Isolation and characterization of lactic acid bacteria and yeasts from the Brazilian grape sourdough

Krischina Singer Aplevicz1*, Jaciara Zarpellon Mazo1, Eunice Cassanego Ilha2, Andréia Zilio Dinon3, Ernani Sebastião Sant’Anna2

1Department Teaching, Research and Extension, Federal Institute of Santa Catarina, Florianopolis, SC, Brazil; 2Department of Food Science and Technology, Federal University of Santa Catarina, Florianopolis, SC, Brazil; 3Department of Engineering of Food, Staty University of Santa Catarina, Florianopolis, SC, Brazil

Sourdough is a mixture of flour and water fermented by lactic acid bacteria and yeast, with a large use in bakery products. This study was developed with Brazilian grape (*Niagara rosada*) sourdough obtained from spontaneous fermentation. The aim of this work was to characterize genotypic and phenotypically lactic acid bacteria and yeasts isolated from sourdough. The phenotypic identification for bacteria and yeasts was performed by using the kit API50CHL and 20CAUX and the genotypic characterization was performed by sequencing method. A total of four isolated strains were analyzed in this study. Two of these strains were phenotypically and genotypic identified as *Lactobacillus paracasei* and one as *Saccharomyces cerevisiae*. Another sample phenotypically identified as *Candida pelliculosa* did not show the same identity by sequencing. It shows the need to use phenotypic and genotypic characterization associated for the correct microorganism identification.

Uniterms: Spontaneous fermentation. Sourdough. Brazilian grape/sourdough *Niagara rosada*/sourdough. Lactic acid bacteria/phenotypic characterization. Lactic acid bacteria/genotypic characterization. Yeast/phenotypic characterization. Yeast/genotypic characterization.

INTRODUCTION

Sourdough has a large application for bakery products, for examples the production of sourdough bread, classical bread, snacks, pizza and sweet baked products (De Vuyst, Vancanneyt, 2007). Production of sourdough can be as simple as mixing flour and water and placing in a warm place. After several replenishments, the sourdough will be formed. The incorporation of sourdough in baking technological traits, enhances sensorial characteristics, increases shelf life and improves nutritional properties (Arendt, Ryan, Dal Bello, 2007; Corsetti et al., 2000). This positive impact of sourdough utilization is attributed
to the microbiota that forms this unique ecosystem and consists of yeasts and lactic acid bacteria (Paramithiotis, Tsiasiotou, Drosinos, 2010).

Mostly yeasted pre-ferments are being used for the production of white bread. The use of lactic acid bacteria (LAB) as starter culture may help to improve the quality and shelf life of the products. The LAB from the sourdoughs naturally fermented may be used in the production of novel fermented foods such as sourdough bread, which is likely to have superior quality and long shelf life (Saeed et al., 2009). LAB belongs to a group of Gram positive bacteria, catalase-negative, non-motile, non-spore forming rods or coccid and produce lactic acid as the major end product during the fermentation. They are strictly fermentative, microaerophile, acidophilic, salt-tolerant with complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives and vitamins. The natural habitat of these microorganisms includes humans, animals and plants (Holzapfel et al., 2001). The LAB microbiota is predominant (8 log CFU/g) and is represented, principally, by members of the genus Lactobacillus. Yeasts are generally counted in lower levels (7 log CFU/g) and can even be absent. The major part of the yeasts belong to the genera Saccharomyces and Candida (Vera et al., 2012).

Throughout Europe, Italy (Reale et al., 2011), Germany (Vogel et al., 1994) or Belgium (Scheirlinck et al., 2007) sourdoughs have been studied intensively. Characterizations of Turkish (Gület et al., 2005), Thai (Luangsakul et al., 2009), Chinese (Zhang et al., 2011) and French (Vera et al., 2012) sourdoughs have also been reported. However, research published in Brazil about sourdoughs is still scarce, even though the bread is widely consumed. The objective of this study was to develop a Brazilian grape sourdough and to identify genotypic and phenotypically four LAB and yeasts for further applications in breads.

