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Trehalose as a cryoprotectant in freeze-dried wheat sourdough production

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ABSTRACT

Sourdough, a mixture of wheat flour and water, fermented by the action of lactic acid bacteria (LAB) and yeasts, presents some technological advantages, such as improvement in dough structure, flavor, aroma, bread texture, and shelf life. Few studies related to methods of preservation of sourdoughs are currently available. This work aimed to test the cryoprotective effect of trehalose on microorganism survival and its effect on freezing, freeze-drying and storage of freeze-dried sourdough, and to molecularly identify predominant bacteria and yeasts. Refined and whole wheat flour were used to prepare the sourdough. On the 14th day of production, varying amounts of trehalose were added (0, 10 and 15%) and the sourdough was freeze-dried. The cryoprotective effect of trehalose was evaluated before and after freezing, after freeze-drying, and after 15, 30 and 45 days of storage. Predominant microorganisms were molecularly identified through amplification and sequencing of rDNA fragments. Addition of trehalose promoted a cryoprotective effect survival of microorganisms, and it was more significant for LAB. *Lactobacillus plantarum, Lactobacillus fermentum, Saccharomyces cerevisiae, Pichia anomala* and *Pediococcus pentosaceus,* were the main species recovered.

1. Introduction

Sourdough is a mixture of wheat flour and water fermented by the action of lactic acid bacteria (LAB) and yeasts present in flour and in the environment (Rinaldi et al., 2015). Sourdough is a specific and stressful ecosystem characterized by low pH, high carbohydrate concentrations, low oxygen and higher cell counts of LAB [$> 10^8$ colony forming units (CFU)/g] compared with yeasts ($< 10^7$ CFU/g) (De Vuyst et al., 2014). Wheat breads made with sourdough show technological advantages compared with breads made with baker's yeast, such as improvement in dough structure (Arendt, Ryan, & Dal Bello, 2007), as well as in flavor, aroma, bread texture and shelf life (Chavan & Chavan, 2011).

Sourdough is advantageous because of its nutritional properties and beneficial effect on health by decreasing or increasing levels of certain compounds (antinutritive factors, phenolics and sterols). It also enhances or retards the bioavailability of nutrients (minerals, vitamin and dietary fibers) (Poutanen, Flander, & Katina, 2009). Sourdough presents artisan characteristics (natural status), traditional value and gastronomic quality (De Vuyst et al., 2014), besides the additive-free image (Katina, Heinio, Autio, & Poutanen, 2006).

Three types of sourdough can be distinguished according to its manufacturing process (Corsetti & Settanni, 2007; De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009). The first type (Type I) represents "spontaneous" sourdough (SS) fermentation processes based on back-slopping, i.e. the repeated cyclic re-inoculation of a new batch of flour and water from a previous dough derived from the so-called mother dough (De Vuyst et al., 2014). Another way of sourdough fermentation (Type II) results from the addition of a starter culture to the flour/water mixture (Gaggiano et al., 2007). Microbial strains such as *Pediococcus pentosaceus* and *Lactobacillus rossiae* are used in the fermentation process, which usually takes one to three days (Gaggiano et al., 2007; Nionelli et al., 2014; Rizzello, Lorusso, Montemurro, & Gobbetti et al., 2016). The third type (Type III) of sourdough fermentation process represents a mixture of Type I and Type II processes (i.e., sourdough initiated with a starter culture followed by traditional back-slopping; De Vuyst et al., 2014).

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Microbial stability of the fermented dough is influenced by several factors. Among the aspects that affect microbial stability, we can highlight those related to environmental contamination and use of different batches of flour during propagation time (Lattanzi, Minervini, & Gobbetti, 2014). Daily back-slopping process is a fastidious procedure that demands time and skilled workforce. Hence, some bakers prefer to preserve the sourdough at low temperatures or in dried form. It is important to observe that besides rheological properties relevant for bread quality, high survival rates during freezing or drying are necessary for the economic production of dried sourdough (Brandt, 2007). An alternative to obtain dried sourdough would be using freeze-drying technology. However, few studies related to this method of sourdough drying are available in current literature.

Freeze-drying is widely used as a long-term preservation technique for bacteria and yeast, where they need to be previously frozen and water is removed by sublimation without passing through liquid phase (Santo, Lima, Torres, Oliveira, & Ponsano, 2013). Low temperatures, especially below freezing point, may cause severe damage to microorganisms due to intracellular ice crystals formation (Momose, Matsumoto, Maruyama, & Yamaoka, 2010). Thus, a cryoprotective agent may be added before the freeze-drying process, because it plays a significant role in microorganism preservation (Morgan, Herman, White, & Vesey, 2006).

Several studies suggest using trehalose as a cryoprotective agent due to high microorganism survival rates after freezing and freeze-drying processes (Bandara, Fraser, Chambers, & Stanley, 2009; Nakamura, Takagi, & Shima, 2009). Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide of glucose, and its most significant function is to protect proteins and lipids included in the membrane structure against different kinds of stress conditions such as heat and freeze-thaw (Yoshiyama et al., 2015). The main advantage of using trehalose compared to other sugars, such as sucrose and lactose, is its water-binding ability, consequently preventing intracellular and extracellular ice crystals formation (Costa, Usall, Teixidó, Garcia, & Viñas, 2000). Also, trehalose is stable, colorless, odor-free, only 45% as sweet as sucrose, and prevents browning of the product during processing (Maillard reaction) (Schiraldi, Di Lernia, De Rosa, & De Rosa, 2002).

