. Uncultured bacterium clones were prevalent after 27 and 45 days of fermentation. Figueroa *et al.* (10) suggested that the traditional sour cassava starch fermentation process is characterized by a succession of microbial populations. Ampe *et al.* (3) suggested that this succession could be determined by the varying sensitivities of microorganisms to the acidic conditions that develop during the fermentation process. Our results show that unculturable bacteria were found at a high frequency during the middle and at the end of the sour cassava fermentation process. These microorganisms may potentially play an important role in the fermentation process that should be examined further.

Most of the clones obtained during the fermentation of cachaça corresponded to lactic acid bacteria belonging to the genus *Lactobacillus*. The bacterial communities present in this fermentation process are very similar regardless of the raw material used and are characterized by the presence of lactic acid species, especially those from the genera *Lactobacillus*, *Leuconostoc*, *Weissella* and *Lactococcus* (14). Many species from these genera are characteristically resistant to low pH and high alcohol concentrations, which explains their presence and wide distribution in different fermentation processes, including both alcoholic and lactic fermentation processes (15). After 7 days of fermentation, the most prevalent species was *Lb. nagelli*, representing 76.9% of the total clones analyzed. The other clones were *Lactobacillus* sp. and *Lactobacillus*

*paracasei* subsp. *paracasei*. After 21 days, *Lb. harbinensis* was the most prevalent species, representing 75% of the total clones. *Lb. paracasei* subsp. *paracasei*, *Lb. nagelli* and *Lb. satsumensis* were found at lower frequencies. After 35 days of fermentation, *Lb. buchneri* was the most prevalent species, representing 57.9% of the clones analyzed, followed by *Lb. harbinensis* (21%). *Lb. paracasei* subsp. *paracasei*, *Streptococcus salivarius*, *Lb. acetolerans* and *Lb. plantarum* were also found at lower frequencies. *Leuconostoc mesenteroides*, *Lb. plantarum*, and *Lb. brevis* were described as contaminants of the cachaça fermentation vats (23, 27). Carvalho-Netto *et al.* (6) also characterized the bacterial community during cachaça production in the state of São Paulo using partial 16S rRNA gene sequencing. Their analysis included 587 sequences and revealed the presence of 170 operational taxonomic units. The genus *Lactobacillus* was the most predominant, accounting for approximately 66% of the sequences, and *Lb. hilgardii* and *Lb. plantarum* were the species most frequently found. Other species were found at lower frequencies, such as *Curtobacterium flaccumfaciens*, *Lactobacillus casei*, *Leuconostoc mesenteroides* and *Ln. citreum*. Gomes *et al.* (14) identified populations of culturable lactic acid bacteria involved in cachaça fermentation using both physiological and molecular methods. A high frequency of lactic acid bacteria was found during the entire fermentation process, and the species *Lb. plantarum* and *Lb. casei* were the most prevalent in the vats. These differences may be related to the different approaches used and the regional differences in the composition of the lactic acid bacterial community; Carvalho-Netto *et al.* (6) worked with fermentations from the state of São Paulo, whereas our work was performed with distilleries from the state of Minas Gerais.

The characterization of the bacterial communities involved in the production of cachaça using culture-dependent and culture-independent techniques shows the prevalence of a variety of *Lactobacillus* species during the fermentation process. *Lactobacillus plantarum* was the only common

species found during cachaça fermentation using both techniques. Although lactic acid bacteria are considered to be contaminants in cachaça fermentation, it is important to understand the influence these microorganisms have on the cachaça fermentation process (14). Lactic acid bacteria ferment glucose and produce lactic acid as their main metabolic by-product, thus reducing the yield of alcohol. The microbial community present during fermentation is also responsible for producing secondary compounds that determine the flavor of the beverage. The presence of certain concentrations of bacteria during fermentation is not considered a contaminating factor but rather an essential component of the final quality of the beverage (6). However, there is little information available regarding the composition and concentration of these microorganisms during cachaça production. Therefore, a better understanding of the relationship between the microorganisms involved in the fermentation process and the final chemical quality of the beverage is needed.