**MATERIAL AND METHODS**

**Sourdough preparation**

Sourdough has been developed using Brazilian grape juice from “Niagara rosada” as substrate. The substrate was mixed with water and spontaneously fermented for 3 days at 25°C for the initial fermentation (IF). Thereafter, it was realized the first mixing with addition of 100% IF, 100% wheat flour, 10% of rye flour and 90% water, standing 24 hours at room temperature to origin the preferment. Following intervals of 24 hours, it was performed a second, a third and a fourth mixture using 100% of preferment, 50% wheat flour, 10% of rye flour and 40% water. The sourdough remained for 8 hours at 25°C, and 16 hours at 5°C after each daily alimentation. The sourdough was refreshed regularly during one year.

**Isolation of lactic acid bacteria and yeast**

The counting of LAB and yeast was performed using the pour plate method. Aseptically, 25 g of sourdough was mixed with 225 mL of 0.1 g 100 g⁻¹ of peptone water in BagMixer (model P, Interscience, St.Nom, France). From this dilution were realized subsequent necessary dilutions. The isolation of LAB was realized in MRS agar (Difco, Sparks, USA) with incubation at 3°C under aerobiosis for 48 h and yeasts were grown in PDA Agar (Himedia, Mumbai, India) and tartaric acid 10% under aerobic conditions at 25°C for 72 h. After incubation, isolated LAB colonies were randomly selected and were transferred to tubes containing MRS broth (Difco, Sparks, USA). Morphology was observed by Gram coloration and catalase test. Colonies isolated for yeasts grewed in malt extract broth (Himedia, Mumbai, India) (Apha, 2001). The colonies were subjected to microscopic test to detect the presence of hyphae or pseudo-hyphae.

**Phenotypic characterization of LAB and yeast in sourdough**

The identification of lactic acid bacteria was done by phenotypic analysis using the kit API50CHL (BioMérieux, Marcy-l’Etoile, France) according Saeed et al. (2009) and Lu et al. (2008). The API 50CHL system allows the species identification of LAB according to the biochemical profile of carbohydrate fermentation. Readings were taken after 24 and 48 hours of incubation at 30°C. The identification of yeasts was performed using the kit API 20CAux (BioMérieux, Marcy-l’Etoile, France) according Saeed et al. (2009) and the readings were made after 48 and 72 hours of incubation at 30°C.

**Genotypic characterization of LAB and yeast in sourdough**

Two samples phenotypically characterized as LAB and two samples from yeasts were selected and sent for DNA sequencing at Genotyping Biotecnologia (Botucatu, São Paulo, Brazil). The genomic DNA of the isolates was extracted with 10% Chelex (Biorad, Hercules, CA), according manufacturer’s protocol. The extracted genomic DNA was amplified by the universal 16S primer (16S f AACCGGAAGAACCTAC
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and 16S F C G GT G T C A A G A C C C) set for bacteria and with ITS primer (ITS 4 T C T C C G C T T A T T G A T T A and ITS 5 GGAAGTGAAAAGGCTTAAACAAGG) set for fungi. PCR products were treated with ExoSAP-IT (USB, Ohio, USA) according to the manufacturer’s instructions and directly sequenced with Applied Biosystems® 3500 Genetic Analyzer (Foster City, CA). The nucleotide sequence of the sequencing products was determined by using the Sanger’s chain termination method (Sanger, Níclen, Coulson, 1977). The identity of the isolates was analyzed by using the Blast tool (Basic Local Alignment Search Tools Nucleotide) from the National Center for Biotechnology Information (NCBI).

Statistical analysis

All tests were performed in triplicate and the data expressed as mean ± standard deviation. The genus and species of LAB and yeast isolated were interpreted by the statistical program APIWEB 50CHLv5.1 and APIWEB 20CAuxv 4.0, respectively.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria and yeast

The counting of aerobics LAB at 30°C was 7.52 ± 0.07 log CFU/g and yeasts was 7.62 ± 0.29 log CFU/g. After incubation, 10 isolated LAB colonies were transferred to tubes containing MRS broth. A total of twenty-five colonies were isolated for yeasts and grown in malt extract broth.