Since sourdough is a stressing ecosystem and there is no clear relationship between a typical sourdough and its microbiota, the identification of microorganisms involved in this process is fundamental to verify the relationships between LAB and yeast species (De Vuyst et al., 2014). Molecular DNA-based methods (DNA fingerprinting and sequencing) have become essential for the identification of LAB and yeasts (Lhomme et al., 2016). Compared with phenotyping methods, molecular DNA-based identification methods offer a much higher taxonomic resolution of species up to strain level (De Vuyst et al., 2014).

Several studies (De Vuyst et al., 2009; De Vuyst et al., 2014; Ercolini et al., 2013; Gaggiano et al., 2007) described the microorganisms present in sourdough. However, research involving the production of freeze-dried sourdough (FSS), as well as its microbiota, is uncommon (Meuser, Barber, & Fischer, 1995). Therefore, this work aimed to test the cryoprotective effect of trehalose on microorganism survival in an artisanal FSS. We also identified bacteria and yeasts present in FSS by molecular techniques.

2. Materials and methods

2.1. Material

Commercial refined wheat flour (RWF) was provided by a local supplier (Ipiranga mill, Santa Maria, Brazil) in a 50 kg-batch of Antoniazzi Massa Tipo 1 (06-03-2013); whole wheat flour (WWF) was purchased from Cisbra® (Panambi, Brazil) in a 5 kg-batch of PM1-5 (030105). Flours used for preparing the sourdough were stored in a freezer (−20 °C) in plastic bags and thawed at room temperature before use. Malt extract (Unimalt-WC 15826501) was purchased from Myler (São Paulo, Brazil). Trehalose was provided by Prozyn® (São Paulo, Brazil).

RWF presented the following characteristics: moisture, 12.4% ± 0.1%; protein (N x 5.40), 10.5% ± 0.2%, ash content of dry matter (d.m.) was 0.53%. WWF contained 9.6% ± 0.4% of moisture, 11.9% (d. m.) of protein, and 1.5% of ash content. Farinograph characteristics of RWF were absorption of 59.5% and stability of 8.7 min. Values for dough deformation energy (*W*), representing the energy necessary to inflate the dough bubble to rupture point, was 251×10^{-4} J for RWF, and 199×10^{-4} J for WWF, respectively. Falling number values were 280 s for RWF, and 267 s for WWF. LAB and yeasts were counted in RWF and in WWF before sourdough preparation. For these analysis, results were expressed in CFU per mL. For RWF, values were 2.90 log CFU/mL and 1.63 log CFU/mL, respectively; for WWF, they were 3.41 log CFU/mL and 4.25 log CFU/mL, respectively (data not shown).

2.2. Production and propagation of sourdough

Dough was prepared and sourdough propagated according to traditional protocols (Minervini et al., 2012), based on back-slopping without using starter cultures or baker's yeast (Fig. 1). Dough preparation was made by mixing RWF (50 g) and WWF (50 g) with malt extract (5 g) and tap water (120 mL) for 5 min. Dough was incubated in a microbiological chamber (CIENLAB, CE-210/80, São Paulo, Brazil) at 30 °C for 72 h, and was considered dough prior to fermentation and before becoming sourdough. Each sourdough was propagated every 12 h at 30 °C for 14 days, and the final dough was called mother dough.

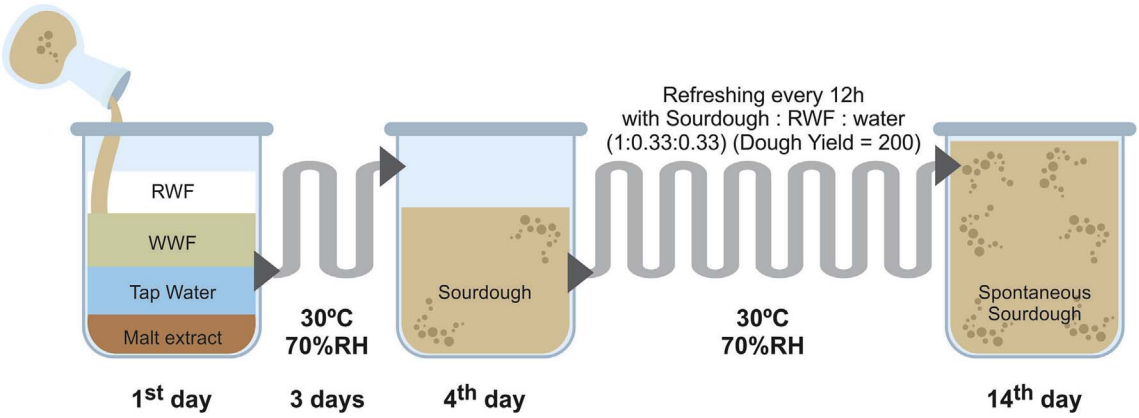


Fig. 1. Production and propagation of a "spontaneous" sourdough from the blend of refined wheat flour (RWF) and whole wheat flour (WWF) during 14 days. Relative humidity (RH).

