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PhD thesis

PRESERVATION OF SELECTED SOURDOUGH: COMPARISON OF FREEZING, FREEZE DRYING, DRYING AND SPRAY DRYING TECHNIQUES

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PREFACE

Spontaneous sourdough fermentation is one of the oldest methods used in the bakery industry for the production of homemade bread and various sweets typical of the Italian tradition such as Panettone, Pandoro and Colomba (DM 22 July 2005). The modern biotechnology of baked goods largely uses fresh sourdough (namely sourdough of type I) as a natural leavening agent because of the many advantages it offers over baker's yeast resulting in a final product with high sensory quality. Type I sourdough has the largest application and resembles the traditional processes. It is fully known that sourdough is characterized by a heterogeneous microbial consortium, mainly represented by lactic acid bacteria and yeasts, whose fermentation confers to the bakery product its features such as improved taste, texture and aroma, high palatability, delayed staling and increased shelf-life. It is characterized by continuous (daily) propagation to keep the microorganisms in an active state. Furthermore, the ecosystem of sourdough type I can easily undergo modification due to factors affecting the management and preservation of the dough itself, such as modification of the ingredients used and the type of flour, the change in the storage temperature, the number of refreshments made, the hygienic conditions of the processing environment and the operator. Propagation of sourdough type I is achieved by daily back-slopping, using the mother sponge taken from the preceding fermentation, mixing it with water and flour, and allowing to ferment for at least 6 h at 30°C. So, the maintaining of a fresh sourdough starter requires a little extra time and effort because daily or weekly refreshments are necessary to guarantee its good vitality.

For these reasons, numerous industrial bakeries use sourdough of type III, that is the most convenient way to introduce authentic bread taste into nowadays high-tech bakery industry. Sourdough of type III consist mainly in different preparations of dried or lyophilized sourdoughs which are often characterized by reduced microbial vitality and which almost always include the addition of "*Saccharomyces cerevisiae*" yeast, which is presented as an "activator" but which actually is responsible only for the leaven, often causing flattening of the aromatic characteristics of the finished products.

A proper stabilization of the sourdough of type I over time could represent not only an important milestone in economic terms in the bakery industry but also a prerequisite for the protection of typical and traditional bakery products, without the use of baker's yeast activator.

Aim of PhD thesis was, therefore, to compare different techniques in order to identify the one that could better preserve the microbial characteristics of the sourdough of type I and thus the

finished baked products. To this end, a sourdough of type I has been subjected to different storage modes: freezing, drying, freeze-drying and spray drying. Sourdough was selected among 28 typical sourdoughs, previously characterized for lactic acid bacteria and yeast count, pH and Total Titratable Acidity (TTA) value and volatile organic composition. After the preservation treatments, microbial vitality and chemical-physical properties of the doughs were assessed. Then, the stabilized sourdough starter were used in breadmaking manufacture to assess the ability to leaven and ferment the doughs. The breads obtained were subjected to sensorial analysis and digital image analysis. Positive and encouraging results were obtained mainly with the sourdough spray-dried.

Among the different storage techniques tested, spray drying showed the highest survival both of yeasts and lactic acid bacteria respect to the other techniques. Furthermore, the results suggested that spray-dried sourdough, opportunely refreshed, can be successfully used for breadmaking, leading to bread with sensorial characteristics comparable to those produced using fresh sourdoughs.

The activities of comparison of preservation techniques, breadmaking experiment and sensorial analysis were carried out in the Technology laboratory of Department Agriculture, Environmental and Food Science, University of Molise, Campobasso.

Sourdough sampling and characterization of typical sourdoughs and isolation, identification and characterization of lactic acid bacteria were carried out in collaboration with ISA-CNR, Avellino, within the Project “Irpinia in fermento”.

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INTRODUCTION

SOURDOUGH: MICROBIOLOGICAL AND TECHNOLOGICAL ASPECTS**1.1 Introduction**

Bread is one of the most widely consumed food products in the world and bread making technology is probably one of the oldest technologies known. Findings suggest that people of Babylon, Egypt, Greece, and Rome used bread as part of their diet long before the A.D. period.

Bread is consumed in large quantity in the world in different types and forms depending on cultural habits. Flat breads are the oldest, most diverse, and most popular product in the world. It is estimated that over 1.8 billion people consume various kinds of flat breads all over the world. Bread products and their production techniques differ widely around the world. The objective of bread making is to convert cereal flours into attractive, palatable, and digestible food. The foremost quality characteristics of leavened wheat breads are high volume, soft and elastic crumb structure, good shelf life, and microbiological safety of the product (Cauvain *et al.*, 2003; Chavan & Jana, 2008).

Southern Italy boasts numerous traditional and typical breads obtained using traditional recipes, high quality ingredients and sourdough fermentation. “Traditional” is the definition used for foods that historically are part of the cultural heritage of people living in a certain geographical area (Ministero per le Politiche Agricole. 1999. Decreto ministeriale 8 Settembre 1999, no. 350. Gazzetta Ufficiale della Repubblica Italiana 12 Ottobre 1999, no. 240). “Typical” is the attribute used for a food produced using one or more ingredients having characteristics strictly depending on the geographical area it comes from (Minervini *et al.*, 2012).

Sourdough fermentation is one of the oldest biotechnological processes in cereal food production. As reported in numerous studies present in literature (Minervini *et al.*, 2014; Lattanzi *et al.*, 2013) during sourdough fermentation different activities and metabolic interactions occur and a complex and unique microbial ecosystem composition establishes. The sourdough microflora is dominated by lactic acid bacteria that, along with yeasts, play a key role in fermentation process and in the definition of the final products. The sourdough fermentation has a number of beneficial effects that include prolonged shelf life, accelerated volume gain, delayed staling, improved bread flavor and texture, and enhanced nutritional value (Chavan & Chavan, 2011).

1.2 Properties of Sourdough

Sourdough is a mixture of flour, water, and other ingredients that is fermented by naturally occurring lactic acid bacteria (LAB) and yeasts. These microorganisms originate mainly from flours and processing equipment, but the resulting composition of the sourdough microbiota is determined by endogenous (e.g., chemical and enzyme composition of the flour) and exogenous (e.g., temperature, redox potential, water content, and duration of the fermentation process) factors (Hammes & Gänzle, 1998). In mature sourdoughs, LAB dominate, occurring at concentrations $>10^8$ cfu/g, whereas the number of yeasts is commonly one/two logarithmic cycles lower (Ehrmann & Vogel, 2005). Sourdough fermentation positively influences all aspects of baked goods quality such as texture, aroma, nutritional properties, and shelf life. Recently, sourdough has been successfully applied for the improvement of the quality of naturally gluten-free (GF) bread due to the complex metabolic activity of LAB.

Today, the acid mixture is used in many craftsmanships such as panettoni, pandas, doves, but also typical regional specialties such as croissants and brioche (Foschino *et al.*, 1999; Zorzanello & Sugihara, 1982) and in the production of homemade bread.

Recipes and technology for obtaining sourdough and fermented baked goods are however different and closely related to the place of production, local habits and the type of product that is intended to obtain (Hammes & Gänzle, 1998; Onno & Roussel, 1994; Ottogalli *et al.*, 1996).

To obtain sourdough, it is necessary to carry out continuous refreshments, adding new water and flour, and allowing the mass to rest to allow the present microflora to develop and colonize the dough.

The sourdough fermentation affects dough rheology at 2 levels, in sourdough itself, and in bread dough-containing sourdough. In dough, fermentation decreases elasticity and viscosity, whereas the addition of sourdough to final bread dough results in less elastic and softer dough. The level of rheological changes taking place in these dough and its influences on bread quality can be controlled by adjusting fermentation time and the ash content of flour during the prefermentation process (Clarke *et al.*, 2004). Many inherent properties of sourdough rely on the metabolic activities of its resident LAB: lactic fermentation, proteolysis, and synthesis of volatile compounds, antimould, and antiropiness production is among the most important activities during sourdough fermentation (**Figure 1**) (Hammes & Ganzle, 1998; Gobbetti *et al.*, 1999).

Moreover, endogenous factors in cereal products (carbohydrates, nitrogen sources, minerals, lipids, and free fatty acids, and enzyme activities) and process parameters (temperature, dough yield [DY], oxygen, fermentation time, and number of sourdough propagation steps) markedly

influence the microflora of sourdough and the features of leavened baked goods (Hammes & Ganzle, 1998).

Some of the factors are explained as follows:

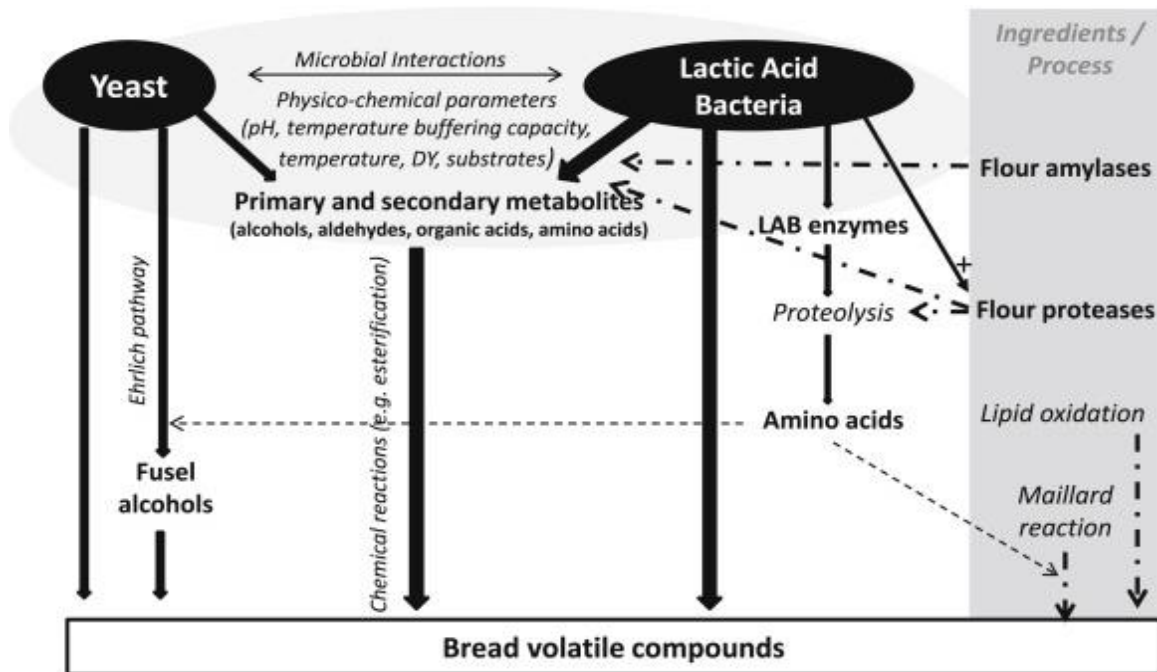


Figure 1. Sourdough fermentation (adapted from Pétel *et al.*, 2017).

Sourdough can be different consistency. The sourdough fermentation can be performed as firm dough or as a liquid suspension of flour in water. This proportion between flour and water is called the DY and is defined as:

$$\text{Dough yield} = (\text{amount of flour} + \text{amount of water}) \times 100 / \text{amount of flour}$$

The DY value of a sourdough will significantly influence the flavor profile of the sourdough. The firmer the sourdough (lower DY value), the more acetic acid is produced and the less lactic acid. The acidification rate is also influenced by the DY of a sourdough. The higher the DY, the faster the acidification will occur, most probably due to the better diffusion of the produced organic acids into the environment (Spicher & Stephan 1999).

Temperature is the utmost important factor, as it influences DY more than acidification rate and also has an influence on the microbial composition of the sourdough. If backslopping is used where a part of the previous sourdough is used to inoculate the next fermentation, temperature plays a critical role because part of the microflora can be lost over the different sourdough refreshments if it is not controlled (Spicher & Stephan, 1999). Optimum temperatures for the

growth of lactobacilli are 30°C to 40°C depending on strain and for yeasts 25°C to 27°C. In general, a higher temperature, a higher water content of sourdough, and the utilization of wholemeal flour enhance the production of acids in wheat sourdoughs (Brummer & Lorenz, 1991).

The titratable acidity and pH of the dough are important during sourdough fermentation. The acidification process of the doughs is almost strictly dependent on the kind of flour used and to the microbial species present in the dough. The substrate, mainly flour, used for sourdough fermentation is another parameter that significantly influences the sourdough. Ash content is important to determine flour grade and extraction rate, since the ash content of the bran is about 20 times that of endosperm. The ratio of bran to endosperm is higher in small kernels (Posner, 2000). The bran fraction contains more minerals and micronutrients that are important for the growth of LAB. The ash also influences the buffering capacity of the sourdough system that makes possible to reach a higher total titratable activity. The falling number of the flour is an indicator for the enzymatic activity of the flour. The lower the value the more amylase activity is present in the flour.

1.3 Classification of Sourdough

Sourdoughs, based on the protocol of propagation and metabolic activities of the main lactic acid bacteria have been grouped into three types (I, II e III) (**Figure 2**). Type I sourdough has the largest application and resembles the traditional processes. It is characterized by continuous (daily) propagation to keep the microorganisms in an active state, as indicated by high metabolic activity. Propagation of sourdough type I is achieved by daily back-slopping, using the mother sponge taken from the preceding fermentation, mixing it with water and flour, and allowing to ferment for at least 6 h at 30°C (De Vuyst & Neysens, 2005; Minervini *et al.*, 2010).

Type II sourdough is propagated at high temperatures (>30 °C) with long fermentation time (up to 5 days) and high water content, and it is mainly used as acidifying and aroma carrier. Type III is a dried sourdough in powder form, that is fermented by defined starter cultures. It is used as acidifier supplement and aroma carrier during breadmaking. In contrast to type I, type II and III sourdoughs often require the addition of baker's yeast (*Saccharomyces cerevisiae*) for leavening (De Vuyst & Neysens, 2005). The Type III sourdoughs are the most convenient to introduce authentic bread taste into the nowadays high-tech bakery industry. In industry, a lot of Type III sourdoughs are available (Bocker *et al.*, 1995). The doughs of Types II and III require the

addition of baker's yeast (*S. cerevisiae*) as leavening agent, whereas Type I sourdoughs do not require this addition.

Sourdough LAB, consisting of obligate and facultative heterofermentative and obligate homofermentative species associated with sourdoughs of Types I, II, and III.

Different techniques are used to obtain sourdough type III. In spray-drying, the liquid sourdough is pulverized in a hot air stream. The water content (about 90%) is evaporated, while the sourdough droplets are falling down in the hot air. Due to the presence of evaporating water in the falling hot droplets, the product itself is cooled down during the process thus avoiding browning of the powder. In the drum-drying technology, stainless steel cylinders are heated with steam. A thin film of product is spread over the cylinder and almost immediate evaporation occurs.

The rest of the residence time of the semi-dry product on the drum will be used to allow Maillard reactions. Dependent on the temperature/time combination, the end sourdough can be more or less caramelized or toasted. Using the previous stabilization processes, there is a loss of volatile flavor compounds during the evaporation of the water. A way to prevent this and to achieve more complete flavor properties is to keep the sourdough in a liquid form and to stabilize the sourdough by pasteurization or by cooling. Most volatile compounds remain present in the product. An advantage for the industrial application is the pumpability of such a product and also it is very accurate and has a constant quality at any time.