Phenotypic characteristics

The results obtained by the isolates tested with API 50CHL were shown in Table I. LAB samples 1 and 2 (Table I) of Lactobacillus paracasei (LAB samples 1 LAB obtained 6.36 ± 0.18 and LAB sample 2 obtained 8.34 ± 0.15 log CFU/g) were selected to proceed this study due the high reliability of identification that was 99.5% and 99.9%, respectively. Lactobacillus paracasei was the dominant species with 60% of identification among the LAB of Brazilian grape sourdough. The species Lactobacillus paracasei (LAB sample 1) achieved 99.5% reliability of identification, while the Lactobacillus paracasei (LAB sample 2) showed 99.9%. It proves that sourdough is more dominated by heterofermentative LAB in agreement with a previous study (De Vuyst, Neysens, 2005). Endo, Futagawa-Endo, Dicks (2011) reported that the carbohydrates have a big impact on the isolation of a variety of LAB in fermented food. It is observed in this study that after one year of sourdough cultivation, Lactobacillus brevis was the dominant species, with 70% of identification among 10 LAB samples analyzed. Scheirlinck et al. (2009) indicated that specific strains of LAB persist in artisanal dough after many years and they circulate in the bakery environment. Furthermore, the air is a potential carrier of LAB in artisanal bakery environments.

Mugula, Naryhus, Sørhaug (2003) identified that starter cultures of LAB (Lactobacillus brevis, Lactobacillus celllobiosus, Lactobacillus fermentum, Lactobacillus plantarum and Pediococcus pentosaceus) and yeasts (Candida pelliculosa, Candida tropicalis, Issatchenkia orientalis and Saccharomyces cerevisiae) were isolated from native togwa.

The result of the fermentation of carbohydrates to yeasts is illustrated in Table II. The yeast samples selected for this study were samples 1 and 2, both with 99.9% reliability of identification. Saccharomyces cerevisiae (yeast sample 1) and Candida pelliculosa

| Samples | Identification | % of reliability of identification | Samples | Identification after 1 year | % of reliability of identification |
|---------|----------------|----------------------------------|---------|-----------------------------|----------------------------------|
| 1       | Lactobacillus paracasei | 99.5                | 1       | Lactobacillus paracasei | 99.8                        |
| 2       | Lactobacillus paracasei | 99.9                | 2       | Lactobacillus brevis       | 93.3                        |
| 3       | Lactobacillus brevis    | 91.9                | 3       | Lactobacillus brevis       | 95.8                        |
| 4       | Lactobacillus paracasei | 95.5                | 4       | Lactobacillus brevis       | 98.3                        |
| 5       | Lactobacillus brevis    | 95.5                | 5       | Lactobacillus paracasei    | 99.1                        |
| 6       | Lactobacillus paracasei | 91.9                | 6       | Lactobacillus brevis       | 91.6                        |
| 7       | Profile dubious         |                     | 7       | Lactobacillus brevis       | 99.8                        |
| 8       | Lactobacillus delbruecki| 95.6                | 8       | Lactobacillus brevis       | 95.9                        |
| 9       | Lactobacillus paracasei | 95.5                | 9       | Lactobacillus brevis       | 99.7                        |
| 10      | Lactobacillus paracasei | 99.1                | 10      | Lactobacillus delbruecki   | 92.5                        |
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samples 2) obtained 7.54 ± 0.18 and 7.34 ± 0.06 log CFU/g, respectively. *Candida pelliculosa* was the dominant species with 48% of identification among yeasts isolated in Brazilian grape sourdough, followed by 28% of identification for *Saccharomyces cerevisiae*. Yeasts *Candida famata* and *sphaerica* showed 4% of identification. After one year of cultivation, it was observed that *Saccharomyces cerevisiae* was the dominant species.