For refreshment procedure (Ercolini et al., 2013), dough from the day before was used as starter inoculum to ferment the new mixture of RWF (100 g), and sterile tap water (50 mL), having a dough yield of 200 (DY = dough weight x 100/flour weight). Preparation and propagation were repeated four times.

2.3. Determination of pH and total titratable acidity during back-slopping

Dough pH and total titratable acidity (TTA) was determined on 10 g of dough homogenized with 90 mL of deionized water at room temperature, and expressed as g of lactic acid/g of dough (Gamel, Abdelaal, & Tosh, 2015). pH measurement was done using a pH meter (Digimed®, DM-22, SP, Brazil), and TTA was determined by standard method 02–31.01 (AACC, 2010) using 0.1 M NaOH and phenolphthalein as indicator (pH 8.3). These analyses were carried out during 14 days of back-slopping.

2.4. LAB and yeast cell counting

LAB and yeast cells were counted in the sourdough produced during back-slopping on days 1, 4, 6, 8, 10 and 14; samples were taken prior to each back-slopping step. Ten grams of sourdough were homogenized with 90 mL of sterile peptone water (1% [wt/vol] of peptone and 0.9% [wt/vol] of NaCl solution). LAB were counted using MRS not supplemented (Oxoid, Basingstoke, Hampshire, United Kingdom) through pour plate method (Rizzello, Lorusso, Montemurro, & Gobbetti, 2016). Plates were incubated at 30 °C for 48 h, under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid). Yeasts were counted on acidified (pH 3.5; 10% tartaric acid) Potato Dextrose agar (PDA) (Difco, Detroit, Mich.) using spread plate technique (Coda et al., 2011) at 25 °C for 24–72 h.

2.5. Freeze-drying of sourdough

On the 14th day of production, sourdough was divided among three containers with 600 g per container (Fig. 2). In each container, a different concentration of trehalose was added: 0% of trehalose (control - SS0), 10% (60 g) of trehalose (SST10) and 15% (90 g) of trehalose (SST15). Thus, treatments were divided into 12 individual fractions, containing 50 g each. Freezing was performed (at −80 °C) using an ultra-low temperature freezer (Thermo Scientific, FormaTM 900 Series, USA) for 24 h.

After 24 h of freezing, cultures were transferred to a freeze-dryer (Terroni, LS 3000, São Paulo, Brazil). Freeze-drying was carried out for 60 h and was performed at −37 °C, at chamber pressure of 40 Pascal (Pa). Freeze dried sourdoughs samples were ground in a basic analytical mill (Deleo, EDB-5, Porto Alegre, Brazil) and stored at room temperature. Samples were stored in metallic bags (8 cm × 12.5 cm) (Tradbor, 80TZ, São Paulo, Brazil) in a dry, odor-free and ventilated room for 45 days.

2.5.1. Evaluation of the cryoprotective effect of trehalose

To evaluate the cryoprotective effect of trehalose on samples of sourdough, total viable counts of LAB and yeast were measured according to the item §2.4. Analysis were realized before and after freezing, after freeze-drying and after 15, 30 and 45 days of storage (Bosnea, Moschakis, & Biliaderis, 2014) on samples of sourdough with or without trehalose (SS0; SST10; SST15).

2.6. Molecular analysis

2.6.1. Bacteria and yeast isolation

Bacteria and yeast selection was performed for samples of RWF, WWF, freeze-dried sourdough without trehalose (SS0) and freeze-dried sourdough with 10% trehalose (SST10). Isolation and identification analysis were done in duplicate. Sourdoughs were freeze-dried on the 14th day of production.

Bacteria counts were performed on samples of RWF, WWF, and sourdough (SS0 and SST10), by diluting 1 g of each sample in 10 mL of 0.85% saline solution, in a sterile flow hood, performing serial dilutions up to 10^{-8}. From each dilution, 100 µL were inoculated in duplicate in culture media M17, MRS and PDA (Merck, Darmstadt, Germany). Cultures were incubated for 24–48 h in an anaerobic chamber, except those in PDA medium, at 37 °C. After that, colonies of each morphological type obtained were isolated in Petri dishes containing M17, MRS or PDA, according to their original medium of preference for growth.

For yeast count on samples of RWF, WWF, and sourdough (SS0 and SST10), aliquots of 0.1 mL from serial dilutions were spread in duplicate on acidified YM agar medium (1% glucose, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 2% agar, 400 mg/L of chloramphenicol, pH 4.0) or acidified YEPG agar medium (0.5% yeast extract, 2% glucose, 1% peptone, 2% agar, 400 mg/L of chloramphenicol, pH 4.0). After incubation at 22–25 °C for 3–5 days, yeast colonies were counted and results were expressed as CFU/g of flour or freeze-dried sourdough sample. Representative colonies of each morphological type obtained were isolated and purified in Petri dishes containing YEPG medium (Landell, Hartfelder, & Valente, 2006). Strains were maintained in GYP medium (0.5% glucose, 2% malt extract, 0.5% yeast extract, 0.2% monobasic sodium phosphate, 2% agar), slants covered