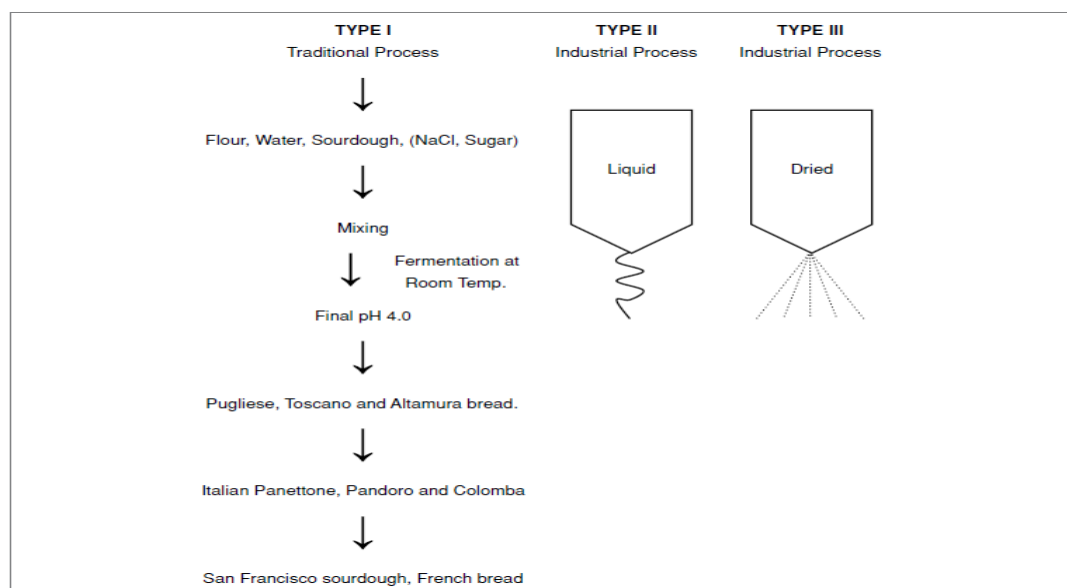


Figure 2. Scheme of sourdough production processes (adapted from Chavan & Chavan, 2011).

1.4 Sourdough Microbiota

Sourdough is a complex ecosystem in which lactic acid bacteria (LAB) and yeast interact together and with the ingredients depending on the process parameters (Zotta *et al.*, 2006). Sourdoughs fermentation, are dominated by specifically adapted LAB occurring at numbers above 10^8 CFU/g, which may be in coexistence or possibly in symbiosis with typical yeasts whose numbers are orders of magnitude lower (Gobbetti *et al.*, 1999; Vogel *et al.*, 2002).

Microbiological studies revealed that more than 50 LAB species have been isolated from this ecosystem, mainly belonging to the genus *Lactobacillus* (about 30 species) and more than 20 yeast species belonging to the genera *Saccharomyces* and *Candida* (Iacumin *et al.*, 2009; De Vuyst & Vancanneyt 2007; Reale *et al.*, 2011; De Vuyst *et al.*, 2002). However, several works report also the presence of non-*Lactobacillus* species, such as *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* (Corsetti & Settanni 2007; Valmorri *et al.*, 2006).

During sourdough fermentation, a selection of microbial population with specific nutrient requirements and growth conditions occurs; lactobacilli, among all the bacteria inhabitant of sourdoughs, are highly adapted to the environmental conditions (temperature, pH, acidity, antimicrobial products, etc.) of sourdough (Vera *et al.*, 2009). For this reason, lactobacilli represent the dominant microbial group and commonly they occur with the highest concentration, especially in mature sourdoughs (Reale *et al.*, 2011). Lactobacilli are classified, according to their ability to ferment different types of sugars, in three groups.

First group: obligate HOMOFERMENTATIVE lactic acid bacteria; starting from glucose mainly produce lactic acid through the EMP (Embden-Meyerhof-Parnas) glycolytic pathway, they are not able to ferment pentose and do not produce carbon dioxide (eg *Lb. helveticus*).

Second group: facultative HETEROFERMENTATIVE lactic acid bacteria produce, besides lactic acid, CO₂, acetic acid and/or ethanol (depending on the presence of additional substrates acting as electron acceptors) through 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (heterolactic fermentation).

Sometimes, in the absence of sugars, they also produce acetic acid, ethanol and formic acid (eg *Lb. plantarum*).

Third group: obligate HETEROFERMENTATIVE; produce equimolar amounts of lactic acid, carbon dioxide, acetic acid and/or ethanol (eg *Lb. sanfranciscensis*).

Table 1 shows the lactic bacteria prevalently found in sourdough fermentation or found in products derived from them. Some species have a wide spread and have been found in samples of different origin.

Obligately heterofermentative	Facultatively heterofermentative	Obligately homofermentative
<i>Lb. acidifarinae</i>	<i>Lb. plantarum</i>	<i>Lb. amylovorus</i>
<i>Lb. brevis</i>	<i>Lb. pentosus</i>	<i>Lb. acidophilus</i>
<i>Lb. buchneri</i>	<i>Lb. alimentarius</i>	<i>Lb. delbrueckii subsp. delbrueckii</i>
<i>Lb. fermentum</i>	<i>Lb. paralimentarius</i>	<i>Lb. farciminis</i>
<i>Lb. fructivorans</i>	<i>Lb. casei</i>	<i>Lb. mindensis</i>
<i>Lb. frumenti</i>		<i>Lb. crispatus</i>
<i>Lb. hilgardii</i>		<i>Lb. johnsonii</i>
<i>Lb. panis</i>		<i>Lb. amyolyticus</i>
<i>Lb. pontis</i>		
<i>Lb. reuteri</i>		
<i>Lb. rossiae</i>		
<i>Lb. sanfranciscensis</i>		
<i>Lb. siliginis</i>		
<i>Lb. spicheri</i>		
<i>Lb. zymae</i>		

Table 1. *Lactobacillus* species generally associated with sourdough fermentation or found in fermented sourdough (adapted from Chavan & Chavan, 2011).

Lactic acid bacteria cause acidification of the medium through the production of organic acids. The most prevalent products are lactic acid and lower amounts of acetic acid. The synthesized amounts are 0.80-0.97% and 0.25-0.30%, respectively (Spicher, 1986). Other acids produced in minor quantities are propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid. The production of these compounds results in a pH decrease that in mature sourdoughs ranges between 3.8 and 4.3. These conditions make the environment hostile to the development of many undesirable microbial species. Carbon dioxide is a product that derives from the primary metabolism of yeasts. It is obtained by degradation of glucose through the pathway of the hexoses monophosphate. Obviously, the amount of CO₂ produced is not comparable to that, which is released from the yeast metabolism.

Lactic acid bacteria, in addition to the production of organic acids and carbon dioxide, which metabolites derived from a primary metabolism, are also responsible for protein degradation through secondary metabolic pathways. Proteolytic activity is exemplified by both the flour proteases and the lactic acid proteases. These enzymes are responsible for the presence of free amino acids and peptides within the dough.

The presence of free amino acids: 1) stimulates the growth of LAB, 2) participates in the formation of aromatic compounds.

The proteolytic activity depends on the temperature, the duration of the fermentative process and the amount of proteins present in the flour.

In addition to LAB, yeasts also play an important role in sourdough.

Yeasts are typically mesophilic organisms with an optimum growth temperature of 20°C to 40°C, preferably acidic pH (excellent 3.5 to 4.5), are optional anaerobes. They have simple nutritional needs. The compounds used are carbohydrates, nitrogen compounds and vitamins. Not everyone can use maltose disaccharide.

Yeasts are far less demanding than LAB: they can grow by using ammonium ion as the only source of nitrogen for the synthesis of amino acids, purines and pyrimidines.

Yeasts play a key role in producing carbon dioxide and are considered the agents responsible for the leavening dough. Other metabolism products are ethanol, which evaporates during cooking, and aromatic compounds such as glycerin, acetaldehyde, formic acid etc. By using an oxidative deamination process, higher alcohols can be produced from amino acids.

The majority of yeasts found in sourdoughs have been allotted to the species *Candida milleri*, *C. holmii*, *S. exiguus*, and *S. cerevisiae* (Hammes & Ganzle, 1998). Yeasts are often associated with LAB in sourdough and the yeasts/LAB ratio is generally 1:100 (Gobbetti *et al.*, 1994; Ottogalli *et al.*, 1996). Typical yeasts associated with LAB in sourdoughs are *S. exiguus*, *C. humilis* (formerly described as *C. milleri*), and *Issatchenkia orientalis* (*C. krusei*) (Spicher & Schroder 1978; Gobbetti *et al.*, 1995a, 1995b; Succi *et al.*, 2003). Other yeast species detected in sourdough ecosystem are *Pichia anomala* as *Hansenula anomala*, *Saturnispora saitoi* as *P. saitoi*, *Torulasporea delbrueckii*, *Debaryomyces hansenii*, and *P. membranifaciens* (Gobbetti *et al.*, 1994; Foschino & Galli 1997; Succi *et al.*, 2003). The variability in the number and type of yeast species in dough are affected by many factors such as dough hydration, quality and the type of cereal used, the leavening temperature, and the sourdough maintenance temperature (Gobbetti *et al.*, 1994).

Most yeast species completely ferment common sugars such as glucose, fructose, maltose, sucrose and maltotriose, while others ferment mannose, galactose and xylulose. Some yeasts often found in acidic mixtures, such as *C. milleri* and *S. exiguus*, do not ferment maltose.

Both bacteria and yeasts require their metabolic activity as sugars, carbon sources, amino acids as a source of nitrogen, and vitamins and mineral salts as growth factors. Flour and, therefore, doughs are certainly an ideal substrate for the growth of microbial flora, as they contain all the essential elements for the development of microorganisms.

During the fermentation of a dough, however, strong competition between the various microbial species that colonize the medium can start if an essential nutrient element for growth becomes insufficient for both. In this case, the most competitive species with greater adaptation potential

will be able to take advantage of the smallest available metabolites and to take over on or on other species. The less competitive species is therefore repressed or unable to compete for the supply of nutrients or because it is inhibited by toxic metabolites produced by other microorganisms.

The presence, in the same environment, of different microbial species can also have positive effects on the development of both microorganisms that establish a true proto-cooperation relationship.

In the definition of the sensory and structural characteristics of baking products obtained through the use of sourdough, particular emphasis has been placed on interaction relationships between yeasts and LAB.

In fact, these interactions interfere both in the metabolism of carbohydrates and nitrogen compounds, in the production of carbon dioxide and other volatile compounds, and in the production of antimicrobial compounds.

The choice of a microbial species during the preparation of a sourdough can be closely governed by a multitude of external factors such as temperature, fermentation time, number of refreshments and raw material used as well as the ability of the microorganism to adapt the environment, compete for nourishment and produce inhibitory substances.

It follows that each acid mixture is characterized by a singular and varied pool of microbial species that play a primary role in defining the sensory and nutritional characteristics of bakery products. The presence of such microorganisms has been found in sourdough of different types and from different parts of the world (Italy, Greece, Spain, Germany, France, California, Morocco, Holland, etc.). In Europe, and in particular in Italy, there are hundreds of traditional breads and different natural yeasts. They differ in the type of flour, other ingredients, the applied technology and the fermentation process. For example, rye is widely used for bread production in Germany, Poland, Russia and Scandinavia (Bushuk, 2001).

The biodiversity of LAB may be considered narrow or wide. In the case of limited biodiversity, a limited but growing number of LAB species is responsible for the fermentation process. In the case of large biodiversity, microorganisms may form microbial consortia and establish relationships of particular microbial interactions (De Vuyst & Neysens, 2005).

Table 2 shows LAB biodiversity in sourdough from different geographic areas.

Country	Product/method of isolation and identification	Lactic acid bacteria	Reference
Belgium	Wheat/rye sourdoughs polyphasic approach	<i>L. brevis</i> , <i>Lactobacillus</i> spp. ^a , <i>L. plantarum</i> , <i>L. sanfranciscensis</i> , <i>L. paralimentarius</i> , <i>P. pentosaceus</i> , <i>L. helveticus</i>	Unpublished results
Denmark	Rye sourdough phenotypical	<i>L. reuteri</i> , <i>L. panis</i> , <i>L. amylovorus</i>	Rosenquist and Hansen (2000)
Finland	Sour rye dough phenotypical	<i>L. acidophilus</i> , <i>L. plantarum</i> , <i>L. casei</i>	Salovaara and Katunpää (1984)
France	Wheat bread phenotypical	<i>L. plantarum</i> , <i>L. casei</i> , <i>L. delbrueckii</i> subsp. <i>delbrueckii</i> , <i>L. acidophilus</i> , <i>L. brevis</i> , <i>Leuc. mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Leuc. mesenteroides</i> subsp. <i>dextranicum</i> , <i>P. pentosaceus</i> , <i>L. curvatus</i>	Infantes and Tourneur (1991)
Germany	Wheat sourdough phenotypical	<i>L. delbrueckii</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>L. fermentum</i> , <i>L. buchneri</i> , <i>L. brevis</i>	Spicher (1959)
	Rye bread phenotypical	<i>L. acidophilus</i> , <i>L. farciminis</i> , <i>L. alimentarius</i> , <i>L. casei</i> , <i>L. plantarum</i> , <i>L. brevis</i> , <i>L. sanfranciscensis</i> , <i>L. fructivorans</i> , <i>L. fermentum</i> , <i>L. buchneri</i>	Spicher and Schröder (1978) and Spicher et al. (1979)
	Rye sourdough phenotypical	<i>L. acidophilus</i> , <i>L. casei</i> , <i>L. plantarum</i> , <i>L. farciminis</i> , <i>L. alimentarius</i> , <i>L. brevis</i> , <i>L. buchneri</i> , <i>L. fermentum</i> , <i>L. fructivorans</i> , <i>L. sanfranciscensis</i> , <i>Pediococcus</i> spp.	Spicher (1984)
	Wheat sourdoughs (Panettone, wheat bread) phenotypical	<i>L. plantarum</i> , <i>L. casei</i> , <i>L. farciminis</i> , <i>L. homohiochii</i> , <i>L. brevis</i> , <i>L. hilgardii</i> (spontaneous); <i>L. sanfranciscensis</i> , <i>L. brevis</i> , <i>L. hilgardii</i> , <i>W. viridescens</i> (masa madre)	Spicher (1987)
	Rye sourdough RAPD-PCR	<i>L. amylovorus</i> , <i>L. pontis</i> , <i>L. frumenti</i> , <i>L. reuteri</i>	Müller et al. (2001)
	Rye bran PCR-DGGE	<i>L. sanfranciscensis</i> , <i>L. mindensis</i> (type I rye sourdough); <i>L. crispatus</i> , <i>L. pontis</i> , <i>L. panis</i> , <i>L. fermentum</i> , <i>L. frumenti</i> (type II rye sourdough); <i>L. johnsonii</i> , <i>L. reuteri</i> (type II rye bran sourdough)	Meroth et al. (2003)
Greece	Wheat sourdoughs polyphasic approach	<i>L. sanfranciscensis</i> , <i>L. brevis</i> , <i>Lactobacillus</i> spp. ^a , <i>L. paralimentarius</i> , <i>W. cibaria</i>	De Vuyst et al. (2002)
Italy	Panettone phenotypical	<i>L. brevis</i> , <i>L. plantarum</i>	Galli and Ottogalli (1973)
	Panettone, Brioche phenotypical	<i>L. sanfranciscensis</i> , <i>L. fermentum</i> , <i>L. plantarum</i> , <i>Leuc. mesenteroides</i> , <i>Pediococcus</i> spp.	Galli et al. (1988)
	Umbrian wheat sourdoughs phenotypical	<i>L. sanfranciscensis</i> , <i>L. plantarum</i> , <i>L. farciminis</i>	Gobbetti, Corsetti, Rossi, La Rosa, and De Vincenzi (1994)
	Pizza (Naples) phenotypical	<i>L. sakei</i> , <i>L. plantarum</i> , <i>Leuc. gelidum</i> , <i>Leuc. mesenteroides</i>	Coppola et al. (1996)
	Verona sourdoughs RAPD-PCR	<i>L. sanfranciscensis</i>	Zapparoli et al. (1996, 1998)
	Lombardian mother sponges species-specific PCR	<i>L. sanfranciscensis</i>	Foschino et al. (1999)
	Apulian wheat sourdoughs 16S rDNA sequencing 16S/23S rRNA spacer region PCR	<i>L. sanfranciscensis</i> , <i>L. alimentarius</i> , <i>L. brevis</i> , <i>Leuc. citreum</i> , <i>L. plantarum</i> , <i>L. lactis</i> subsp. <i>lactis</i> , <i>L. fermentum</i> , <i>L. acidophilus</i> , <i>W. confusa</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Corsetti et al. (2001)
Iran	Sangak phenotypical	<i>Leuc. mesenteroides</i> , <i>L. plantarum</i> , <i>L. brevis</i> , <i>P. cerevisiae</i>	Azar et al. (1977)
Mexico	Pozol (maize) 16S rDNA sequencing	<i>L. lactis</i> , <i>S. suis</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>L. alimentarius</i> , <i>L. delbrueckii</i>	Escalante et al. (2001)
Morocco	Sourdough ferments traditional starter sponges phenotypical	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. buchneri</i> , <i>L. casei</i> , <i>Leuc. mesenteroides</i> , <i>Pediococcus</i> sp.	Boraam et al. (1993)
	Soft wheat flour phenotypical	<i>L. plantarum</i> , <i>L. delbrueckii</i> , <i>L. buchneri</i> , <i>L. casei</i> , <i>L. sanfranciscensis</i> , <i>Leuc. mesenteroides</i> , <i>P. pentosaceus</i>	Faid et al. (1994)
Portugal	Broa phenotypical	<i>Leuconostoc</i> spp., <i>L. brevis</i> , <i>L. curvatus</i> , <i>L. delbrueckii</i> , <i>L. lactis</i> subsp. <i>lactis</i> , <i>E. casseliflavus</i> , <i>E. durans</i> , <i>E. faecium</i> , <i>S. constellatus</i> , <i>S. equinus</i>	Rocha and Malcata (1999)
Russia	Rye sourdough phenotypical	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. fermentum</i>	Kazanskaya et al. (1983)
Spain	Wheat sourdough phenotypical	<i>L. brevis</i> , <i>L. plantarum</i>	Barber et al. (1983)
	Wheat sourdough phenotypical	<i>L. brevis</i> , <i>L. plantarum</i> , <i>L. cellobiosus</i> , <i>Leuc. mesenteroides</i>	Barber and Báguena (1988, 1989)
Sudan	Kisra (sorghum sourdough)	<i>L. fermentum</i> , <i>L. reuteri</i> , <i>L. amylovorus</i>	Hamad et al. (1992)
	Kisra RAPD	<i>E. faecalis</i> , <i>L. lactis</i> , <i>L. fermentum</i> , <i>L. reuteri</i> , <i>L. vaginalis</i> , <i>L. helveticus</i>	Hamad et al. (1997)
Sweden	Rye/wheat phenotypical	<i>L. fermentum</i> , <i>L. delbrueckii</i> , <i>L. acidophilus</i> , <i>L. plantarum</i> , <i>L. rhamnosus</i> , <i>L. farciminis</i> , <i>L. fermentum</i> , <i>L. sanfranciscensis</i> , <i>L. brevis</i> , <i>W. viridescens</i>	Spicher and Lönner (1985)
	Rye sourdough phenotypical	<i>Lactobacillus</i> sp., <i>P. pentosaceus</i>	Lönner, Welander, Molin, Dostálek, and Blickstad (1986)
USA	San Francisco sourdough French bread phenotypical	<i>L. sanfranciscensis</i>	Kline and Sugihara (1971)