*Saccharomyces cerevisiae* is the most common yeast used in bread making. Yeast cells metabolize fermentable sugars (glucose, fructose, sucrose and maltose) under anaerobic conditions producing carbon dioxide (CO₂) as a residual product, which acts as a leavening agent and enhances dough volume (Chavan, Jana, 2008; Giannou, Kessoglou, Tzia, 2003). Rosenquist and Hansen (2000) reported that *Saccharomyces cerevisiae* was the yeast species isolated only from sourdoughs.

Lu *et al.* (2008) isolated and characterized phenotypically 170 LAB and 96 yeasts in sour *Mifen*, a traditional fermented rice noodle from China. *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were identified as predominant. The microflora of twenty five wheat sourdoughs from the Apulia region, southern Italy, was characterized for Corsetti *et al.* (2001). Edema and Sanni (2008) reported that for sour maize bread, 30% of the isolates were identified as *Lactobacillus sanfranciscensis*, 20% as *Lactobacillus alimentarius*, 14% as *Lactobacillus brevis*, 12% as *Leuconostoc citreum*, 7% as *Lactobacillus plantarum*, 6% as *Lactococcus lactis* subsp. *lactis*, 4% as *Lactobacillus fermentum* and *Lactobacillus acidophilus*, 2% as *Weissella confuse* and 1% as *Lactobacillus delbrueckii* subsp. *delbrueckii*.

Qureshi, Masud, Sammi (2007) isolated and characterized yeast strains on the basis of the capacity to use maltose during bread fermentation. From these

**TABLE II - Yeasts isolated from brazilian grape sourdough identified by API 20CAux gallery kit**

| Samples | Identification | % of reliability of identification | Samples | Identification after 1 year | % of reliability of identification |
|---------|---------------|-----------------------------------|---------|-----------------------------|----------------------------------|
| 1       | *Saccharomyces cerevisiae* | 99.9 | 1 | *Candida pelliculosa* | 93.9 |
| 2       | *Candida pelliculosa* | 99.9 | 2 | *Saccharomyces cerevisiae* | 99.9 |
| 3       | *Saccharomyces cerevisiae* | 99.9 | 3 | *Saccharomyces cerevisiae* | 96.2 |
| 4       | *Saccharomyces cerevisiae* | 96.2 | 4 | *Candida guilliermondii* | 89.9 |
| 5       | *Candida famata* | 96.2 | 5 | - | Profile dubious |
| 6       | *Candida sphaerica* | 98.5 | 6 | *Saccharomyces cerevisiae* | 99.9 |
| 7       | - | Profile dubious | 7 | *Candida sphaerica* | 99.8 |
| 8       | *Candida pelliculosa* | 88.8 | 8 | *Candida sphaerica* | 98.5 |
| 9       | *Saccharomyces cerevisiae* | 96.2 | 9 | - | Profile dubious |
| 10      | - | Profile dubious | 10 | - | Profile dubious |
| 11      | *Candida pelliculosa* | 93.1 | 11 | *Saccharomyces cerevisiae* | 99.2 |
| 12      | - | Profile dubious | 12 | *Saccharomyces cerevisiae* | 99.9 |
| 13      | *Candida pelliculosa* | 99.3 | 13 | *Saccharomyces cerevisiae* | 99.7 |
| 14      | *Saccharomyces cerevisiae* | 99.9 | 14 | *Saccharomyces cerevisiae* | 99.7 |
| 15      | - | Profile dubious | 15 | *Candida guilliermondii* | 84.3 |
| 16      | *Candida pelliculosa* | 93.1 | 16 | *Candida pelliculosa* | 99.8 |
| 17      | *Candida pelliculosa* | 88.8 | 17 | *Cryptococcus albidus* | 99.8 |
| 18      | *Candida pelliculosa* | 99.3 | 18 | *Saccharomyces cerevisiae* | 99.9 |
| 19      | *Candida pelliculosa* | 99.9 | 19 | *Saccharomyces cerevisiae* | 99.9 |
| 20      | *Candida pelliculosa* | 93.1 | 20 | - | Profile dubious |
| 21      | *Saccharomyces cerevisiae* | 99.9 | 21 | *Saccharomyces cerevisiae* | 99.9 |
| 22      | *Candida pelliculosa* | 88.8 | 22 | *Saccharomyces cerevisiae* | 99.7 |
| 23      | *Candida pelliculosa* | 88.8 | 23 | *Saccharomyces cerevisiae* | 99.2 |
| 24      | *Saccharomyces cerevisiae* | 99.9 | 24 | *Saccharomyces cerevisiae* | 99.9 |
| 25      | *Candida pelliculosa* | 99.8 | 25 | *Saccharomyces cerevisiae* | 99.9 |
TABLE III - Genotypic identification of lactic acid bacteria and yeasts isolated from Brazilian grape sourdough