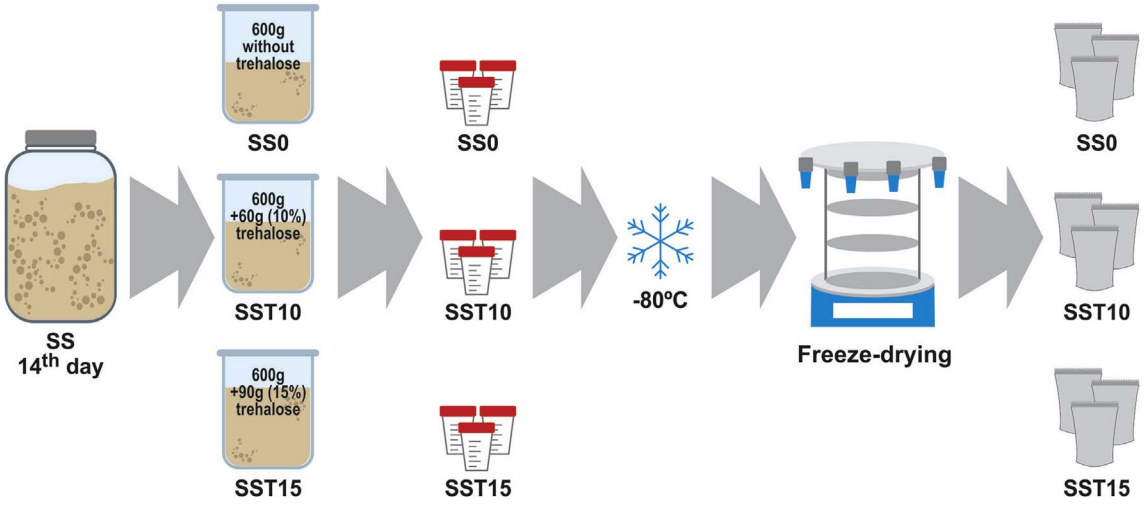


Fig. 2. Process of freeze-drying of "spontaneous" sourdough (SS). SS0: "spontaneous" sourdough without trehalose; SST10: "spontaneous" sourdough with 10% trehalose; SST15: "spontaneous" sourdough with 15% trehalose.

with a layer of sterile mineral oil, and kept in the refrigerator.

2.6.2. Bacteria and yeast DNA extraction and sequencing

Total DNA from isolated colonies of bacteria was extracted using a traditional phenol-chloroform method (Sambrook & Russell, 2006). Bacteria species were identified through amplification and sequencing of a fragment of the 16S rDNA gene, using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) (Lane, 1991). Samples were incubated at 94 °C for 4 min, followed by 30 cycles consisting of 1 min at 94 °C, 30 s at 55 °C, and 2 min at 72 °C, and a final extension step at 72 °C for 10 min.

Total genomic DNA from yeast colonies was extracted as described by Osorio-Cadavid, Ramirez, Lopez, and Mambuscay (2009), with some modifications (Mattanna et al., 2014). Sequencing of D1/D2 domain of the large subunit (26S) ribosomal DNA was performed according to O'Donnell (1993), using NL-1 (5′-GCATATCAATAAGCGGAGGAAAGG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers. The PCR mix contained DNA Taq polymerase (1U) (Invitrogen), 1× Buffer, $MgCl_2$ (3 mM), primers (70 μM), dNTPs (10 μM) and DNA (30 ng). Amplification conditions were: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 15 s, 55 °C for 45 s, extension at 72 °C for 90 s, and final extension at 72 °C for 6 min.

2.6.3. Molecular identification

Sequences were obtained by automated sequencer ABI-PRISM 3100 Genetic Analyzer (Life Technologies Corp., USA), and protocols established by Ludwig Biotech Brazil company (Alvorada, RS, Brazil) were used. Obtained sequences were aligned using CLUSTAL-X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and edited in BioEdit 7.0.5.3 (Hall, 1999). Sequences were assembled and compared with sequences reported in GenBank (bacteria and yeasts sequences) and in Mycobank and Yeast IP (yeast sequences), using basic local alignment search tool (BLAST) algorithm for identification. After identification, lineages were deposited in the Microorganism Collection Nucleus of the Adolfo Lutz Institute under the access code IAL 4533, IAL 4539–4543.

2.7. Statistical analysis

Results were initially submitted to Levene's test to check for homogeneity of variances, followed by one-way ANOVA and parametric Tukey's HSD test. The software SASr Studio 3.5–2016 (SAS Software, Cary, NC, USA) was used, at a 5% significance level, to check if there were significant differences between treatments.

3. Results and discussion

Sourdough microbiota can develop spontaneously, originating from flour and/or other ingredients (De Vuyst et al., 2014). WWF showed a slightly higher level of microbiological counts for yeasts (4.25 log CFU/g) than RWF (1.63 log CFU/g). However, microbiological counts for LAB did not differ by more than 1 log cycle. Values for LAB were 3.41 log CFU/g and 2.90 log CFU/g for WWF and RWF, respectively.