We obtained 40 clones from cheese samples after 7 and 60 days of ripening. After 7 days of ripening, 9 clones of *Lc. lactis* and 1 clone of both *Lb. plantarum* and *S. salivarius* were found. After 60 days of ripening, *S. salivarius* was the most prevalent, representing 34.5% of the clones obtained. *Lactobacillus arizonensis* was the second most prevalent species, representing 24.1% of the total clones. *Lactobacillus plantarum* and *Lc. lactis* were also isolated at this ripening time with other bacteria, such as *Lactobacillus brevis*, *Sphingomonas* sp. and an uncultured *Streptococcus* sp. Lactic acid bacteria found during cheese ripening are collectively referred to as non-starter lactic acid bacteria, and they are considered to be the species involved mainly in flavor development. The composition of these bacterial species varies with the method used in milk processing and the age of the cheese (21). The presence of lactic acid bacteria during cheese maturation has been described in several reports (5, 11, 21, 24). Duthoit *et al.* (8) used clone libraries and 16S rRNA gene sequencing to show that the lactic acid bacteria *Lc. lactis*, *Lb.*

*plantarum*, *S. termophilus*, *Enterococcus faecium*, *Ln. mesenteroides*, *Ln. pseudomesenteroides* and *Lb. pentosus* were the most predominant during the manufacturing and ripening of Salers, an artisanal cheese produced in France. The first two species were also found during the ripening of Canastra Minas cheese in our study.

The succession of the bacterial populations observed using an analysis of a 16S rRNA gene clone library showed that the fermentation of sour cassava starch, cachaça and Minas cheese is mediated by complex microbial ecosystems, which include both lactic acid and uncultured bacteria. The acidic conditions found during these fermentation processes may be the most important factor in determining the prevalence of the different bacterial species during traditional fermentation. Lactic acid bacteria were the most prevalent species during the fermentation processes for cachaça and Canastra Minas cheese production. Unculturable bacteria predominated during the middle and at the end of sour starch cassava fermentation. These unculturable bacteria may play an important role in the fermentation process, and more studies are necessary to understand the contribution these microorganisms make to the quality of this product.

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#### REFERENCES

1. Abriouel, H.; Platero, A.M.; Maqueda, M.; Valdivia, E.; Bueno, M.M. (2008). Biodiversity of the microbial community in a Spanish farmhouse cheese as revealed by culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 127, 200-208.
2. Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a

