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Description of the microflora of sourdoughs by culture-dependent and culture-independent methods

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ABSTRACT

Four types of sourdoughs (L, C, B, Q) from artisanal bakeries in Northern Italy were studied using culture-dependent and culture-independent methods. In all samples, the yeast numbers ranged from 160 to 10^7 cfu/g, and the numbers of lactic acid bacteria (LAB) ranged from 10^3 to 10^9 cfu/g. The isolated LAB were sequenced, and a similarity was noted between two samples (C, Q), both in terms of the species that were present and in terms of the percentage of isolates. In these two samples, *Lactobacillus plantarum* accounted for 73% and 89% of the bacteria, and *Lactobacillus brevis* represented 27% and 11%. In the third sample (B), however, the dominant LAB isolate was *Lb. brevis* (73%), while *Lb. plantarum* accounted for only 27%. The fourth sourdough (L) was completely different from the others. In this sample, the most prominent isolate was *Weisella cibaria* (56%), followed by *Lb. plantarum* (36%) and *Pediococcus pentosaceus* (8%). In three out of four samples (L, C and Q), all of the yeasts isolated were identified as *Saccharomyces cerevisiae*, yet only *Candida humilis* (90%) and *Candida milleri* (10%) were isolated in the fourth sample (B). The microbial ecology of the sourdoughs was also examined with direct methods. The results obtained by culture-independent methods and DGGE analysis underline a partial correspondence between the DNA and RNA analysis. These results demonstrate the importance of using a combined analytical approach to explore the microbial communities of sourdoughs.

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1. Introduction

Sourdough can be defined as "dough whose microorganisms originate from sourdough or a sourdough starter and are metabolically active or can be reactivated, upon addition of flour and water they continue to produce acid" (Anonymous, 1994). Sourdoughs have been classified into types I, II and III based on the kind of technology used in their production (Böcker et al., 1995; Hammes and Gänzle, 1998; Vogel et al., 1996; De Vuyst and Neysens, 2005; Stolz et al., 1995).

Sourdoughs are very complex biological ecosystems, where LAB are the dominant organisms, and, in many cases, they co-exist with yeasts, which are also present in elevated numbers (Vogel et al., 1999). Microbiological studies have revealed that more than 50 species of LAB, mostly species of the genus *Lactobacillus*, and more than 20 species of yeasts, especially species of the genera *Saccharomyces* and *Candida*, occur in this ecological niche (De Vuyst et al., 2002; De Vuyst and Vancanneyt, 2007; De Vuyst and Neysens, 2005; Di Cagno et al., 2007; Scheirlinck et al., 2007a,b; Van der Meulen et al., 2007; Corsetti et al., 2007; Ehrmann et al., 2007; Randazzo et al., 2005; Ferchichi et al., 2007). It is generally considered that, in sourdoughs, the ratio of LAB to yeast should be 100:1 for optimal activity (Gobbetti et al., 1994b).

These microorganisms usually originate from flour, dough ingredients, or the environment (De Vuyst and Vancanneyt, 2007). Whereas homofermentative LAB play an important role in the majority of fermented foods, heterofermentative LAB are dominant in sourdough, especially when traditionally prepared (Corsetti et al., 2003, 2001; De Vuyst and Vancanneyt, 2007; Kline and Sugihara, 1971; Meroth et al., 2003; Rocha and Malcata, 1999). The metabolic traits of sourdough LAB highlight their adaptation to the sourdough environment (Vrancken et al., 2008; Hassan and Bullerman, 2008; Bullerman et al., 2006; Stolz, 1999; Stolz et al., 1995, 1996; De Angelis et al., 2001; Gänzle and Vogel, 2002; Gobbetti, 1998; Hammes and Gänzle, 1998; Messens and De Vuyst, 2002).

Concerning yeast strains in sourdoughs manufactured by traditional procedures, Gobbetti et al. (1994a) isolated, in sourdoughs from Central Italy, yeast microflora characterized mostly by

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Saccharomyces cerevisiae (66%), *Candida krusei* (17%), *Saccharomyces exiguus* (16%) and *Hansenula anomala* (1%). The dominant presence of *S. cerevisiae* was also confirmed by Rossi (1996), who found *S. exiguus*, *Candida krusei*, *Pichia norvegensis* and *H. anomala* in sourdoughs from the Umbria region. *Saccharomyces cerevisiae*, until a short time ago, was considered a contaminant from other products because it is frequently used in the baking industry. In contrast, molecular analyses have demonstrated that strains in sourdough have a high intraspecific variability ratio; this means that these strains are not derived from compressed yeast but are autochthonous strains (Antoniazzi, 2005). Results obtained from the phenotypic and molecular studies by Gullo et al. (2002) suggest that more than 95% belong to the species *Candida humilis* in Italian durum wheat bran sourdoughs.