The predominance of species strongly depends on condition of dough propagation, type of flour or environment of each bakery. It was observed that in each sample of flour or freeze-dried sourdough, an association of LAB and yeast developed, which was characteristic of a specific type of substrate with regard to the number of species as well as to flour composition (Hammes et al., 2005). Besides providing nutrients, WWF is a non-sterile material, and these microorganisms can become dominant in sourdough due to daily and continuous back-slopping (De Vuyst et al., 2009).

3.1. pH and TTA during back-slopping

During the preparation of sourdough (Table 1), pH values decreased ($P < 0.05$) on the first four days (from 5.9 to 3.7), while TTA increased during the 14 days of manufacturing (from 0.27 to 1.25). This pH decrease and hence acidity increase during the first four days is the result of lactic acid production by LAB (Minervini et al., 2012; Vrancken, De Vuyst, Rimaux, Allemeersch, & Weckx, 2011). This fact helps to inhibit growth of undesired microorganisms and contributes to development of sensorial characteristics of the final product (Leroy & De Vuyst, 2004).

Table 1
pH, total titratable acidity (TTA) and counts (Log CFU/mL) of LAB and Yeasts on "spontaneous" sourdough (SS) during the production period (14 days) at 30 °C.

Production day	pH	TTA	LAB	Yeasts
			Log CFU/g	
1	5.91 ± 0.13^{a}	0.27 ± 0.03^{d}	3.11 ± 0.24^{c}	2.63 ± 0.07^{c}
4	3.74 ± 0.11^{b}	0.87 ± 0.10^{c}	8.58 ± 0.41^{b}	6.54 ± 0.44^{b}
6	3.58 ± 0.14^{c}	1.25 ± 0.13^{a}	8.47 ± 0.40^{b}	6.43 ± 0.42^{b}
8	3.63 ± 0.09^{bc}	1.18 ± 0.09^{ab}	8.69 ± 0.13^{ab}	7.27 ± 0.19^{a}
10	3.58 ± 0.08^{c}	1.12 ± 0.04^{b}	8.71 ± 0.12^{ab}	7.31 ± 0.16^{a}
14	3.64 ± 0.14^{bc}	1.23 ± 0.06^{a}	9.02 ± 0.10^{a}	7.44 ± 0.31^{a}

TTA: total titratable acidity (g of lactic acid/100 g dough). LAB: acid lactic bacteria. SS: "spontaneous" sourdough. Mean and standard deviation of four determinations (n = 108 to pH and TTA; n = 33 to LAB; n = 32 to Yeast). Values in the same column with different superscript are significantly ($P \leq 0.05$) different.

Sourdough used for manufacturing traditional Italian breads (Minervini et al., 2012) also showed pH values between 3.70 and 4.28. pH changes observed in our study agree with Vogelmann and Hertel (2011), who observed a sharp drop in pH after 12 h of sourdough fermentation (5.5–3.1), and with Hamad, Dieng, Ehrmann, and Vogel (1997), who reported a drop in pH (4.28–3.35) after 42 h of sourdough fermentation.

In this study, the blend of RWF and WWF in the same proportion resulted in 1.01 g/100 g ash content and presented an adequate acidification. Ash measures the amount of bran present in wheat flour, and according to Chavan and Chavan (2011), this fraction contains more minerals and micronutrients important for the growth of LAB, and consequently affects the acidification properties. Besides, it influences the buffering capacity of the sourdough, reaching a higher TTA.

Mariotti et al. (2014) verified that the higher acid content of WWF is probably attributable to the higher ash content of WWF than RWF, thus causing the higher buffering capacity of WWF.

Katina et al. (2006) studied the effect of ash content (0.6–1.8 g/100 g) on sensory properties of sourdough bread and observed that the ash content of flour is the most important parameter influencing the flavor of the bread. Sourdough bread with higher ash content resulted in enhanced aftertaste, overall intensity, pungent and roasted flavor.

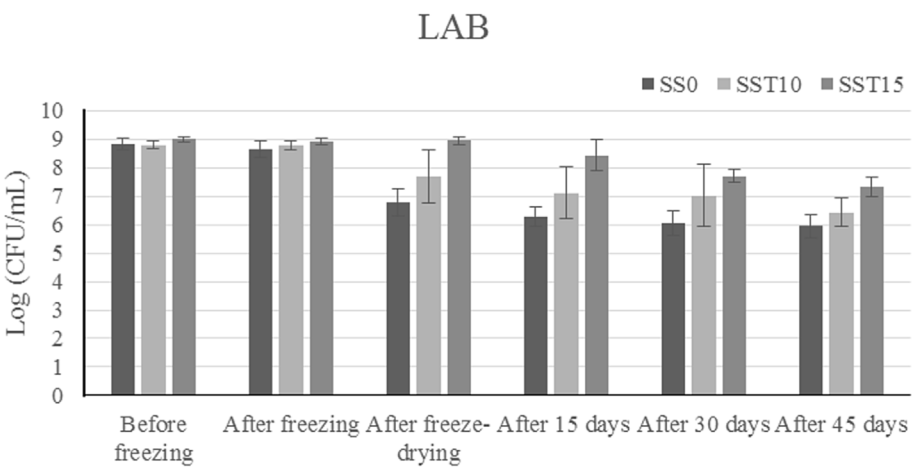
3.2. LAB and yeast cell counting

LAB and yeast cell counting during manufacturing of sourdough is shown in Table 1.