^a Phylogenetically related to *L. brevis*.

Table 2. LAB biodiversity in sourdoughs from different geographic areas.

1.5 Influence of Sourdough on Bread Characteristics

The sourdough fermentation process is strongly influenced by a variety of factors such as the activity of contaminating microorganisms, the characteristics of the raw material (water, flour and other ingredients) and the endogenous enzymatic activity of the flour.

These factors act synergistically by participating in the definition of the processes involved in the formation of the sourdough such as the formation of an acidic environment, the production of Exopolysaccharides (EPS), the production of volatile compounds and the degradation of carbon and nitrogen compounds (Martínez-Anaya, 1996). The level and intensity of these modifications play a predominant role in defining the qualitative characteristics of the finished product.

Lactic and alcoholic fermentations are essential in the formation of sourdough and in the definition of its peculiarities (Katina *et al.*, 2004).

During lactic fermentation there is a lowering of the pH values and an increase in total titratable acidity (TTA). Typical pH and TTA values of a mature sourdough are 3.6-3.8 and 8.13, respectively (Brummer & Lorenz, 1991). The lactic acid content is about 600-800 mg/100 g and acetic acid is 80-160 mg/100 g (Barber *et al.*, 1992; Hansen & Hansen, 1994). Acetic acid positively influences the aroma and together with lactic acid is a catalyst for the Maillard reaction (Seibel & Brummer, 1991).

The production of minor acids such as propionic, isobutyric, butyric, isovaleric, α -methyl n-butyric acid contributes to the formation of aroma and TTA (Galal *et al.*, 1978). Unlike the sourdough, fermented yeast mixtures have typical pH and TTA values of 5.5-6 and 3.9 mL, respectively.

The main factor that regulates the acidification of a dough is the amount of fermentable carbohydrates. White flours are characterized by low volumes of free sugars, about 1.55-1.84% (sucrose, glucose, fructose and oligosaccharides) but the activity of endogenous α -amylase increases maltose levels by ten to fifteen times.

Wholemeal flour, and especially bran, are characterized by the highest α -amylase activity (Martínez-Anaya, 2003).

Sugars are used as a source of energy from LAB. The ability of a lactic bacterium to ferment a sugar rather than another depends on the species and sometimes from the strain. Most of the lactic acid bacteria isolated from the acid mix is able to ferment excessive, pentose, sucrose and maltose. Specifically, some species such as *Lb. sanfranciscensis* specifically ferment maltose; some LAB are negative fructose and grow faster in the presence of maltose and glucose; *Lb.*

plantarum prefers maltose and glucose compared to fructose for rapid growth and ferments poorly sucrose.

Heterofermentative LAB like *Lb. sanfranciscensis* and *Lb. brevis* are stimulated by the presence of oxygen, orientating the metabolism towards acetate production. Fructose, acting as a protons, has a similar effect by directing metabolism to the kinase acetate pathway, producing mannitol traces and an increase in acetic acid. Fructose efficiency as a protons acceptor depends on the concentration, temperature and consistency of the acid mix (Martínez-Anaya, 2003).

Sourdough with heterofermentative lactic bacteria have a higher titratable acidity, a higher content of acetic acid, and a lower concentration of yeast than the homofermentative lactic acid bacteria.

In alcoholic fermentation, conducted under anaerobic conditions, yeasts, as a result of the fermentation of sugars, produce predominantly carbon dioxide and ethanol. Most yeast species completely ferment common sugars such as glucose, fructose, maltose, sucrose, maltotriose. Some species fermented mannose, galactose and xylulose.

Since the sourdough is colonized by both yeast and LAB, interactions between these two groups of microorganisms are important for defining the characteristics of the sourdough. When, in fact, species like *Lb. sanfranciscensis*, *Lb. brevis* subsp. *lindner* or *Lb. plantarum* are found in association with species of maltose negative yeast such as *S. exiguus*, maltose is exclusively used by lactic bacteria that benefit from cellular yield and organic acid production.

In association with yeast *S. cerevisiae*, maltose positive, there is a decrease in bacterial metabolism due to faster consumption of maltose and glucose by yeasts that reduce the availability of sugars (Martínez-Anaya, 2003). It has also been shown that the presence of yeasts in the doughs results in a decrease in the production of organic acids (Brummer, 1991).

The production of organic acids also depends on other factors such as temperature and fermentation time. In general, a higher temperature as well as a higher water content and the use of integral flours favor acid production in the sourdough (Lorenz & Brummer, 2003).

Increased proteolysis is attributed to the activity of LAB and/or the activation of endogenous enzymes of the flours under acidic conditions (Gobbetti, 1998; Gobbetti *et al.*, 1994; Thiele *et al.*, 2002).

Generally, proteolytic enzymes (proteases) are grouped into proteinases and peptidases. Proteinases catalyze protein degradation into smaller peptide fractions; peptidases hydrolyze specific peptide bonds or completely break down peptides to amino acids. The proteolytic

activity of wheat and rye flours is attributable mainly to aspartic proteinases and carboxypeptidase, with both these protease groups active under acidic conditions.

The degradation of wheat and rye proteins is of crucial importance for bread flavor, volume, and texture. Gluten proteins, glutenins and gliadins, are the major storage proteins of the wheat grain. The major storage proteins of rye are the alcohol-soluble secalins. It is generally observed that a limited extent of proteolysis during all sourdough fermentations beneficially improves the bread flavor without adverse effects on texture and volume (Thiele *et al.*, 2002). Amino acids and peptides affect the taste of fermented foods and, in particular, are important precursors for volatile flavor compounds. Amino acids serve as substrates for microbial conversions or are converted to flavor compounds during baking; accordingly, a limited extent of proteolysis during fermentation improves bread flavor (Thiele *et al.*, 2002). The odor of bread crumb is mainly determined by microbial fermentation products, whereas the taste and aroma products originating from thermal reactions dominate in the crust (Kirchhoff & Schieberle, 2001).

The effect of acidification and endogenous wheat proteinases, which have an optimum pH at 3.0 to 4.0, is considered important for proteolysis in the dough, especially for longtime sourdough fermentations. Strain-specific proteolytic activity of LAB may additionally contribute to proteolysis. Microbial acidification and the reduction of disulfide bonds in gluten proteins by heterofermentative Lactobacilli increase the solubility of gluten proteins and make them more susceptible for proteolytic degradation. The hydrolysis of peptides (secondary proteolysis) by sourdough Lactobacilli accumulates amino acids in dough in a strain-dependent manner, whereas yeasts decrease amino acids levels in dough. The metabolic activities during proteolysis are outlined in **Figure 3**.

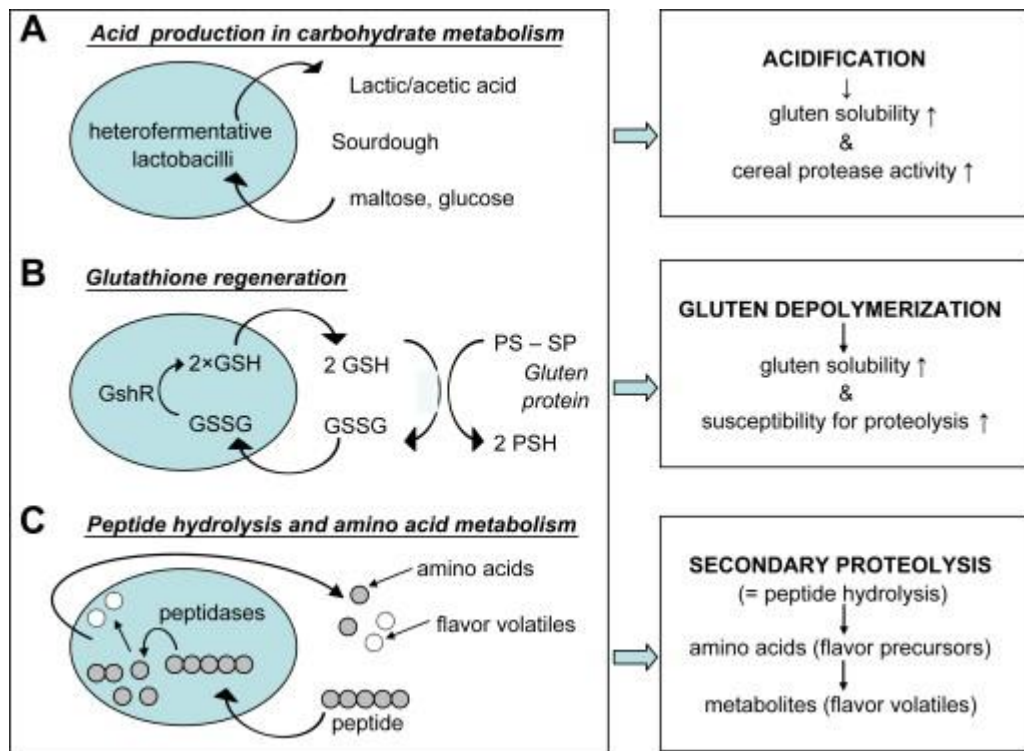


Figure 3. Contribution of lactic acid bacteria to proteolysis in sourdoughs (adapted from Chavan & Chavan, 2011).

It has been extensively reported in the literature that some lactic bacteria are able to produce exopolysaccharides (EPS), substances that affect the bread volume and weaving characteristics and its shelf-life (Korakli *et al.*, 2001; Tieking *et al.*, 2003c).

The EPS are microbial polysaccharides secreted extracellularly, the amount and their structures depend on the particular microorganisms and the available carbon substrate (Korakli *et al.*, 2001). Cereal-associated Lactobacilli produce a large structural variety of EPS and oligosaccharides from sucrose through the activity of glycosyltransferases. EPS are produced by LAB during fermentation, one of the aspects of sourdough technology with the potential for replacement by hydrocolloids. These compounds, commonly called as gums, are used as texturizing, antistaling, or prebiotic additives in bread production (Tieking *et al.*, 2003a, 2003b, 2003c).

Two classes of EPS from LAB can be distinguished, extracellularly synthesized homopolysaccharides and heteropolysaccharides (HePS) with irregular repeating units.

Homopolysaccharides, eg. fructans and glucans, are composed of only one type of monosaccharide, respectively fructose and glucose, and are synthesized by extracellular enzymes such as glucosyltransferase (Gtfs) for the production of glucans and fructosyltransferases (Ftfs)

for fruit production. Both enzymes catalyze the transfer of a sugar, glucose or fructose from sucrose to an accepting molecule resulting in the formation of an (ethero)-oligosaccharide.

It has been shown that some LAB typical of sourdough such as *Lb. reuteri*, *Lb. pontis*, *Lb. sanfranciscensis* and *Lb. frumenti* (Tieking *et al.*, 2003c) produce glucans (reuteran, dextran or mutants) or fructans (levans or inulins) (van Hijum *et al.*, 2002; Tieking & Gänzle, 2005). Generally microorganisms produce these and other substances to protect themselves from drying or other external factors (Salkinoja-Salonen & Lounatmaa, 2002).

Recently, the use of microorganisms able to produce EPS is considered to be of great importance, both because EPS improves the rheological properties of the dough and the weaving of bread and because some of them are considered prebiotic substances.

Fruit-oligosaccharides (FOS) have interesting properties in food applications as they are less sweet than sucrose, essentially calorie-free and non-carcinogenic (Yun, 1996). Inulin and FOS are mainly used for their prebiotic activities (Rhee & Song, 2002).

In the bakery industry, it is common practice to add polysaccharides or hydrocolloids for the production of frozen bread or doughs to improve its rheological and shelf-life characteristics. In order to induce positive changes in bread texture, the dough should contain a quantity of xanthans and dextrans ranging from 0.1 to 2% on dry matter. Studies show that EPS produced by LAB can positively influence one or more of the following technological properties of the dough and bread: water absorption, dough rheology and doughiness, dough stability during freezing, volume increase and delay in refining bread (Tieking & Gänzle, 2005).

The use of baking EPS in bacterial crops may not only facilitate EPS formation, thus eliminating or reducing the use of hydrocolloids usually added to the dough to improve its rheology, but also the formation of EPS flavor and shelf-life of the bread.