- new generation of protein database search programs. *Nucleic Acid Res.* 25, 3389-3402.
3. Ampe, F.; Sirvent, A.; Zakhia, N. (2001). Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. *Int. J. Food Microbiol.* 65, 45-54.
  4. ben Omar, N.; Ampe, F.; Raimbault, M.; Guyot, J.-P.; Tailliez, P. (2000). Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Syst. Appl. Microbiol.* 23, 285-291.
  5. Borelli, B.M.; Ferreira, E.G.; Lacerda, I.C.A.; Franco, G. R.; Rosa, C. A. (2006). Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. *World J. Microbiol. Biotechnol.* 22, 1115-1119.
  6. Carvalho-Netto, O. V.; Rosa, D. D.; Camargo, L. E. A. (2008). Identification of contaminat bacteria in cachaça yeast by 16S RDNA gene sequencing. *Sci. Agric.* 65, 508-515.
  7. Cereda M. P. (1987) Tecnologia e qualidade do polvilho azedo. *Inf. Agrop.* 13, 63-68.
  8. Duthoit, F.; Godon, J.J.; Montel, M. C. (2003). Bacterial community dynamics during production of registered designation of origin salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl. Environ. Microbiol.* 69, 3840-3848.
  9. Figueroa, C.; Davila, A. M.; Pourquié, J. (1995). Lactic acid bacteria of the sour cassava starch fermentation. *Lett. Appl. Microbiol.* 21, 126-130.
  10. Figueroa, C.; Davila, A. M.; Pourquié, J. (1997). Original properties of ropy strains of *Lactobacillus plantarum* isolated from the sour cassava starch fermentation. *J. Appl. Microbiol.* 82, 68-72.
  11. Gala, E.; Landi, S.; Solieri, L.; Nocetti, M.; Pulvirenti, A.; Giudici, P. (2008). Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. *Int. J. Food. Microbiol.* 125, 347-351.
  12. Gallo, C. R. (1992). Identificação de bactérias contaminantes da fermentação alcoólica. *STAB* 4/5, 30-34.
  13. Gomes, F.C.O.; Silva, C.L.C.; Marini, M.M.; Oliveira, E.S.; Rosa, C.A. (2007). Use of selected indigenous *Saccharomyces cerevisiae* strains for the production of the traditional cachaça in Brazil. *J. Appl. Microbiol.* 103, 2438-2447.
  14. Gomes, F. C. O.; Silva, C. L. C.; Vianna, C. R.; Lacerda, I. C. A; Borelli, B. M.; Nunes, A. C.; Franco, G. R.; Mourão, M.; Rosa, C. A. (2010). Identification of lactic acid bacteria associated with traditional cachaça fermentations in Brazil. *Braz. J. Microbiol.* 41, 486-492.
  15. Huhtamella, S.; Leinonen, M.; Nieminen, T.; Fahnert, B.; Myllykoski, L.; Breitenstein, A.; Neubauer P. RNA-based sandwich hybridization method for detection of lactic acid bacteria in brewery samples. *J. Microbiol. Methods* 68, 543-553, 2007.
  16. Jany, J.L.; Barbier, G. (2008). Culture-independent methods for identifying microbial communities in cheese. *Food Microbiol.* 25, 839-848.
  17. Justé, A.; Thomma, B.P.H.J.; Lievens, B. (2008). Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiol.* 25, 745-761.
  18. Lacerda, I.C.A.; Miranda, R.L.; Borelli, B.M.; Nunes, A.C.; Nardi, R.M.D.; Lachance, M.A.; Rosa, C.A. (2005). Lactic acid bacteria and yeast associated with spontaneous fermentations during the production of sour cassava starch in Brazil. *Int. J. Food Microbiol.* 105, 213-219.
  19. Lane, D.J. (1991). 16S-23S rRNA sequencing. In: Stackebrandt, R., Goodfellow, M. (eds). *Nucleic acid techniques in bacterial systematics*. Wiley, New York, USA, p. 117-175.
  20. Lima, C.D.C.; Cerqueira, M.M.O.P.; Ferreira, E.G.; Faria Jr.; C.L.L.; Nelson, D.L.; Carmo, L.S.; Rosa, C.A. (2008). Microbiological, physical-chemical and sensory evaluation of a traditional Brazilian cheese during the ripening progress. *World J. Microbiol. Biotechnol.* 24, 2389-2395.
  21. McSweeney, P.L.H.; Ottogalli, G.; Fox, P.F. (2004). Diversity of cheese varieties: an overview. In: Fox, P.H.; Mc Sweeney, P.L.H.; Cogan, T.; Guinee, T. (Eds). *Cheese: Chemistry, Physics and Microbiology*, Elsevier Academic Press, San Diego, USA, p.1-22.
  22. Morlon- Guyot, M. J.; Guyot, J. P.; Pot, B.; Haut, J.; Raimbault, M. (1998). *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolysin lactic acid bacterium isolated during cassava sour starch fermentation. *Int. J. Syst. Bacteriol.* 48, 1101-1109.
  23. Oliva-neto, P.; Yokoya, F. (1994). Evaluation of bacterial contamination in a fed-batch alcoholic fermentation process. *World J. Microbiol. Biotechnol.* 10, 697- 699.
  24. Randazzo, C.L.; Torriani, S.; Akkermans, A.D.L.; Vos, W.M.; Vaughan, E.E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ. Microbiol.* 68, 1882-1892.
  25. Rosa, C.A.; Soares, A.M.; Faria, J.B. Cachaça production. (2009). In: Ingledew, W.M.; Kelsall, D.R.; Austin, G.D.; Kluhspies, C. (eds). *The Alcohol TextBook*: Nottingham University Press, Nottingham, UK, p. 481-490.
  26. Silva, C.L.C.; Vianna, C. R.; Cadete, R. M.; Santos, R. O.; Gomes, F. C. O.; Oliveira, E. S.; Rosa, C. A. (2009). Selection, growth, and chemosensory evaluation of flocculent starter culture strains of *Saccharomyces cerevisiae* in the large-scale production of traditional Brazilian cachaça. *Int. J. Food Microbiol.* 131, 203-210.
  27. Schwan, R.F.; Mendonça, A.T.; Silva-Júnior, J.J.; Rodrigues, V.; Wheals, A.E. (2001) Microbiology and physiology of cachaça (aguardente) fermentations. *Antonie van Leeuwenhoek* 79, 89-96.
  28. Yang, C.H.; Crowley, D.E.; Borneman, J.; Keen, N.T. (2001). Microbial phyllosphere populations are more complex than previously realized. *Proc. Natl. Acad. Sci. USA* 98, 3889-3894.