There was a marked LAB and yeast growth ($P < 0.05$) in the first four days of SS production. On the 14th day, LAB and yeast counts were 9.02 log CFU/mL and 7.44 log CFU/mL, respectively. LAB and yeast counts of sourdough elaborated with RWF and WWF mix were similar to the results of Minervini et al. (2012), who found 9.01 and 7.30 log CFU/mL for LAB and yeast counts, respectively. According to Belz et al. (2012), a sourdough is stable and ready to be used when it presents microbiological counts of about 2×10^{8} UFC/g, pH ≤ 3.90 and TTA ≥ 14 mL as quality parameters.

3.3. Effect of trehalose

The cryoprotective effect of trehalose and survival of LAB and yeast are shown in Fig. 3. According to microbiological counts of FSS with (SST10 and SST15) and without trehalose (SS0), it was possible to



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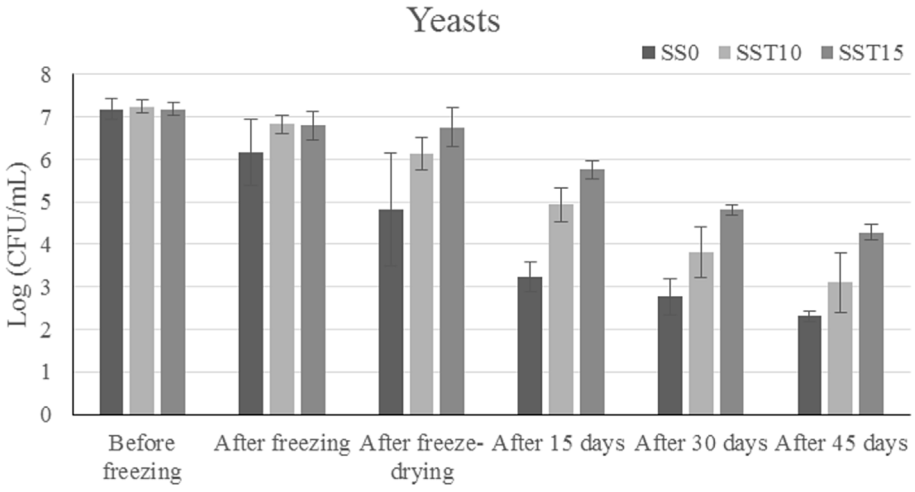

b

Fig. 3. Survival of LAB (a) and yeasts (b) measured at total viable counts before freezing, after freezing, after freeze-drying, and after 15, 30 and 45 days of storage and expressed as the logarithm of CFU/mL. * Experiment was carried out in triplicate and repeated three times. SS0: "spontaneous" sourdough without trehalose; SST10: "spontaneous" sourdough with 10% trehalose; SST15: "spontaneous" sourdough with 15% trehalose.

verify that trehalose effect on LAB and yeast survival after the freeze-drying process was directly proportional to the quantity of trehalose added. Survival rates for SS0, SST10 and SST15 were around 67%, 73% and 81%, respectively. The same behavior was described by Leslie, Israeli, Lighthart, Crowe, and Crowe (1995) where the microorganisms *Escherichia coli* DH5a and *Bacillus thuringiensis* HD-1 showed an increased tolerance to freeze-drying when dried in the presence of disaccharides trehalose and sucrose.

Reductions in microbiological counts were greater in yeast, indicating that the cryoprotective effect was more significant for LAB. Whereas reduction in LAB after 45 days of storing was around 3.2, 2.3 and 1.5 log CFU/mL for SS0, SST10 and SST15, respectively. In yeast, the observed reduction was 5.0, 4.0 and 3.0 log CFU/mL. The cryoprotective effect of trehalose is due to its ability to inhibit intracellular ice crystals formation (Nakamura et al., 2009). Streeter (2003) showed that trehalose added into a culture medium was able to increase survival of *Bradyrhizobium japonicum* after 24 h of desiccation.

Bread flavor has a major influence of raw material and ingredient employed, enzymatic reactions occurring during dough fermentation by yeast and/or LAB, and thermal reactions induced during baking, mainly through caramelization and Maillard reactions (Cho & Peterson, 2010).

According to literature (Schiraldi, Di Lernia, & De Rosa, 2002), trehalose (α-D-glucopyranosyl α-D-glucopyranoside) presents no significant impact on bread aromatic profile, because this sugar is among the most chemically unreactive sugars. It is a stable, colorless, odor-free, and non-reducing disaccharide that is widespread in nature. Trehalose is only 45% as sweet as sucrose, and unlike other disaccharides, it does not engage in chemical reactions with amino acids or proteins, thus preventing browning of the product during processing.

A previous sensorial test related to this study was carried out to evaluate technological performance and sensorial pattern of breads containing lyophilized sourdough with zero (control), 10 and 15% of trehalose (Stefanello, Machado, Menezes, & Fries, 2017). As expected in the sensorial preference ordering test, results showed no significant difference among trials, demonstrating that the addition of trehalose had no impact on flavor and aroma intensity of breads produced.