In recent years, the use of molecular methods for the detection, identification and characterization of microorganisms in foods has attracted a lot of attention, and these methods are currently considered an indispensable tool that permits the accurate description of the microbial ecology of foods (De Vuyst and Vancanneyt, 2007). Several studies have employed molecular techniques (mainly species-specific PCR, RAPD, rep-PCR, multiplex PCR and PCR–DGGE, AFLP) for the identification of isolates and for their characterization at the subspecies level (Ferchichi et al., 2008; Di Cagno et al., 2007; Scheirlinck et al., 2007a,b; Corsetti et al., 2007; Ehrmann et al., 2007; De Angelis et al., 2007; Kitahara et al., 2005; Catzeddu et al., 2006; Ehrmann and Vogel, 2005; Meroth et al., 2003; Müller et al., 2000, 2001; Randazzo et al., 2005; Vogel et al., 1999). In particular, DGGE and TTGE has been widely used to study the ecology of fermented foods, such as sourdoughs (Scheirlinck et al., 2008; Garofalo et al., 2008; Van der Meulen et al., 2007; Ferchichi et al., 2007; Randazzo et al., 2005; ben Omar and Ampe, 2000; Cocolin et al., 2000, 2001, 2004; Mills et al., 2002; Randazzo et al., 2002; van Beek and Priest, 2002) and to profile pathogens directly in food samples (Cocolin et al., 2002), and its use in food microbiology was recently reviewed (Ercolini, 2004).

In this study, we applied culture-dependent and -independent methods at both the DNA and RNA levels to study the ecology of sourdoughs produced in four different plants in the north of Italy. Sourdoughs were collected at the end of the production process, and we performed traditional microbiological analysis to identify the main microbial groups present. Representative LAB and yeast strains were randomly selected and isolated, and they were identified through molecular methods. At the same time, the total DNA and RNA were directly extracted from the sourdoughs and PCR–DGGE was performed using universal primers for bacteria and yeast. This allowed a clear view of the ecology of the LAB and yeast present, both inactive and active. Finally, the analysis allowed us to complement previously performed studies on the microbial ecology of sourdoughs.

2. Materials and methods

2.1. Sourdough samples

Four local plants in Northern Italy were selected to carry out the study. They are called plants Q, C, L and B. For the production of sourdoughs, traditional techniques were employed without the use of starter cultures. Samples were called with the name of the plant where they came from. Sourdoughs products in plants Q, C and B were classified as type I, whereas sample L was classified as type III, because it was a dry sourdough. Durum wheat flour, used to produce all the sourdoughs tested, was kneaded with warm water and the doughs were incubated for 16 h (plants Q and B) and 14 h (plants C and L) at 22 °C. The first dough was again mixed with flour, water and incubated for 16 h at the same temperature (first refreshment). For each plant four samples were collected at the end of the production process (before baking) and subjected to chemical and microbiological analysis.

2.2. pH measurements

Potentiometric measurements of pH were carried out with a pin electrode of a pH meter (Radiometer Copenhagen pH M82, Cecchinato, Italy). Three independent measurements were performed on each sample and means were calculated.

2.3. Total acidity measurements

Ten grams of samples were diluted in 90 ml of distilled water. The solution was titrated with NaOH 0.1 N at pH 8.5, under shaking. The total acidity was expressed as NaOH 0.1 N volume (ml) used. Three independent measurements were performed on each sample and means were calculated.

2.4. Microbiological analysis

Samples were analyzed by traditional microbiological methods, to determine the main microbial population and the hygienic quality of the sourdough. One batch for each plant was probed and three sourdough samples at each sampling point were used for microbiological analysis. Twenty-five grams of each sample were transferred into a sterile stomacher bag, 225 ml of saline-peptone water (8 g l^{-1} NaCl, 1 g l^{-1} bacteriological peptone, Oxoid, Milan, Italy) were added and mixed for 1.5 min in a Stomacher machine (PBI, Milan, Italy). Further decimal dilutions were made in the same solution and the following microbiological analyses were performed in duplicate agar plates: i) total aerobic bacterial count on Gelisate agar (Oxoid, Milan, Italy) incubated for 48–72 h at 30 °C; ii) mesophilic and thermophilic lactobacilli on double layer mMRS agar (Oxoid, Milan, Italy) modified by adding 1% maltose and 5% FYE (Corsetti et al., 2001), incubated at 30 °C and 42 °C respectively, for 48 h and on double layer SDB (Kline and Sugihara, 1971) incubated at 30 °C for 48 h; iii) lactococci on double layer M17 agar (Oxoid, Milan, Italy), incubated at 30 °C for 48 h; iv) faecal enterococci on Kanamycin Aesculin azide agar (KA, Oxoid, Milan, Italy) incubated at 42 °C for 24 h; v) total enterobacteria on Violet Red Bile Glucose agar (VRBG, Oxoid, Milan, Italy), incubated at 37 °C for 48 h; vi) yeasts and moulds on Malt Extract agar (Oxoid, Milan, Italy), supplemented with tetracycline (10 mg ml^{-1}, Sigma, Milan, Italy), incubated at 25 °C for 48–72 h; vii) coliforms and *Escherichia coli* on ColiID medium (Bio-Merieux, Marcy d'Etoile, France). Each analysis was conducted in triplicate. After counting means and standard deviations were calculated. An average of 20 colonies per sourdough sample were selected from each one of these medium: mMRS agar at 30 °C and 42 °C, M17 agar, SDB and ME agar. The colonies were random collected from the agar plates conteining between 30 and 300 colonies. Colonies were selected independently of their morphology, colour or size. A total of 320 lactic acid bacteria isolates and 80 yeasts isolates were obtained. Isolates were streaked on the corresponding fresh medium and then stored at −20 °C in the appropriate broth, supplemented with glycerol (30% final concentration). The isolates were then identified molecularly.

2.5. DNA extraction from pure culture

One milliliter of an overnight culture was centrifuged at 14,000×*g* for 10 min at 4 °C to pellet the cells and the pellet was subjected to DNA extraction according to Andrighetto et al. (2001), with the addition only of lysozyme (50 mg/ml, Sigma) for bacterial cell lysis.

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