| Samples                                      | Sequence coverage | Percent of identity | Genotypic identity                        |
|----------------------------------------------|-------------------|---------------------|-------------------------------------------|
| Lactobacillus paracasei (LAB 1)              | 93%               | 99%                 | Lactobacillus casei, L. rhamnosus and L. paracasei |
| Lactobacillus paracasei (LAB 2)              | 97%               | 98%                 | Lactobacillus casei, L. rhamnosus and L. paracasei |
| Saccharomyces cerevisiae (yeast 1)           | 93%               | 88%                 | Saccharomyces cerevisiae                   |
| Candida pelliculosa (yeast 2)                | 89%               | 99%                 | Saccharomyces cerevisiae                   |

40 strains, 14 were identified as *S. cerevisiae*, 12 as *S. kluveri*, 4 as *S. exigus* and *S. dairnensis*, 2 as *S. ludwigii*, *S. octosporus* and *S. unisporus*, respectively. Later on, 14 isolates of *S. cerevisiae* were assessed for their maltose utilization capacity for bread production.

**Genotypic identification**

Four representative LAB cultures and yeasts phenotypically characterized in this study were genotypically analyzed by sequencing of 16S rRNA region for LAB and ITS for fungi detection. The sequencing product resulted in a main amplicon of 600 bp for LAB samples and 800 bp for yeasts samples. The result of the genotypic identification is illustrated in Table III.

The sample phenotypically characterized as *Lactobacillus paracasei* (LAB sample 1) showed 93% of sequence coverage with 99% of identity for *Lactobacillus casei*, *L. rhamnosus* and *L. paracasei*. The isolated culture phenotypically identified as *Lactobacillus paracasei* (LAB sample 2) showed 97% of sequence coverage with 98% of identity for *Lactobacillus casei*, *L. rhamnosus* and *L. paracasei*.

The partial sequence results for one of the isolated culture phenotypically characterized as *Saccharomyces cerevisiae* (yeast sample 1) obtained 88% percent of identity for *Saccharomyces cerevisiae* with 93% of sequence coverage. The isolated culture phenotypically identified as *Candida pelliculosa* (yeast sample 2) showed 99% percent of identity for *Saccharomyces cerevisiae* with 89% of sequence coverage.

These sequence results suggested that all LAB samples showed major identity with *Lactobacillus casei*, *L. rhamnosus* and *L. paracasei*. Moreover, the phenotypic characterization suggested that LAB samples were *L. paracasei*.

According to the phenotypic and genotypic results for yeast samples, it was possible to confirm the identification of *Saccharomyces cerevisiae* for yeast sample 1. Therefore, yeast sample 2 showed phenotypic identification as *Candida pelliculosa* and genotypic identification as *Saccharomyces cerevisiae*. Compared with PCR, the phenotypic characterization by carbohydrates fermentation is less sensitive due to natural variations in this process (Barros et al., 2009). According to Nigatu (2000), main differences between genotypic and phenotypic analysis suggested that results from API kit should be complemented with genetic results to give an accurate result.

**CONCLUSION**

This study allowed to isolate and to identify four microorganisms in Brazilian grape sourdough with technological potential for sourdough applications. The phenotypic and genotypic characterization suggested that two samples were *Lactobacillus paracasei* and one sample is *Saccharomyces cerevisiae*. One sample phenotypically identified as *Candida pelliculosa* was genotypic identified as *Saccharomyces cerevisiae*. It suggests that phenotypic results should be confirmed by genotypic analysis.

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