3.4. Molecular identification

Species of LAB and yeast isolated and identified in the freeze-dried sourdough (SS0; SST10) are listed in Table 2. Bacteria species were identified using a fragment of the 16S rDNA gene occurring in sourdough and flour samples. Seven different species of bacteria were identified in flour samples (RWF and WWF) and freeze-dried sourdough (SS0 and SST10), three of them being LAB (*Lactobacillus fermentum, Lactobacillus plantarum* and *P. pentosaceus*). Among the three different isolates found in freeze-dried sourdough, *L. plantarum* was found only in SST10, and only *P. pentosaceus* was also found in WWF.

Table 2
Species of bacteria and yeasts identified in refined wheat flour (RWF), wholewheat flour (WWF), "spontaneous" sourdough without trehalose (SS0) and "spontaneous" sourdough with 10% of trehalose (SST0) through the sequencing of a fragment of the gene 16S rDNA and of the D1/D2 domain of the large subunit (LSU) rDNA region for bacteria and yeasts, respectively.

Isolated number	Source	Identification of specie	Medium for isolation	Fragment size (bp)	Sequence Identity (%)
Bacteria					
1	RWF	*Streptomyces* sp.	PDA	1332	100%
2	RWF	*Enterobacter cloacae*	PDA	1296	99%
3	RWF	*Pseudomonas* sp.	PDA	1215	99%
4	WWF	*Enterobacter* sp.	MRS	945	100%
5	WWF	*Enterobacter* sp.	M17	932	87%
6	WWF	*Enterobacter cloacae*	M17	1364	99%
7	WWF	*Escherichia coli*	M17	981	91%
8	WWF	*Pediococcus pentosaceus*	MRS	1196	99%
9	WWF	*Pediococcus pentosaceus*	MRS	1041	100%
10	SS0	*Pediococcus pentosaceus*	M17	1344	100%
11	SS0	*Lactobacillus fermentum*	M17	1383	100%
12	SST10	*Lactobacillus fermentum*	M17	1346	100%
13	SST10	*Pediococcus pentosaceus*	M17	778	100%
14	SST10	*Lactobacillus plantarum*	MRS	911	100%
Yeasts					
15	RWF	*Saccharomyces cerevisiae*	YM/YEPG	602	99%
16	RWF	*Saccharomyces cerevisiae*	YM/YEPG	556	99%
17	RWF	*Candida glabrata*	YM/YEPG	508	99%
18	WWF	*Pichia anomala*	YM/YEPG	515	99%
19	WWF	*Pichia anomala*	YM/YEPG	508	98%
20	WWF	*Saccharomyces cerevisiae*	YM/YEPG	585	99%
21	WWF	*Saccharomyces cerevisiae*	YM/YEPG	536	99%
22	SS0	*Candida glabrata*	PDA	511	99%
23	SS0	*Meyerozyma guilliermondii*	PDA	540	82%
24	SS0	*Saccharomyces cerevisiae*	YM/YEPG	509	99%
25	SS0	*Rhodotorula mucilaginosa*	YM/YEPG	529	99%
26	SS0	*Saccharomyces cerevisiae*	YM/YEPG	528	99%
27	SS0	*Pichia anomala*	YM/YEPG	597	99%
28	SS0	*Saccharomyces cerevisiae*	YM/YEPG	523	98%
29	SST10	*Saccharomyces cerevisiae*	YM/YEPG	526	99%
30	SST10	*Candida glabrata*	YM/YEPG	516	98%
31	SST10	*Pichia anomala*	YM/YEPG	604	99%
32	SST10	*Saccharomyces cerevisiae*	YM/YEPG	546	99%

We used non-supplemented MRS for LAB selection, and presumably because of this, some microorganisms were not isolated and identified due to the inability of certain LAB to grow in these media. *L. fermentum* and *L. plantarum* are highly adapted to hostile and nutrient-poor environments (Vogelmann & Hertel, 2011). These acid-tolerant species of LAB were dominant in freeze-dried sourdough as well as in most Italian sourdough (Minervini et al., 2012), Altamura sourdough (Perricone, Bevilacqua, Corbo, & Sinigaglia, 2014) and organic French sourdough (Lhomme et al., 2016). In another study (Lhomme et al., 2016), six different LAB species were identified as dominant (*Lactobacillus sanfranciscensis, L. plantarum, L. kimchi, L. sakei, L. hammesii* and *L. pentosus*) using the sequencing of the 16S rRNA gene of 520 isolates from organic French sourdough.

As the preparation process of sourdough is affected by physical-chemical parameters such as pH and acidity (Minervini et al., 2012), heterofermentative LAB play a significant role in sourdough microbiological stability (Vrancken et al., 2011). *Saccharomyces cerevisiae* is not so much tolerant at lower pH. This may be another reason why this species of yeast did not dominate spontaneous fermentations in laboratory, since it could not overcome more acid tolerant strains of *Pichia anomala* (also known as *Hansenula anomala* or *Wickerhamomyces anomalus*) (Vrancken et al., 2010).