Sourdough fermentation has a well-established role in improving flavor and structure of rye and wheat breads (Brummer & Lorenz, 2003). Bread obtained using sourdough has a higher content of volatiles and, also, achieves higher scores in sensory tests compared to, for example, bread chemically acidified with lactic and acetic acid (Hansen & Hansen, 1996). During sourdough fermentation are produced two categories of flavour compounds: volatile and nonvolatile compounds. Nonvolatile compounds include organic acids, produced by homo- and heterofermentative bacteria (Gobbetti *et al.*, 1995a e b) that acidify, decrease pH, and contribute aroma to the bread dough. Volatile compounds that include ester and acetates, alcohols, aldehydes, ketones, and sulfur are produced by biological and biochemical actions during fermentation.

The generation of sufficient amounts of volatile compounds during fermentation needs a multiple-step process of about 12 to 24 h, while fermentation by baker's yeast alone is finished within a few hours. The generation of volatiles in sourdoughs is clearly influenced by the activities of the LAB and the sourdough yeasts. Factors influencing their activity, such as temperature and water content, will consequently influence the amounts of the metabolites formed.

Many bakery products, such as bread, have a significant content of IP6 *myo*-Inositol hexaphosphate largely known as the storage form of phosphorus in seeds, is particularly abundant in many cereal grains, oilseeds, and legumes and is contained in flours and brans of different cereals (Reale *et al.*, 2004). IP6 is often reported as antinutrient since it forms complexes with dietary minerals hindering their absorption and hence reducing their bioavailability. On the other hand, it is also reported that lower inositol phosphate derivatives can have health benefits in the protection against colon cancer, arteriosclerosis, neural tissue, and coronary heart diseases.

In this regard it has been reported that lactic fermentation significantly reduces the content of phytates in fermented cereal foods with a concomitant improvement in mineral solubility.

From studies present in literature, it can be seen that dephosphorylation of phytates takes place in particular thanks to the action of endogenous flour enzymes, called phytases, which are activated at relatively acidic pH. The reduction of phytates seems to be due to the activation of endogenous phytases or to the coprecipitation of the phytate-protein complex as result of the acidification activity of LAB (Reale *et al.*, 2004; Leenhardt *et al.*, 2005).

Most seeds and legumes have phytase that act at pH values between 3 and 10 with a maximum activity of between 4 and 5 (Greiner & Konietzny, 2006).

Some studies, however, show that different LAB typical of sourdough are able to phytate degradation and for this aspect the use of LAB in the breadmaking is finally suggested.

The application of LAB in the form of sourdough has a positive effect also on bread staling. One such effect is an improvement in loaf-specific volume, which is associated with the reduction in the rate of staling (Axford *et al.*, 1968). The breads containing sourdough can decrease the staling rate as measured by differential scanning calorimetry (Corsetti *et al.*, 2001).

Sourdough associated LAB produce many antimicrobial substances, such as organic acids, CO₂, ethanol, hydrogen peroxide, diacetyl, fatty acids, phenyllactic acid, reuterin, and fungicins (Messens & De Vuyst, 2002; Schnürer & Magnusson, 2005). Among the organic acids, acetic and propionic acid produced by heterofermentative LAB are more effective than LAB(Schnürer

& Magnusson, 2005). Caproic acid produced by *L. sanfranciscensis* CB1, together with a mixture of acetic, formic, propionic, butyric, and n-valeric acids, play a key role in inhibiting *Fusarium*, *Penicillium*, *Aspergillus* and *Monilia* growth in bread. Also, *L. plantarum* shows very broad antimicrobial activity, and the antifungal compounds 4-hydroxyphenyllactic and especially phenyllactic acids have been identified as responsible for fungal inhibition (Dal Bello *et al.*, 2007; Lavermicocca *et al.*, 2000; Ryan *et al.*, 2009).

A synergistic effect was found when sourdough fermented with antifungal *L. plantarum* strains was used in combination with calcium propionate for production of wheat bread (Ryan *et al.*, 2008). Sourdough-associated LAB are also effective against rope spoilage of bread induced by *Bacillus* spp., probably due to production of organic acids and other still unknown antibacterial substances (Katina *et al.*, 2002; Valerio *et al.*, 2008).

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SOURDOUGH CONSERVATION METHODS

2.1 Introduction

Fresh sourdough starter is a great resource. Many breads, pancakes, waffles and cakes are produced with sourdough. However, maintaining sourdough starter requires time, effort and experience. If stored at room temperature sourdough, in fact, need daily refreshment, but for most home bakers, daily feeding is impractical; so often it is kept in the refrigerator, and it is fed once a week.

Fortunately, progress in biotechnology led to the application of sourdough in frozen and freeze dried and lyophilized forms for direct incorporation into the food formulation, avoiding continuous refreshment and allowing to store sourdough starter for long time. However, these techniques have advantages and disadvantages.

The application of freezing or freeze-drying techniques to produce sourdough or pure culture starter eliminates in-plant sub-culturing, reduces the costs associated with bulk culture preparation and lowers the risk of bacteriophage infection (Desmod *et al.*, 2002). Very low transportation and storage temperatures are the main commercial disadvantages of frozen starter cultures (Ghandi *et al.*, 2012). Besides the risk of thawing, high transportation costs may limit the use of frozen starter cultures in distant areas or countries.

In spite of being efficient methods, freezing and freeze drying have high manufacturing costs and energy consumption. The costs of different drying methods are shown in **Table 1** (Santivarangkna *et al.*, 2007).

Drying processes	Fixed costs (%)	Manufacturing costs (%)
Freeze drying	100.0	100.0
Vacuum drying	52.2	51.6
Spray drying	12.0	20.0
Drum drying	9.3	24.1
Fluidized bed drying	8.8	17.9
Air drying	5.3	17.9

Table 1. Costs of drying processes referenced to that of freeze drying (adopted from Santivarangkna *et al.*, 2007).

Thus, many attempts have been made to develop alternative drying processes at lower cost, and some authors have reported reasonable cell viability after drying. For this reason, increasing attention has been paid on alternative drying processes such as spray drying, fluidized bed drying and vacuum drying.

2.2 Spray drying

Spray drying is considered a good long-term preservation method for lactic acid and probiotic cultures (Riveros *et al.*, 2009). The spray drying of microorganisms dates back to 1914 to the study of Rogers on dried LAB cultures. The speed of drying and continuous production capability are very useful for drying large amounts of starter cultures.

Since then, much research has been reported on the spray drying of bacteria without loss of cell activity in order to overcome the difficulties involved in handling and maintaining liquid stock cultures (Boza *et al.*, 2004).

The low production cost of spray drying makes it more energy efficient compared with freeze drying (**Table 1**). Nevertheless, compared with other drying methods (e.g., freeze drying), spray drying of microbial cultures has been less developed commercially.

The reasons for this are mainly low survival rates during drying of the cultures, low stability under storage and the difficulty in rehydrating the product (Ananta *et al.*, 2005; Boza *et al.*, 2004; Chavez & Ledebøer, 2007).

Spray drying is a unique process, in which particles are formed at the same time as they are dried (Barbosa-Canovas *et al.*, 2005). It is very suitable for the continuous production of dry solids in powder, granulate or agglomerate form from liquid feed stocks as solutions, emulsions and pumpable suspensions.

The end product of spray drying must comply with precise quality standards regarding particle size distribution, residual moisture content, bulk density, and particle shape.

In the spray drying process, dry granulated powders are produced from a slurry solution, by atomizing the wet product at high velocity and directing the spray of droplets into a flow of hot air, (e.g. 150-200 °C).

The atomized droplets have a very large surface area in the form of millions of micrometer-sized droplets (10e200 mm), which results in a very short drying time when exposed to hot air in a drying chamber (Morgan *et al.*, 2006; Santivarangkna *et al.*, 2007).

Dehydrated enzymes, detergents, coffee extracts, and isolated proteins are examples of products produced by spray drying. This process is also widely used in the production of LAB cultures and dehydrated probiotic bacteria (Barbosa Canovas & Vega Mercado, 1996; Riveros *et al.*, 2009).

The concept of spray drying was first patented by Samuel Percy in 1872, and its industrial application in milk and detergent production began in the 1920s.

2.2.1 Spray Drying Equipment

Spray drying involves atomization of a liquid feedstock into a spray of droplets and contacting the droplets with hot air in a drying chamber. The sprays are produced by rotary (wheel) or nozzle atomizers. Evaporation of moisture from the droplets and formation of dry particles proceed under controlled temperature and airflow conditions. Powder is discharged continuously from the drying chamber. Operating conditions and dryer design are selected according to the drying characteristics of the product and powder specifications.

Every spray dryer consists of a feed pump, atomizer, air heater, air disperser, drying chamber and equipment for product discharge, transport, packaging and removing air (**Figure 1**) (Devakate *et al.*, 2009).

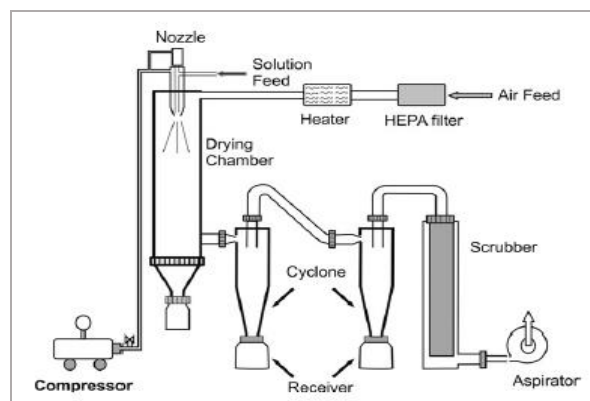


Figure 1. Schematic of spray dryer (adopted from Devakate *et al.*, 2009).

A complete air exhaust system contains fans, wet scrubbers, dampers and ducts.

The atomization process is the most important part of the spray dryer. In any type of atomization, energy is needed to break up liquid bulk to create individual droplets.

2.2.2 Classification of Atomizers

Depending on the type of energy used to produce the spray particles, atomizers can be classified into four main categories: centrifugal, pressure, kinetic and sonic (Barbosa-Canovas *et al.*, 2005).

- 1) In pressure nozzles, the feed forced through an orifice under pressure and readily disintegrates into a spray. Pressure nozzles are small, simple to maintain, easy to replace, and low in cost. They are not applicable for viscous liquids and have clogging problems.
- 2) Rotary atomizers or centrifugal nozzles use the energy of a high speed-rotating wheel to break up liquid bulk into droplets. They are flexible and also easy to operate and maintain. Rotary atomizers have no blockage problems and can be run for a long time

without operator interface. They operate under low feed pressure and can handle abrasive feeds. However, they cannot be used in horizontal dryers because the liquid is thrown horizontally. Rotary atomizers produce large quantities of fine particles, which can result in pollution control problems. They are also expensive compared to other types of atomizers.

- 3) In kinetic energy atomizers, the liquid feed and the compressed air are passed separately to the nozzle head and then the feed is broken down into small droplets. These atomizers are useful for high viscous feeds and require a smaller drying chamber. They are often used in laboratory and pilot plant spray dry applications. They are expensive to operate and require two or three times more energy than that of pressure nozzles.
- 4) In sonic atomization, a sonic generator is a part of the nozzle head; when the feed passes through the head it breaks up the liquid into droplets. This device is suitable for droplets below 50 microns. The disadvantages of the sonic atomization are its capacity restrictions, low rate feeds and acoustic environmental problems (Barbosa-Canovas *et al.*, 2005; Gohel *et al.*, 2009).

The nature and viscosity of the feed and the desired characteristics of dried product influence the choice of atomizer configuration (Gharsallaoui *et al.*, 2007).

The classification of atomizers is summarized in **Figure 2**.

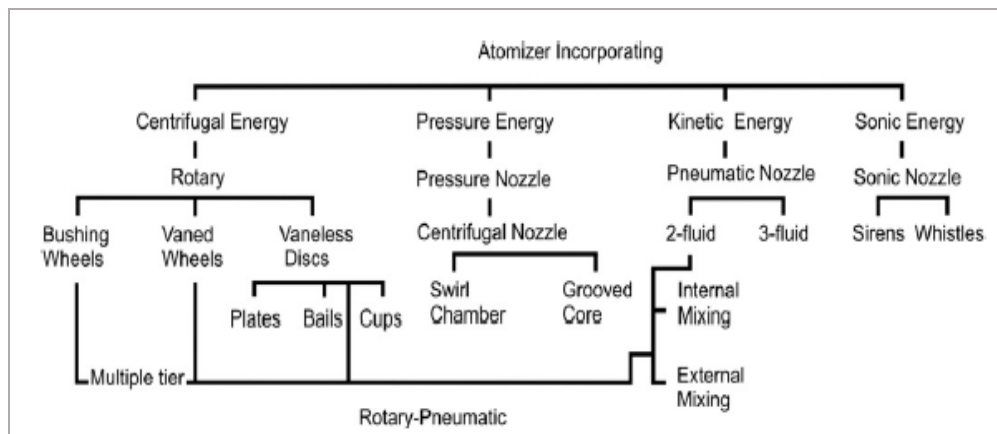


Figure 2. Classification of atomizers (adopted from Vega-Mercado *et al.*, 2001).

2.2.3 Classification of Spraying flow

There are three types of product-air flow pattern in spray dryers: co-current, counter-current, and mixed flow.

- 1) In the co-current process, the droplets and air pass through the dryer in the same direction. The droplets meet the air at the highest temperature. This causes rapid surface evaporation, while it is still wet, providing safe conditions for heat-sensitive materials.
- 2) The counter-current configuration sprays the droplets in the opposite direction to the hot air flow, which exposes the dry product to high temperatures. This design can only be used for non-heat-sensitive materials and is less commonly used than the co-current configuration (Oakley, 1997).
- 3) Mixed flow is a combination of co-current and counter-current flow patterns. A nozzle is positioned in the bottom of the chamber, forcing the spray to travel upward until overcome by gravity and the downward flow of the drying medium. Mixed flow is a good method for drying relatively coarse droplets in a small chamber at small production rates because the spray has a long path through the chamber (Barbosa-Canovas *et al.*, 2005).

Typical product-air flow patterns in spray dryers are shown in **Figure 3** (Vega-Mercado *et al.*, 2001).

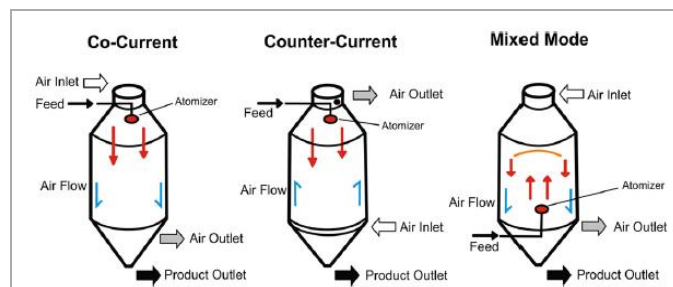


Figure 3. Typical product-air flow patterns in spray dryers (adopted from Vega-Mercado *et al.*, 2001).

2.2.4 Influence of Spray Drying Conditions on Survival of Bacteria

Spray drying is a common industrial and economic process for the preservation of microorganisms and for the preparation of starter cultures. The survival of LAB is an important issue when spray drying is used for the preparation of microbial cultures.

However, biological activity of a LAB starter, which includes cell viability and physiological state, is a criterion for evaluating starter quality.

Biological activity is defined as the ability of a lactic acid starter to acidify a certain medium. Activity tests for lactic acid bacteria are normally based on measurements of the increase in titratable acidity or the decrease in pH during incubation of inoculated milk for 3 - 5 h. These

tests are long and laborious. Therefore, other methods, such as impedimetric methods have been developed. Carvalho et al. (2003) analyzed the residual activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* cultures using pH and various impedimetric methods to quantify the loss in activity following freeze drying. They reported that the measurement of capacitance can be used as an alternative method for estimating the residual activity of freeze-dried preparations of *Lactobacillus bulgaricus*.

Preparation of dried starter cultures is a long process beginning with cell cultivation to storage of the dried powder. Many factors are involved in the viability of the cultures during spray drying (Boza et al., 2004; Corcoran et al., 2004; Santivarangkna et al., 2007).

Although it is reported that an increase in inlet air temperature decreases cell viability (Mauriello et al., 1999), the higher temperature is not directly correlated to the inactivation and has only a slight effect (Kim & Bhowmik, 1990).

This may be because the extent of inactivation of bacteria during spray drying depends on the temperature time combination.

Various investigators have also reported that increasing outlet air temperature reduces the survival of microorganisms after spray drying (Kim & Bhowmik, 1990; Lian et al., 2002; To & Etzel, 1997).

Water content and temperature are controlling parameters for inactivation of microorganisms.

In spray drying, removal of moisture takes place quickly and hence the temperature change, and moisture concentration change, and inactivation processes are also fast.

The inactivation rate reduces as moisture content decreases. It is shown that the rate-dependence of microbial inactivation is greater at higher drying rate conditions and also during the early stage of the drying (Chen & Patel, 2007).

The outlet air temperature is believed to be the major drying parameter affecting the viability of spray-dried starter cultures. This parameter depends on the inlet air temperature, air flow rate, product feed rate, medium composition, and atomized droplet size (Boza et al., 2004; Santivarangkna et al., 2008b; 2007).

However, the proper settings for these variables are difficult to calculate in advance, and this may lead to great variation in the viability of the dried culture (Roelans & Taeymans, 1990).

Many researchers have obtained higher viability of microbial cells at lower outlet air temperatures (Ananta et al., 2005; Bielecka & Majkowska, 2000).

It is believed that two principal mechanisms are responsible for viability deterioration during convective drying of bacterial cultures: inactivation due to dehydration and inactivation due to

temperature (Janning & in'T Veld, 1994). It is not clear which of these mechanisms is more damaging, as they generally occur simultaneously. In spray drying, LAB starter cultures are sprayed into a flow of hot air. Therefore, inactivation of cells due to high temperature can occur besides the dehydration inactivation.

Inactivation in LAB starter cultures is not only induced by dehydration inactivation but also by thermal- and cryo-injuries depending on the drying processes employed.

Lian et al. (2002) spray-dried Bifidobacteria cultures with various carrier media. Comparison of different carrier concentrations revealed that using 10% (w/w) gelatin, Arabic gum or soluble starch led to the highest survival of bacterial cultures. It was concluded that the viability of Bifidobacteria is highly dependent on the type of carriers and varies with strains.

The concentration of carrier medium can also affect the survival of bacteria after spray drying. In the study of Lian *et al.* (2002), increasing the concentration of gelatin, Arabic gum or soluble starch from 10 to 20% (w/w) or more caused reduced survival of Bifidobacteria.

However, higher solid content of carrier medium would result in larger particles that require longer drying times. Thus, microorganisms entrapped in the particles would be subjected to more heat damage, leading to less viability of bacterial cultures (Santivarangkna *et al.*, 2007).

2.2.5 Biological Parameters in Spray Drying

The viability of distinct species of a given genus or even distinct strains of a given species differs under the same drying or storage conditions (Simpson & Ross, 2005). It has been reported that the reduction in survival rate of some starter cultures is dependent on the species and method of preservation (To & Etzel, 1997).

This indicates that thermal tolerance alone is not an accurate predictor of performance during spray drying; other phenomena, such as dehydration affect cell viability during drying.

Thus, sensitivity to dehydration differs among LAB starter cultures and it is an individual trait (Santivarangkna *et al.*, 2007).

Growth conditions and media can affect the viability of lactic acid starter cultures during spray drying or the subsequent storage period. For instance, it has been shown that compatible solutes such as amino acids, quaternary amines (e.g., glycine, betaine, carnitine), and sugars in the medium increase the viability of lactic acid starter cultures during the drying process (Kets *et al.*, 1996).

During drying, microorganisms face decreasing water activity. Under these conditions, some microorganisms accumulate compatible solutes in order to maintain osmotic balance with the

highly concentrated extracellular environment (Morgan *et al.*, 2006; Santivarangkna *et al.*, 2007).

LAB do not synthesize compatible solutes and therefore are dependent on the environment to take up these solutes. Thus, these solutes can help microbial cultures to stabilize proteins and the cell membrane during osmotic stress conditions brought on by low water activity during the drying process (Morgan *et al.*, 2006).

Carvalho *et al.* (2004) studied the role of various sugar substrates in the growth medium. Presence of glucose, fructose, lactose, mannose or sorbitol in the drying medium mostly led in the enhancement of protection during storage, to a degree that was growth medium-dependent.

The optimal growth phase of cells for starter culture production depends on the specific microorganism (Morgan *et al.*, 2006).

Generally, LAB are harvested either in the late log phase or early stationary phase. It is shown that harvesting bacteria cells at the stationary phase leads to enhanced viability after spray drying (Corcoran *et al.*, 2004; Teixeira *et al.*, 1995; Van de Guchte *et al.*, 2002). The depletion of nutrients and glucose starvation in bacterial cells that occurs in the stationary phase of growth provide conditions that cause the cells to be resistant to many stresses, such as osmotic and heat stress (Van de Guchte *et al.*, 2002).

2.2.6 Pre-treatments in Spray Drying

The addition of protective agents is considered as a common method for protecting starter cultures during drying and storage. Protective agents may be simple or complex components.

Different sugars (e.g., glucose, fructose, lactose, mannose, sucrose, sorbitol, adonitol, trehalose) and compounds such as skim milk, acacia gum, monosodium glutamate, starch and oligosaccharides have been investigated for their protective properties on bacterial cells during drying (Desmond *et al.*, 2002; Santivarangkna *et al.*, 2008b; 2007; Sunny Roberts & Knorr, 2009).

Sugars are preferable as protective agents because of their relatively low price, chemically innocuous nature, and common use in the food industry (Santivarangkna *et al.*, 2008a). The presence of different fermentable sugars in the growth medium leads to the formation of metabolites, such as mannitol, which can enhance the viability of bacterial cultures during drying.

Non-fermentable sugars exert a hyperosmotic stress on cells. This can induce accumulation of compatible solutes, which make cells resistant to the osmotic stress during drying (Santivarangkna, *et al.*, 2008b).

According to Chavez & Ledebøer (2007), sugars, especially disaccharides, can replace water molecules and preserve membrane structures. They also retard protein denaturation (preserve cell structure) by forming hydrogen bonds with proteins.

Two hypotheses, the so-called water replacement and vitrification, were proposed to explain the mechanism of membrane stabilization by sugars (Santivarangkna *et al.*, 2008a). Vitrification hypothesis is based on glass formation in a dry state by sugars (Roos, 1995). According to water replacement hypothesis, specific and particular interactions between phospholipids and sugars are required for the protective effect.

A combination of different protectants can be used to improve the survival of spray-dried probiotics. It is important to be noticed that the best protection medium for the drying process may not be the optimum for protection of microbial cells during storage (Santivarangkna *et al.*, 2007).

2.2.7 Post Drying Condition

Rehydration is considered to be a critical step in the recovery of spray-dried LAB starter cultures. The solution used for rehydration and the rehydration conditions may affect the survival rate of dried microbial cultures.

Another factor that must be taken into account is the rate of rehydration. Slow rehydration (soaking) led to higher cell viability, possibly because the soaking method limits the amount of osmotic shock (Teixeira *et al.*, 1995).

Storage and packaging conditions affect cell viability after drying. Different studies have shown that temperature is an important parameter for microbial survival during storage.

As can be expected, the stability of spray-dried samples decreases during storage, and low storage temperatures lead to higher microbial survival rates (Boza *et al.*, 2004; Corcoran *et al.*, 2004; Desmond *et al.*, 2002; Silva *et al.*, 2002).

Proper packaging for storage of the cultures is also important. Packages under vacuum or nitrogen replacement are suitable for storing anaerobic probiotics such as Bifidobacteria. Vacuum storage was shown to be better than nitrogen and air (Chavez & Ledebøer, 2007).

Water content is an important parameter for the stability of dried cultures (Wang *et al.*, 2004; Santivarangkna *et al.*, 2007). The optimum residual moisture content depends on the composition of the fluid, in which the bacteria are dried, the storage atmosphere, and the species of bacteria (Wang *et al.*, 2004).

The moisture content and water activity of dried probiotic cultures must be kept constant in order to achieve long-term storage stability (Chavez & Ledebøer, 2007). As mentioned, relative humidity greatly affects survival of dried starter cultures during storage.

High relative humidity causes caking phenomenon in dried powders. Caking is one of the most undesired conditions for the survival of probiotics.

Therefore, it is essential that the surrounding relative humidity is kept under the critical equilibrium value that corresponds to the glass/rubber transition. Since vacuum packaging also removes air humidity, packaging of dried probiotics under vacuum is suggested (Chavez & Ledebøer, 2007).

2.3 Freeze-Drying

Freeze-drying is the most frequently employed technique for drying of microorganisms.

Lyophilization or freeze drying is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). It is a drying process applicable to manufacture of certain pharmaceuticals and biologicals that are thermolabile or otherwise unstable in aqueous solutions for prolonged storage periods, but that are stable in the dry state. The term “lyophilization” describes a process to produce a product that “loves the dry state” (Akers MJ, 1987).

The main principle involved in freeze drying is a phenomenon called sublimation, where water passes directly from solid state (ice) to the gaseous state without passing through the liquid state. Sublimation of water can take place at pressures and temperature below triple point, i.e. 4.579 mm of Hg and 0.0099 °C (Chien & Yiew, 1981).

The material to be dried is first frozen and then subjected under a high vacuum to heat (by conduction or radiation or both of them) so that frozen liquid sublimates leaving only solid, dried components of the original liquid. The concentration gradient of water vapor between the drying front and condenser is the driving force for removal of water during lyophilization.

To extract water from foods, the process of lyophilization consists of :

1. Freezing the food so that the water in the food become ice.
2. Under vacuum, sublimating the ice directly into water vapour.
3. Drawing off the water vapour.

4. Once the ice is sublimated, the foods are freeze-dried and can be removed from the machine (Neema, 1997).

Traditional lyophilization is a complex process that requires a careful balancing of product, equipment, and processing techniques.

For nearly 30 years, lyophilization has been used to stabilize many types of chemical components. In their liquid form, many such biochemicals and chemical reagents are unstable, biologically and chemically active, temperature sensitive, and chemically reactive with one another. Because of these characteristics, the chemicals may have a very short shelf life, may need to be refrigerated, or may degrade unless stabilized. When performed properly, the process of lyophilization solves these problems by putting reagents into a state of suspended activity (Greiff, 1992).

Lyophilization gives to unstable chemical solutions a long shelf life when they are stored at room temperature. The process gives the product excellent solubility characteristics, allowing for rapid reconstitution. Heat- and moisture-sensitive compounds retain their viability.

Most proteins do not denature during the process, and bacterial growth and enzyme action, which normally occur in aqueous preparations, can be eliminated. Thus, lyophilization ensures maximum retention of biological and chemical purity (Carpenter *et al.*, 1997).

Freeze drying is mainly used to remove the water from sensitive products, mostly of biological origin, without damaging them, so they can be preserved easily, in a permanently storable state and be reconstituted simply by adding water (Craig *et al.*, 1999).

Examples of freeze dried products are: antibiotics, bacteria, sera, vaccines, diagnostic medications, protein-containing and biotechnological products, cells and tissues, and chemicals.

There are four stages in the complete drying process: pretreatment, freezing, primary drying, and secondary drying.

2.3.1 Pretreatment

Pretreatment includes any method of treating the product prior to freezing. This may include concentrating the product, formulation revision (i.e., addition of components to increase stability and/or improve processing), decreasing a high vapor pressure solvent or increasing the surface area. In many instances the decision to pretreat a product is based on theoretical knowledge of freeze-drying and its requirements, or is demanded by cycle time or product quality considerations (Wang *et al.*, 2000).

Methods of pretreatment include: Freeze concentration, Solution phase concentration, Formulation to Preserve Product Appearance, Formulation to Stabilize Reactive Products, Formulation to Increase the Surface Area, and Decreasing High Vapor Pressure Solvents.

2.3.2 Freezing

Freezing is a critical step, since the microstructure established by the freezing process usually represents the microstructure of the dried product. The product must be frozen to a low enough temperature to be completely solidify.

Since freeze drying is a change in state from the solid phase to the gaseous phase, material to be freeze-dried must first be adequately pre-frozen. The method of prefreezing and the final temperature of the frozen product can affect the ability to successfully freeze dry the material (Rey *et al.*, 2004). Rapid cooling results in small ice crystals, useful in preserving structures to be examined microscopically, but resulting in a product that is, more difficult to freeze dry.

Slower cooling results in large ice crystals and less restrictive channel in the matrix during the drying process.

Products freeze in two ways, the majority of products that are subjected to freeze-drying consists primarily of water, the solvent and materials dissolved or suspended in the water, the solute.

Most samples that are to be freeze dried are eutectics, which are mixtures of substances that freeze at lower temperature than the surrounding water.

This is called the eutectic temperature. Eutectic point is the point where all the three phases' i.e. solid, liquid and gaseous phases coexist.

It is very important in freeze-drying to pre freeze the product to below the eutectic temperature before beginning the freeze-drying process.

The second type of frozen product is a suspension that undergoes vitrification during the freezing process. Instead of forming eutectics, the entire suspension becomes increasingly viscous as the temperature is lowered.

Finally, the products freeze at the glass transition point forming a vitreous solid. This type of product is extremely difficult to freeze dry (Serigo *et al.*, 2003, Lam *et al.*, 2004).

2.3.3 Primary Drying

After prefreezing the product, must be established the sublimation condition to remove the ice by the frozen product, resulting in a dry ,structurally intact product.

This requires to control very carefully two parameters: Temperature and Pressure involved in freeze-drying system.

The rate of sublimation of ice from a frozen product depends upon the difference in vapor pressure of the product compared to the vapor pressure of the ice collector. Molecules migrate from the highpressure sample to a lower pressure area. Since vapor pressure is related to temperature, it is necessary that the product temperature is warmer than the cold trap (ice collector) temperature. It is extremely important that the temperature at which a product is freeze dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This balance is key to optimum drying.

A third component essential in freeze-drying system is energy. Energy is essential in the form of heat. Much energy is required to sublime a gram of water from the frozen to the gaseous state such as is required to freeze a gram of water, (2700 joules per gram of ice).

Heat must be applied to the product to encourage the removal of water in the form of vapor from the frozen product. The heat must be very carefully controlled, as applying more heat than the evaporative cooling in the system can warm the product above its eutectic or collapse temperature.

Heat can be applied by several means, one method is to apply heat directly through a thermal conductor shelf, such as is used in tray drying. Another method is to use ambient heat as in manifold drying (Rendolph *et al.*, 2005).

2.3.4 Secondary Drying

After primary freeze-drying is complete, and all ice has sublimed, bound moisture is still present in the product. The product appears dry, but the residual moisture content may be as high as 7-8% continued drying is necessary at warmer temperature to reduce the residual moisture content to optimum values. This process is called 'Isothermal Desorption' as the bound water is desorbed from the product (Charles *et al.*, 2005).

Secondary drying is normally continued at a product temperature higher than ambient but compatible with the sensitivity of the product.

In contrast to process conditions for primary drying which use low shelf temperature and moderate vacuum, desorption drying is facilitated by raising shelf temperature and reducing chamber pressure to a minimum. Care should be exercised in raising shelf temperature too highly; since, protein polymerization or biodegradation may result from using high processing

temperature during secondary drying. Secondary drying is usually carried out for approximately 1/3 or 1/2 the time required for primary drying.

The general practice in freeze-drying is to increase the shelf temperature during secondary drying and to decrease chamber pressure to the lowest attainable level. The practice is based on the ice is no longer present and there is no concern about “melt track” the product can withstand higher heat input (Swarbrick *et al.*, 2004). Also, the water remaining during secondary drying is more strongly bound, thus requiring more energy for its removal. Decreasing the chamber pressure to the maximum attainable vacuum has traditionally been thought to favor desorption of water.