The greatest ability of yeast growth occurs when they are associated with homofermentative LAB compared to heterofermentative LAB (Gobbetti, Corsetti, & Rossi, 1995; Hansen, Lund, & Lewis, 1989). The result found by Iacumin et al. (2009) confirmed a previous study of Hansen et al. (1989), which demonstrated that in samples where *S. cerevisiae* was present, *L. plantarum* showed a high percentage of isolation. Heterofermentative LAB utilize lactic acid and glucose as substrates to produce acetic acid (Mari, Schmidt, Nussio, Hallada, & Kung, 2009), which are effective for enterobacteria and yeast control (Zoppolatto, Daniel, & Nussio, 2009).

Yeast species were identified by sequencing the D1/D2 domain of the large subunit (26S) ribosomal DNA, and results of the identification are exhibited in Table 2. Four different species of yeast were identified among the 16 isolates found in sourdough and flour samples. Nine were *S. cerevisiae,* four were *P. anomala*, two were *Candida glabrata* and one was *Rhodotorula mucilaginosa*. Interestingly, only *R. mucilaginosa* was found in SS0, while the other species were found in RWF and WWF. Utilization of the divergence region D1-D2 within the large ribosomal RNA subunit, which is part of the rDNA gene complex, was suggested as a nuclear marker for species identification (Sonnenberg, Nolte, & Tautz, 2007).

Regarding yeast presence in sourdough manufactured by traditional procedures in Italy, Gobbetti, Corsetti, and Rossi (1994) isolated yeast microbiota from Central Italy, mainly composed by *S. cerevisiae* (66%), *Candida krusei* (17%), *Saccharomyces exiguus* (16%) and *H. anomala* (1%). Presence of *S. cerevisiae* and *H. anomala* was also confirmed by Rossi (1996) in sourdough from Umbria region.

An investigation of microbial composition of 21 artisan sourdough from 11 different Belgian bakeries yielded 127 yeast isolates (Vrancken et al., 2010). Dominant species in the bakery sourdough were *S. cerevisiae* and *P. anomala*, while dominant species in the laboratory sourdough fermentations were *P. anomala* and *C. glabrata,* and there was an occasional detection of *S. cerevisiae* (in only three samples).

The ability of all *P. anomala* and *S. cerevisiae* isolates to strongly

assimilate maltose and sucrose, the major carbohydrates of flour, might be considered as an advantage of these species, leading to their dominance in laboratory sourdough fermentations and a frequent occurrence in bakery sourdough fermentations (Vrancken et al., 2010).

Organic French sourdough preserve special yeast communities in which *Kazachstania bulderi* (48% of the isolates) and *Candida humilis* (24%) are common, while *S. cerevisiae* (less than 0.2%) is rare (Lhomme et al., 2016). Relevant isolates from Altamura sourdough were identified as *S. cerevisiae* and *C. humilis* (Perricone et al., 2014). Different combinations of strains from four yeast species (*Kazachstania unispora*, *S. cerevisiae, Candida krusei* and *C. glabrata*) were detected in rye sourdough propagated at 30 °C based on 26S rRNA partial gene sequencing (Bessmeltseva, Viiard, Simm, Paalme, & Sarand, 2014).

In "spontaneously" fermented mass, fermentation is caused mainly by "wild" flour microbiota, which is well adapted to the ecosystem and evolves during fermentation. Strains that develop spontaneously are variable according to geographic region, because LAB and yeast species depend on ecological factors (De Vuyst & Neysens, 2005).

Ecological factors are also important for stability and competitiveness of microbial associations between LAB and yeast in sourdough (Vogelmann & Hertel, 2011). However, the competition between these two types of microorganisms is not specific, as recently shown for some strains of *L. sanfranciscensis* and *L. plantarum* (Minervini, Pinto, Di Cagno, De Angelis, & Gobbetti, 2011; Siragusa et al., 2009) in sourdough made with wheat flour.

Presence of *P. anomala* can have an important role in stability and improvement of shelf life of breads. Among 60 different species of yeast, *P. anomala* was the species that showed the greatest capacity to inhibit growth of *Penicillium roqueforti,* one of the main spoilage microorganisms in bakery products (Druvefors, Passoth, & Schnurer, 2005). The use of *P. anomala* as a starter for fermentation was studied by Coda et al. (2011) to extend shelf life of baked goods while improving flavor and structure.

4. Conclusion

The artisanal sourdough produced had its pH stabilized after four days of preparation, while TTA increased during the 14 days of sourdough production. Sourdough was possibly mature and ready to use or freeze-dry from the sixth day, as means for LAB and yeast did not differ significantly after this period.

Addition of 10% and 15% trehalose to sourdough prior to freeze-drying had a cryoprotective effect on survival of microorganisms, and such an effect was directly proportional to the added concentration of trehalose. Cryoprotective effect was more significant for LAB than for yeast.

Yeast species *S. cerevisiae* and*P. anomala*, along with LAB *P. pentosaceus*, *L. plantarum* and *L. fermentum* were identified as the main species present in freeze-dried sourdough, using molecular techniques. Studies of the application of sourdough in bread and panettone are undergoing in our laboratory.

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