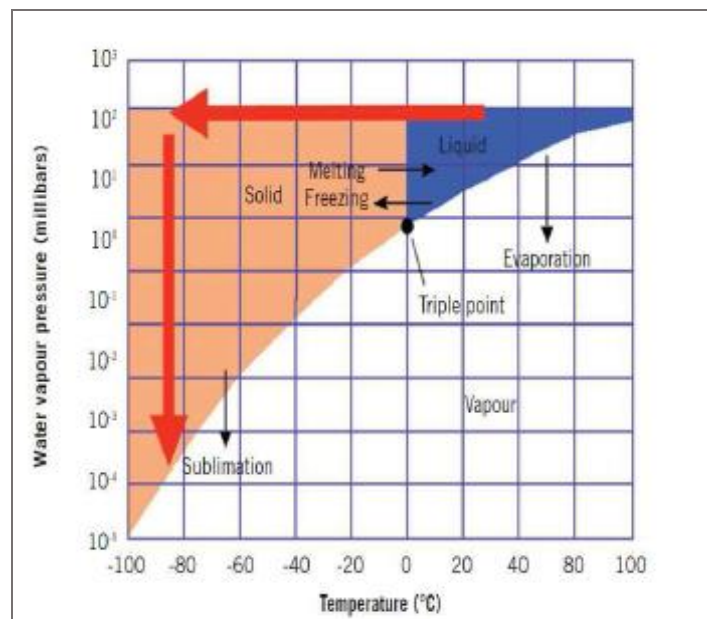


Figure 4. Freeze-drying process. (Nireesha *et al.*, 2013).

2.3.5 Freeze-Drying Equipment

There are essentially three categories of freeze-dryers: the manifold freeze-dryer, the rotary freeze-dryer and the tray style freeze-dryer.

Two components are common to all types of freeze-dryers: a vacuum pump to reduce the ambient gas pressure in a vessel containing the substance to be dried and a condenser to remove the moisture by condensation on a surface cooled to -40°C to -80°C (-40 to -112°F).

A lyophilizer consists of a vacuum chamber that contains product shelves capable of cooling and heating containers and their contents. A vacuum pump, a refrigeration unit, and associated controls are connected to the vacuum chamber.

Chemicals are generally placed in containers, such as glass vials that are placed on the shelves within the vacuum chamber. Cooling elements within the shelves freeze the product. Once the product is frozen, the vacuum pump evacuates the chamber and the product is heated.

Heat is transferred by thermal conduction from the shelf, through the vial, and ultimately into the product (Tang *et al.*, 2004).

2.3.6 Freeze-Drying Product Characteristics, Advantages and Disadvantages

Characteristics

1. Long stability.
2. Minimum reconstitution time.
3. Elegant cake appearance.
4. Maintain original dosage form characteristics upon reconstitution, including solution properties; structure and conformation of proteins; and particle-size dispersion in suspensions.
5. After reconstitution isotonicity maintained.

Advantages

1. Decomposition of chemical is minimized.
2. Water removed without excessive heating.
3. Increase stability of product in a dry state.
4. Processing a liquid is and simplifies aseptic handling.
5. As compare to dry powder filling compatible to sterile operations
6. Fast dissolution of reconstituted products.
7. From heat sensitive material water can be removed by avoiding damage.
8. If freeze-dry product has a high specific surface area, which facilitates rapid, complete rehydration of the solid.
9. Freeze-dried dosage forms allow drug to be filled into vials as a solution. This make more precise than filling of powder in to vials.

Disadvantages

1. Handling and processing time increases.
2. Volatile compounds may be removed by only vacuum.

3. For reconstitution need sterile diluents.
4. Cost and complexity of equipment.
5. Many drugs, particularly biological products such as proteins, liposomes, and vaccines, are damaged by freeze-drying process.
6. Stability of a drug in the solid state depends on its physical state.

2.4 Drying

Drying is the process wherein moisture is removed from the food material as a result of concurrent heat and mass transfer (Sontakke & Salve, 2015).

Heat is applied through conduction, convection and radiation to force water to vaporise, whilst removal of vapours is achieved by employing forced air.

Dehydration preserves food in a stable and safe condition by reducing water activity, extending the shelf life much longer than that of fresh produce (Zhang *et al.*, 2006).

Conventional hot air drying allows to extend the shelf life for long periods of time, however, the quality of the products obtained is drastically reduced when compared to traditional drying.

Drying by convection may, however, offer an efficient alternative in terms of costs and transport.

Drying methods can be broadly classified into natural and artificial methods of drying. The natural method of drying utilizes the solar energy to remove the moisture content in the food, with a disadvantage of dependence on weather condition and poor operational performance (Toshniwal & Karale, 2013).

Artificial methods of drying have advantages over natural method of drying. Artificial methods have the capacity to remove large amount of moisture efficiently (Okoro & Madueme, 2004).

In addition to this controlling various factors involved such as temperature, drying air flux and time of drying is also possible.

Artificial drying is done with the help of mechanical or electrical equipment.

2.4.1 Natural Drying Methods: Solar drying

Sun is an inexhaustible and free source of energy, utilized for drying of foods since ancient times. Solar drying can be classified into direct and indirect method of drying.

Direct method

In open sun drying the food to be dried is left exposed to the sun for a number of days to achieve the desired moisture content. In developing countries where fuel is scarcely available to farmers

due to high cost, open sun drying is the most popularly used method of drying since it is simple and only requires sunlight (Okoro & Madueme, 2004).

Insect infestation, dust and dirt contamination, long time for drying, over heating due to direct exposure, quality deterioration and low rate of transmission of heat due to condensation of the evaporated moisture are some the major problem faced during open air drying (Sontakke & Salve, 2015).

Indirect method

Indirect solar dryer have been developed to overcome the problems encountered by the direct method of drying. Chamber type, chimney type, and wind-ventilated dryers are classified as indirect solar dryers.

In the indirect method of solar drying, the heat acquired by the system is used to heat the air that flows through the product to be dried. The top of the drying chamber is vented to remove the moisture due to evaporation (Toshniwal & Karale, 2013).

2.4.2 Artificial Drying Methods

Freeze drying

Freeze-drying is the most important artificial methods used to storage starter culture and sourdough, as previously discussed in Cap. 1.7.3

Convective drying

Convective method of drying is employed to remove water from the food substances through the application of heat in equipment meant for drying. Hot air is allowed to pass through the product in a manner to transfer the heat to the food and moisture is removed (Brennan & Grandison, 2006).

Drying by radiation

Long drying time and high temperature are the factors responsible for the loss of heat sensitive components of the food. Microwave radiation is an electromagnetic radiation which works in between the frequency range of 300 MHz to 300 GHz and have a wave length of 1mm to 1m.

The propagation of microwave through space is by means of electric and magnetic field. Microwave heating is beneficial as it requires lesser amounts of time and temperature to remove the moisture content in foods (Kahyaoglu *et al.*, 2012).

Scorching is a problem in microwave heating because of the low availability of water content towards the end of the drying process. The major advantage of using microwave is its ability to be combined with other method of drying such as vacuum drying (Borquez *et al.*, 2014).

Osmotic drying

In osmotic drying, foods to be dried is placed in a hypertonic solution which causes a difference in concentration and causes the water content of foods to be driven out from the sample to the solution.

Diffusion of the solutes from the solution into the tissue of the fruits and vegetables also takes place (Mehta *et al.*, 2013). The transfer of mass during osmosis may be responsible for the change in physical, chemical, nutritional values, taste and structural properties of the final product (Shi & Maguer, 2002).

Monosaccharides, disaccharides and salts such as sodium chloride are the most commonly used osmotic ally active solutes. The possibility of the process to be conducted at room temperature makes it more advantageous than other conventional method of drying such as hot air or vacuum method of drying since energy required for carrying out the procedure is significantly less (Chavan & Amarowicz, 2012).

2.4.3 Drying: Advantages and Disadvantages

Advantages

1. It is the simplest of all food preservation methods.
2. Removal of water results in products that weigh less and take up less space. This means dried foods are easier and less expensive to store and transport than other types of preserved foods.
3. Foods can be dried without destruction of the cellular tissue of the food.

Disadvantages

1. Drying is a fairly slow compared to other forms of food preservation. Depending on the product and the drying conditions, drying times can be as long as 12 to 24 hours.
2. Mold can grow on partially dried foods, so once drying is started it should continue without interruption.
3. Not all foods dry well. For some fruits and several vegetables other preservation methods such as canning or freezing are better methods of preservation.

4. Appropriate pretreatment is often necessary to prevent discoloration and other undesirable changes that may occur during drying.
5. Drying causes physical changes, like shrinkage. For example, plums become prunes and grapes become raisins. Due to these changes adding water back the product may not return it to its original state.

2.5 Freezing and Chilling

Chilling and freezing are two of the most common methods for preserving foods. Carried out correctly, they can provide a high-quality, nutritious, and safe product for consumption with a long storage life.

The principal factor controlling the safety and quality of a refrigerated (chilled or frozen) food is its temperature. The principle of the refrigerated preservation of foods is to reduce, and maintain, the temperature of the food such that it stops, or significantly reduces, the rate at which detrimental changes occur in the food. Cryopreservation remains as the main long-term cell preservation method to date due to its high survival rates.

Microorganisms can be cryopreserved at low or ultralow temperature without genetic or phenotypic alterations while maintaining cell viability (Tedeschi & De Paoli, 2011). Whereas cryopreservation of microbial strains at cryogenic temperatures ($<-150\text{ }^{\circ}\text{C}$) generally results in higher survival rates (Heylen *et al.*, 2012) compared to those stored at $-20\text{ }^{\circ}\text{C}$. Cell viability is severely affected due to the formation of large ice crystals at freezing temperature and can lead to mechanical damage of cell membranes (Tedeschi & De Paoli, 2011).

Thus, control of ice crystals is important for improved survival rates. Among the operating conditions, the temperature, freezing rate, and freezing time play a vital role in maintaining the biological activities during storage.

In fact, the lower the temperature and the shorter the duration, the higher acidification activity is preserved in probiotics (Fonseca *et al.*, 2001). Fonseca *et al.* (2001) reported that cell resistance to freezing and frozen storage can be improved by using a high freezing rate ($30\text{ }^{\circ}\text{C}/\text{min}$) and a low storage temperature ($-70\text{ }^{\circ}\text{C}$).

Specifically, the freezing rate determines the outcome of the freezing process since mechanical damages may arise due to the presence of ice crystals either inside or outside of the microbial cells. Whereas cellular damages are caused by the extracellular ice accumulation at low freezing rates, high freezing rates have the advantage of forming a glassy rather than a crystallization state (Fonseca *et al.*, 2001).

2.5.1 Freezing and Chilling System

There are a large number of different chilling and freezing systems for food, based on moving air, wet air, direct contact, immersion, ice, cryogenics, vacuum and pressure shift, some of which are described below.

For the majority of chilled and frozen foods, air systems are used primarily because of their flexibility and ease of use. However, other systems such as immersion, contact and cryogenics can offer much faster and more controlled chilling or freezing.

From a hygiene/safety-based approach, prepacking the food prior to chilling or freezing will lower the risk of contamination/cross-contamination during the chilling process. However, in most cases it will significantly reduce the rate of cooling, due to the insulating effect of the packaging, and this may allow the growth of any microorganisms, if present.

Provided the cooling medium (air, water, etc.) and refrigeration equipment used are kept sufficiently clean, no one cooling method can be said to be intrinsically more hygienic than any other. For unwrapped food, a rapid cooling system allows less time for any contamination/cross-contamination to occur than in slower cooling systems.

It is not unusual for food products (or ingredients found in food products) to be chilled or frozen a number of times before they reach the consumer. For example, during industrial processing, frozen raw material is often thawed or tempered before being turned into meat-based products, (e.g. pies, convenience meals, burgers, etc. or consumer portions, fillets, steaks, etc.).

These consumer-sized portions are often refrozen before storage, distribution, and sale. As long as the maximum food temperatures reached are below those that support pathogen growth, and the exposure times are short, food safety should not be compromised. However, there will be some reduction in the quality of the food. Whether this is commercially important and reduces the practical shelf life will depend on the product and the conditions of exposure (Clark, 2014).

2.5.2 Air Systems

Air is by far the most widely used method of chilling and freezing food, as it is economical, hygienic, and relatively non-corrosive to equipment.

Air systems range from the most basic, in which a fan draws air through a refrigerated coil and blows the cooled air around an insulated room, to purpose-built conveyORIZED air-blast tunnels or spirals.

The big advantages of air systems are their lower cost and versatility compared to immersion, contact and cryogenics, especially when there is a requirement to cool a variety of irregularly shaped products.

In general, relatively low rates of heat transfer are attained from product surfaces in aircooled systems. In standard systems, air speeds are seldom faster than 6 ms⁻¹.

Higher air speeds are not suited for thick products where heat transfer within the product is the rate-limiting factor rather than that between the heat transfer medium and the product (Clark, 2014).

2.5.3 Contact Systems

Contact refrigeration methods are based on heat transfer by contact between products and metal surfaces, which in turn are cooled by either primary or secondary refrigerants.

Contact cooling offers several advantages over air cooling, such as much better heat transfer and significant energy savings. Contact cooling systems include plate coolers, jacketed heat exchangers, belt coolers, and falling film systems. Vertical plate freezers are commonly used to freeze fish at sea, while horizontal systems are commonly used for meat blocks and ready meals. Good heat transfer is dependent on product thickness, good contact, and the conductivity of the product. Plate freezers are often limited to a maximum thickness of 50–70 mm (International Institute of Refrigeration, 2006). Good contact is a prime requirement.

2.5.4 Immersion/Spray Systems

Immersion/spray systems involve dipping products into a cold liquid or spraying a cold liquid onto the food. Cooling using ice or direct contact with a cryogenic substance is essentially an immersion/spray process.

These systems range in size from 2–3m³ tanks used to cool small batches of cooked products to large, continuous chilling systems capable of cooling 10,000 poultry carcasses per hour. This produces high rates of heat transfer due to the intimate contact between product and cooling medium. Both immersion and spray methods offer several inherent advantages over air cooling in terms of reduced dehydration and coil frosting problems (Robertson *et al.*, 1976).

Clearly, if the food is unwrapped, the liquid has to be a substance that is safe to ingest. The freezing point of the cooling medium used dictates its use for chilling or freezing. Obviously, any immersion/spray-freezing process must employ a medium at a temperature substantially

below 0 °C. This necessitates the use of non-toxic salt, sugar or alcohol solutions in water, or the use of cryogenics (Clark, 2014).

2.5.5 Vacuum Systems

Used for food products that having a large surface area to volume ratio.

Since vacuum cooling requires the removal of water from the product, prewetting is commonly applied to prevent the removal of water from the tissue of the product.

Traditionally, this method of cooling has been relatively common for removing “field heat” of leafy vegetables immediately after harvest, but it is also suitable for many other foods, such as baked products, sauces, soups, particulate foods, and meat joints (Zheng & Sun, 2005). It is particularly useful for cooked fillings, stews, sauces and casseroles since pressure cooking and vacuum cooling can be combined in the same vessel, reducing both cooking and cooling times and saving space.

2.5.6 High-Pressure Freezing Systems

High-pressure freezing, and in particular “pressure shift” freezing, is attracting considerable scientific interest (Otero & Sanz, 2012). The food is cooled under high pressure to sub-zero temperatures but does not undergo a phase change and freeze until the pressure is released.

Rapid nucleation yields small homogeneous ice crystals.

2.5.7 Freezing and Chilling: Advantages and Disadvantages

Advantages

1. Simplicity in product preservation;
2. The novel freezing methods of high-pressure freezing accelerate freezing process, forming small and uniform ice crystals. The use of antifreeze and ice nucleation proteins improve freezing process directly by interacting with ice crystals formed.
3. The novel freezing methods reducing drip loss and improving product quality.

Disadvantages

1. Shelf-life of frozen product.
2. High energy costs.
3. Vitality of microorganisms.
4. Quality improving point of view.

5. Availability of frozen products.

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EXPERIMENTAL SECTION

TECHNOLOGICAL CHARACTERIZATION AND EXPLOITATION OF MICROBIAL BIODIVERSITY OF SOUTHERN ITALIAN SOURDOUGHS: THE IRPINIA AREA

The use of sourdough fermentation is one of the oldest biotechnological processes in cereal food production. Currently, for the recognized and documented scientifically valuable characteristics of the bakery products sourdough-based, there is, worldwide, a renewed interest in this process (Hammes & Ganzle 1998). Irpinia area boasts of numerous productions of typical breads, obtained by use of traditional recipes, sourdough fermentation and high quality ingredients. The best-know breads of this area are “*pane di montecalvo*”, included into the traditional food products – dm 1877/2000 ministry of agriculture and forestry, “*pane di calitri*” and “*pane di iurmano*”, pride of the artisan bakers, ancient tradition and popular folklore. These breads are produced, even today, following the ancient procedures of sourdough fermentation, that strongly influence the final quality of the baked products. As reported in numerous study present in literature (Minervini *et al.*, 2014; Lattanzi *et al.*, 2013) during sourdough fermentation complex activities and metabolic interactions occur and a complex and unique microbial ecosystem composition establishes. Aim of this study was to characterize the sourdoughs used for the production of traditional/typical breads from Irpinia area. Although there are numerous papers on the characterization of Italian southern sourdoughs, little information is present in literature about the characteristics of sourdoughs used for the production of these ancient and popular kind of breads of Irpinian area. In detail, microbial quality, lactic acid bacteria biodiversity, chemical and technological characteristics, and volatile organic composition of 28 sourdoughs were defined.

3.1 Materials and Methods

3.1.1 Sourdough Samples

Twenty-eight sourdoughs were collected from different zones of Irpinia area, Campania Region, Italy (**Figure 1**). All sourdoughs, produced by traditional techniques without the use of baker's yeast, were classified as type I, that is the sourdough restarted using a part of the previous fermentation (Chavan & Chavan, 2011). Samples were analyzed for pH, titratable total acidity (TTA), volatile organic compounds (VOCs) and microbial composition. Total mesophilic count, lactic acid bacteria (LAB), yeasts, moulds, enterococci, *Enterobacteriaceae*, total and faecal coliforms were assessed by standard pour-plate technique by using selective media (Oxoid, Milan, Italy). Lactic acid bacteria were isolated and genetically identified.

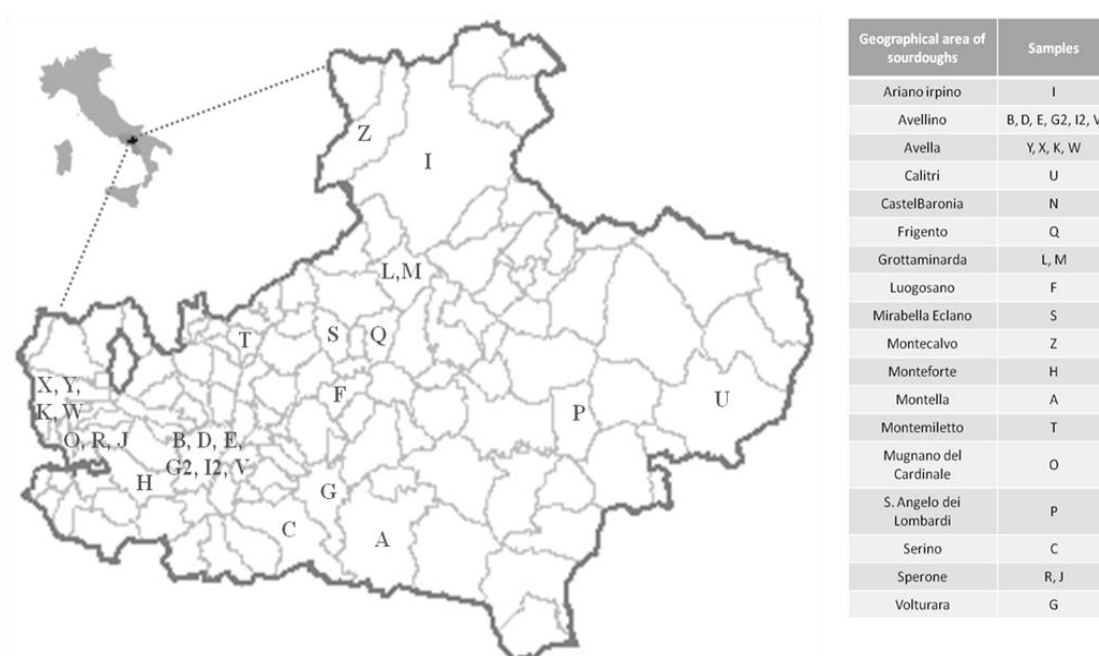


Figure 1. Origin of sourdoughs from Irpinia area.

3.1.2 pH and Total Titratable Acidity Assessment

The pH values were determined with a pHmeter Medidor PH Basic 20 (CRISON, Spain). Three independent measurements were performed on each sample and means were calculated.

Total titratable acidity was measured on 10 g of dough samples, which were homogenized with 90 mL of distilled water for 2 min in a Stomacher laboratory blender (BAG MIXER 400, Interscience, France) and was expressed as the amount (mL) of 0.1 N NaOH necessary to achieve pH 8.3. Three independent measurements were performed on each sample and means were calculated.

3.1.3 Volatile Organic Compounds (VOCs) of Sourdoughs

The volatile fraction of samples was analyzed by headspace sampling, using the solid phase microextraction technique (SPME) (**Figure 2**) according to Reale *et al.* (2016). In detail, for each SPME analysis, 2 g of samples were placed into a 20 mL headspace vial, and added 5 μ L of 4-methyl-2 pentanol (internal standard, 100 mg/L standard solution). The vial was placed in a thermostatic block (40 ° C) on a stirrer and the fibre was inserted and maintained in the sample headspace for 30 min, than it was removed and immediately inserted into the GC/MS injector for the desorption of compounds. For the analyses, a silica fibre, coated with 85 mm of CarboxenePolydimethylsiloxane (Carboxen/PDMS) was used (Supelco, Bellefonte, PA, USA).

3.1.4 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

For VOCs evaluation, an Agilent Technologies (Agilent Technologies, USA) 7890A gas chromatograph coupled to an Agilent Technologies 5975 mass spectrometer equipped with a 30 m x0.25 mm ID, film thickness 0.25 μ m capillary column (HP-INNOWAX, Agilent Technologies, USA) was used. Gas carrier was Helium (flow 1.5 mL/min) and SPME injections were splitless (straight glass line, 0.75 mmI.D.) at 240° C for 20 min during which time thermal desorption of analytes from the fibre occurred. The oven parameters were as follows: initial temperature was 40 ° C held for 3 min, followed by an increase to 240 ° C at a rate of 5 °C/min, then held for 10 min. Injector temperature was 240 ° C. Mass spectrometer operated in scan mode over mass range from 33 to 300 amu (2 s/scan) at an ionization potential of 70 eV. Identification of volatile compounds was achieved by comparing mass spectra with the Wiley library (Wiley7, NIST 05). The data are expressed like relative peak area respect to internal standard. All analyses were performed in duplicate.



Figure 2. SPME-GC/MS Agilent.

3.1.5 Microbial Composition of Sourdoughs and LAB Isolation

Ten grams of each sourdough were diluted in 90 ml of physiological sterile solution (9 g/L NaCl) and homogenized in a Stomacker 400 Lab Blender (PBI International, Milan, Italy) (1 min agitation, 1 min pause, 1 min agitation).

Total mesophilic bacteria were determined on plate count agar (Oxoid) after incubation at 28°C for 48 h. Yeasts and molds were counted on YPD agar (20 g/L peptone, 20 g/L dextrose, 10 g/L yeast extract, and 20 g/L agar) after incubation at 30°C for 72 h. Enterococci were enumerated on Slanetz and Bartley medium (Oxoid) after incubation at 37°C for 48 h. Fecal and total coliforms were counted on violet red bile lactose agar (Oxoid) after incubation for 48 h at 44°C and 37°C, respectively.

For LAB enumeration and isolation several culture media were used, as suggested by other authors (De Vuyst and Vancanneyt 2007). Briefly, LAB were enumerated and isolated by plating serial decimal dilutions on modified MRS medium (MRS-M) and on SDB agar medium (Vera *et al.*, 2009; Kline and Sugihara, 1971). Substrates were supplemented with cycloheximide (0.1g/L). Plates were incubated at 28°C for 72 h under anaerobic conditions using an anaerobic system (Anaerogen, Oxoid, Milan, Italy). Three colonies randomly picked from each medium, were obtained from plates with the highest dilution having positive growth, following the procedure described by Valmorri *et al.* (2006). Isolates were then purified by streaking on the

fresh medium and incubated as above. After morphological examination, presumptive lactobacilli were maintained frozen at -80°C in MRS medium with 15% glycerol.

3.1.6 Genetic Analysis

3.1.6.1 DNA Extraction and Purification from Pure Culture

Lactic acid bacteria communities in twenty-eight sourdoughs from artisanal bakeries of Irpinian area was investigated. 168 LAB were isolated from the samples (about 6 isolated for samples) and genetically identified as described by Reale *et al.* (2011).

Briefly, two milliliters of each overnight culture was centrifuged at 14,000g for 10 min at 4°C to pellet the cells and the pellet was subjected to DNA extraction according to Querol *et al.* (1992), with the addition of lysozyme (25 mg/ml, Sigma) and mutanolysin (10 U/ml, Sigma) for bacterial cell-wall digestion. Quantity and purity of the DNA were assessed by optical reading at 260 and 280 nm, as described by Sambrook *et al.* (1989).

3.1.6.2 DGGE Analysis

The DNA from each strain was prepared for DGGE by amplifying the V1 region of 16S rRNA using the following primers: P1V1 (50-GCG GCG TGC CTA ATA CAT GC-30) (Cocolin *et al.* 2001) and P2V1 (50-TTC CCC ACG CGT TAC TCA CC-30) (Rantsiou *et al.* 2005). A GC clamp (50- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-30) (Sheffield *et al.* 1989) was attached to the 50 end of the P1V1 primer. PCR was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany).

The reaction mixture (50 µl) consisted of 10 mmol/L Tris– HCl (pH 8.3), 50 mmol/L KCl, 200 mmol/L of each dATP, dGTP, dCTP and dTTP, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each primer, 200 ng DNA and 1.25 U Taq-DNA polymerase (Finnzymes, Finland). The amplification program consisted of a 1 min denaturation step at 95 ° C, a 1 min annealing step at 45 ° C and a 1 min extension step at 72 ° C.

The first cycle was preceded by an initial step at 95 ° C for 5 min. After 35 cycles, there was a final 7 min extension step at 72 ° C. Negative controls without DNA template were included in parallel. PCR products were separated in 1.5% (w/v) agarose gel (Sigma-Aldrich, Milan, Italy) by electrophoresis for 45 min at 120 V in TBE 0.5x (Sigma-Aldrich, Milan, Italy) and were subsequently visualised by UV illumination after Gel red 1x staining.

PCR products obtained from amplification of V1 region of 16S rRNA were subjected to DGGE analysis, using a DCode Universal Mutation Detection System (BioRad, Hercules, CA, USA). Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [w/v] acrylamide-bisacrylamide [37.5:1]) by using two different ranges of denaturant to optimise separation of the products. Two denaturant concentrations, from 40 to 60% (100% denaturant was 7 M urea plus 40% [w/v] formamide) increasing in the direction of electrophoresis run, were used. The gels were subjected to a constant voltage of 120 V for 5 h at 60 ° C, and after electrophoresis they were stained for 20 min in 1.25x TAE containing gel red 1x and visualised under UV illumination.

DGGE gels were digitally captured by GEL DOC XR System (Bio-Rad, Hercules, CA, USA) using the software Quantity One Analysis (Bio-Rad) and analysed with the pattern analysis software package, Gel Compare II Version 2.0 (Applied Maths, Kortrijk, Belgium). Calculation of similarities in the profiles of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by mean of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

3.1.6.3 Sequence Analysis

Representative strains of each cluster obtained by DGGE analysis were amplified with primers P1 and P4, as described by Klijn *et al.* (1991), targeting 700 bp of the V1–V3 region of the 16S rRNA gene. After purification, (QIAquick PCR purification kit, QIAGEN GmbH, Hilden), products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany).

Sequences were aligned with those in GeneBank with the Blast program (Altschul *et al.* 1997) to determine the closest known relatives, based on the partial 16S rRNA gene homology.

3.2 Results and Discussion

3.2.1 Microbiological and Physico-Chemical Analysis

Almost all the sourdoughs were characterized by low pH and high values of titratable acidity (**Figure 3**). Most sourdoughs had pH values ranged between 3.5 to 4.0, while just seven samples showed pH values ranged between 4.0 and 5.0. Samples Q, R, Y showed the lowest pH values of 3.5, whereas samples P and M showed the highest pH values of 4.95 and 4.41, respectively.

Titratable acidity values varied from 4 to 15 ml of 0.1N NaOH/10 g sourdough, whereas most sourdoughs were characterized from TTA values ranged between 10 to 15 mL. Samples P, M, S, V, J had the lowest TTA values comprised between 3.8 mL (P sample) and 5.6 mL (J, V). The highest TTA values were recorded for sample G and L.

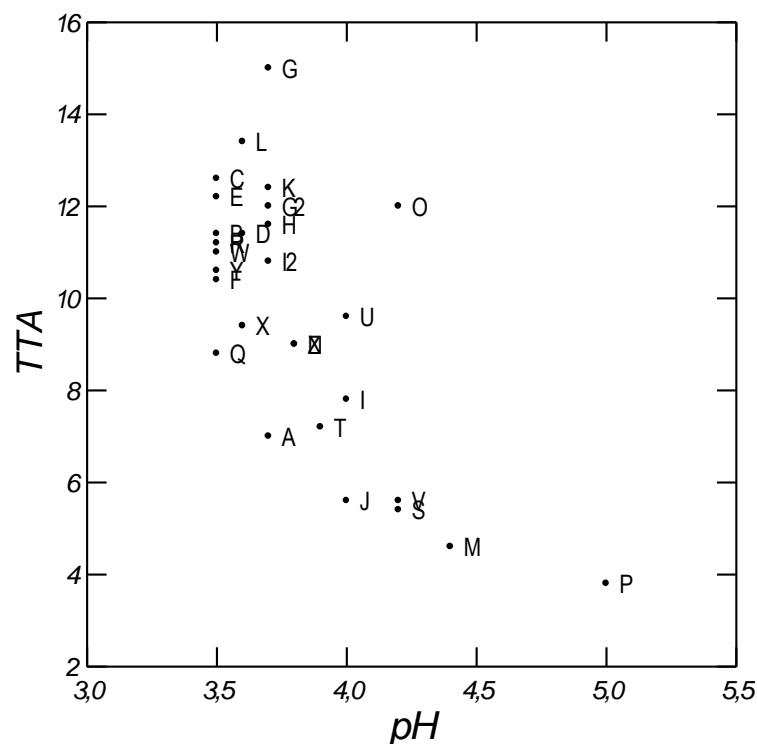


Figure 3. Distribution of 28 sourdoughs on the basis of pH and TTA values.

LAB showed the highest microbial counts almost over time compared with yeast count (Table 1). In the most samples, the cell number of LAB markedly varied between sourdoughs (10^5 to 10^9 ufc/g of dough). In three samples (O, I, G), LAB concentration was less than 3.5 log cfu/g. Seven sourdoughs showed yeast concentration higher compared to LAB count (O, I, G, L, F, N, P) (**Figure 4**). In detail sample O, I and G had very low counts of LAB. On the other hand samples E, I2, G2, Q, S, U, Y showed the highest LAB counts compared to other sourdough samples of more than 8.5 log cfu/g.

Tab. 1 - Mean value (and standard deviation) of cell densities (log CFU/g) of lactic acid bacteria (estimated on MRS-M and SDB agar) and yeast (estimated on YPD) in sourdoughs .

Samples	SDB	MRS-M (UFC/ml)	YPD (UFC/ml)
A	7.2±0.07	7.5±0.19	7.5±0.11
B	7.5±0.26	7.9±0.13	6.9±0.18
C	6.4±0.11	6.7±0.12	7.1±0.09
D	8.0±0.13	8.3±0.18	5.9±0.13
E	8.1±0.03	8.5±0.11	6.5±0.18
F	4.9±0.14	5.6±0.11	6.5±0.18
G	2.0±0.26	2.5±0.17	6.7±0.06
G2	9.1±0.12	9.1±0.04	7.2±0.09
H	8.0±0.01	8.1±0.15	7.6±0.06
I	2.0±0.11	2.3±0.12	5.5±0.13
I2	8.4±0.06	8.9±0.26	7.7±0.05
L	5.2±0.12	5.2±0.09	6.8±0.01
M	7.9±0.18	8.1±0.03	7.6±0.13
N	4.3±0.05	4.5±0.12	7.2±0.09
O	3.2±0.18	3.3±0.22	3.0±0.09
P	4.9±0.22	5.5±0.09	7.9±0.09
Q	8.5±0.05	8.9±0.07	6.8±0.12
R	7.2±0.13	7.2±0.13	5.6±0.22
S	8.3±0.22	8.4±0.13	7.6±0.03
T	7.3±0.03	7.3±0.22	7.7±0.01
U	8.6±0.13	8.8±0.07	7.3±0.07
V	7.9±0.07	8.0±0.03	5.0±0.09
Z	7.1±0.01	7.2±0.03	8.2±0.03
J	6.3±0.07	6.5±0.12	6.9±0.07
Y	8.6±0.01	8.8±0.03	7.2±0.11
X	7.8±0.07	7.8±0.05	7.6±0.11
K	7.0±0.12	7.2±0.07	7.7±0.11
W	6.5±0.22	6.5±0.26	7.9±0.11

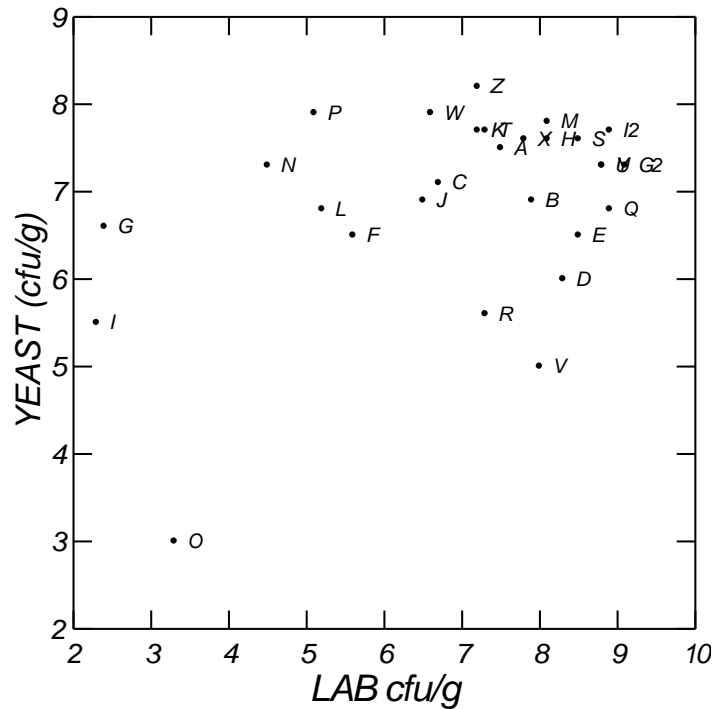


Figure 4. Distribution of 28 sourdoughs on the basis of LAB (estimated on MRS-M agar medium) and yeast counts.

Figure 5 showed total mesophilic counts recorded in sourdoughs. Ten samples (pink lines) had total mesophilic counts comprised between 8.0 to 9.0 log cfu/g, 12 samples (blue lines) had counts comprised between 7.0 to 8.0 log cfu/g, five samples (grey lines) had counts comprised between 6.0 to 7.0 log cfu/g and just one sample (green line) had mesophilic counts lower than 6.0 log cfu/g.

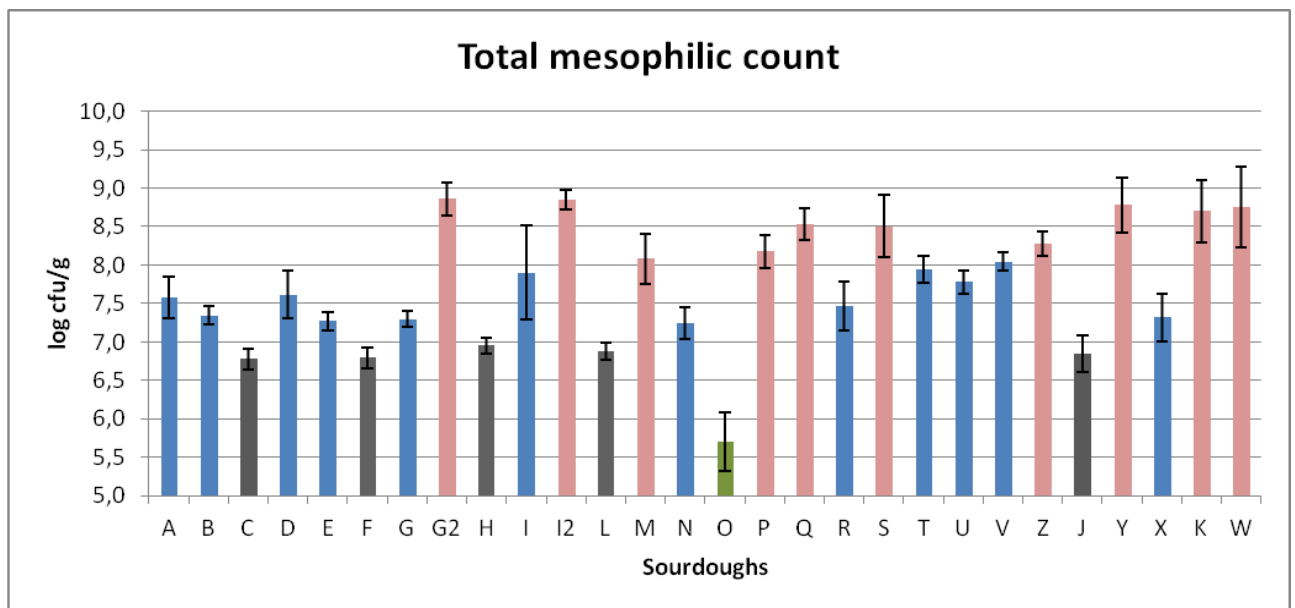


Figure 5. Total mesophilic counts in 28 sourdoughs samples.

Most sourdoughs (A, B, C, D, E, F, G2, H, I, I2, L, Q, R, T, V, J, Y, K, W) resulted of a good microbiological quality (**Table 2**). Enterococci were found in only 4 samples of sourdoughs (N, O, P, T). No moulds were detected in raw sourdoughs. *Enterobacteriaceae* and total coliforms were detected in G, M, O, P, S, Z and X samples. Total and faecal coliforms were detected only in M, O, P, Z samples.

Sourdoughs	Enterococci	Moulds	<i>Enterobacteriaceae</i>	Total coliforms	Faecal coliforms
A, B, C, D, E, F, G2, H, I, I2, L, Q, R, T, V, J, Y, K, W	nd	nd	Nd	nd	nd
G	nd	nd	2.48	nd	nd
M	nd	nd	1.00	2.11	1.00
N	3.38	nd	Nd	nd	nd
O	3.48	nd	3.06	2.46	1.85
P	3.60	nd	2.28	2.54	1.00
S	nd	nd	1.30	nd	nd
T	2.00	nd	Nd	nd	nd
Z	nd	nd	2.80	2.78	1.70
X	nd		2.20	nd	nd

nd= Not detected

Most sourdoughs samples were, therefore, characterized by a good hygienic-sanitary quality. The high acidity and the presence of high concentrations of lactic bacteria allowed, presumably, to contain the development of undesirable microorganisms such as total and faecal coliforms and moulds.

3.2.2 Volatile Organic Compounds (VOCs)

Sourdoughs were characterized by different qualitative and quantitative compositions of VOCs. More than 90 volatile components, which belonged to various chemical classes, were identified through SPME-GC/MS. Peaks with area <1% of the total peak areas and with no significant differences (ANOVA, Tukey's HSD test) in the different conditions were discarded from further statistical and graphical analyses.

Table 3 shows the 69 volatile components that mainly ($p < 0.05$) (ANOVA) characterized and differentiated doughs. The most characteristic ones belonged to eight classes such as aldehydes, ketones, esters, acetates, alcohols, acids, terpens and other minor compounds.

Low amount of aldehydes, ketons and esters compounds was observed in all the samples. In particular, among aldehydes, acetaldehyde, 2-ethylbutanal, hexanal, octanal, (E)2-heptanal,

nonanal, 2-octenal, benzaldehyde, were found in the samples. Most representative ketones found were 4-methyl-2-hexanone, 4-methyl-3-penten-2-one, 2-methyl-4-heptanone, 2,6-dimethyl-4-heptanone, acetoin. Among esters, ethyl hexanoate, isoamyl lactate, ethyl octanoate, ethyl decanoate, ethyl lactate resulted the sole compounds found in appreciable amount. Esters such as acetates, propionates, hexanoates, lactates and octanoates, have been reported as sourdough constituents (Kirchoff & Schieberle, 2002).

Considerable amount of acetates such as ethyl acetate, isobutyl acetate, 2-pentyl-4-methyl acetate, isoamyl acetate, hexylacetate, 2-phenylethylacetate were recorded in all the samples. The acetates compounds that showed the highest values were ethyl acetate and 2-pentyl-4-methyl acetate.

A considerable production of various alcohols was recorded in all the samples. As expected, ethanol compound showed the highest amount in all the samples. The other alcohols that characterized doughs were isoamyl alcohol, 1-hexanol, 1-heptanol, 1-octanol, 2-phenylethanol, 2-hexanol. The development of alcohols can be attributed to the activities of high LAB levels occurring in sourdough, as well as to yeasts activity (Rehman, Paterson, & Piggott, 2006). Apart from ethanol, the most abundant alcohols were 3-methyl-1-butanol (isoamylalcohol) reported as the most important flavour active compounds produced by yeast fermentation (Lund, Hansen, & Lewis, 1988).

Among volatile acids detected by SPME, the main compounds found were acetic, hexanoic, octanoic and 2-methylbutanoic acid. In detail, acetic and hexanoic acids were found in all the samples whereas 2-methylbutanoic and octanoic acids were found in almost all the samples. Moreover, in some samples were detected the presence of 2-methylpropanoic, heptanoic and nonanoic acids.

Among terpenes, low amount of betapinene, limonene and alpha-terpinolene were found in different samples. Furthermore a low amount of 2-pentylfuran and styrene were found.

Table 3-Concentrations of volatile organic compounds identified in the most representative sourdoughs

RI	Compounds	Odor*	A	S	R	M	P	Y	I	Z	U	E
	<i>Aldehydes</i>											
719	Acetaldehyde	Aldehydic, fruity	nd	12.6±0.5	nd	6.6±0.2	7.0±0.1	0.7±0.0	nd	13.7±0.2	8.1±0.5	0.4±0.0
970	2-methylbutanal	Nut, fruity	nd	nd	nd	Nd	1.9±0.0	nd	nd	nd	nd	nd
976	3-methylbutanal	Ethereal, aldehydic	nd	nd	nd	Nd	2.4±0.0	nd	nd	nd	nd	nd
1032	2-ethylbutanal	Sweet, green, fruity	9.2±0.0	1.6±0.1	nd	1.4±0.1	1.4±0.0	1.2±0.1	2.4±0.1	1.3±0.1	1.1±0.1	1.3±0.1
1108	Hexanal	Fresh, Green	nd	10.2±0.5	34.0±1.4	Nd	nd	7.7±0.3	nd	nd	5.2±0.3	9.5±0.2
1235	(E)-2-hexenal	Green, Aldehydic, Cheesy	nd	nd	0.2±0.0	Nd	nd	0.2±0.0	nd	nd	nd	nd
1320	Octanal	Melon, Grass, Floral	0.3±0.0	0.5±0.0	6.1±0.1	0.3±0.0	0.2±0.0	0.2±0.0	0.8±0.0	nd	0.2±0.0	0.6±0.1

1332	(E)-2-heptenal	Sour, Green, Vegetable	nd	1.1±0.1	2.9±0.1	0.6±0.0	0.5±0.0	0.9±0.1	0.6±0.0	nd	nd	0.9±0.1
1412	Nonanal	Aldehydic, Rose	1.3±0.0	0.5±0.0	6.8±0.1	0.4±0.0	0.3±0.0	0.6±0.0	1.5±0.0	0.7±0.0	0.9±0.1	1.2±0.1
1498	(E)-2-octenal	Fresh, Cucumber, Green	0.8±0.0	0.8±0.1	1.5±0.0	0.2±0.0	nd	0.5±0.0	0.5±0.0	nd	nd	0.8±0.1
1560	Benzaldehyde	Almond, Strong	9.7±0.1	0.5±0.0	0.5±0.0	0.2±0.0	0.9±0.1	0.3±0.0	0.1±0.0	0.3±0.0	0.2±0.0	0.1±0.0
	Tot		21.3±0.1	27.7±1.4	52.1±1.0	9.9±0.4	14.6±0.1	12.3±0.6	5.8±0.2	16.1±0.4	15.8±0.9	14.8±0.6
	<i>ketones</i>											
890	2-propanone	Ethereal, Apple	2.0±0.1	1.2±0.1	0.9±0.0	1.2±0.1	0.7±0.0	0.4±0.0	nd	nd	nd	nd
1109	5-methyl-3-hexanone	Fruity	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1124	4-methyl-2-hexanone	Fruity	5.7±0.1	6.3±0.4	2.6±0.1	5.1±0.1	4.5±0.1	6.1±0.3	9.9±0.1	nd	nd	4.4±0.1
1140	4-methyl-3-penten-2-one	Vegetable	10.8±0.1	12.4±0.7	20.1±0.8	10.5±0.3	9.5±0.1	7.3±0.3	21.7±1.5	6.0±0.2	6.2±0.4	7.9±0.2
1148	2-methyl-4-heptanone	Fruity	0.5±0.0	0.6±0.0	nd	0.4±0.0	0.3±0.0	0.4±0.0	1.0±0.0	0.3±0.0	0.2±0.0	0.4±0.0
12210	2,6-dimethyl-4-heptanone	Fruity	15.7±0.1	15.5±1.1	6.2±0.0	9.6±0.2	9.5±0.1	12.5±0.4	14.6±0.1	3.9±0.1	4.5±0.3	11.4±0.2
1220	2-heptanone	Fruity, Spicy	nd	nd	4.8±0.0	nd	nd	nd	nd	nd	nd	nd
1310	acetoin	Sweet, Butter	nd	7.6±0.5	nd	0.4±0.0	0.3±0.0	nd	nd	1.1±0.1	3.1±0.2	0.3±0.0
1490	2-octanone	Earthy, Grass	0.3±0.0	nd	0.4±0.0	1.0±0.0	0.4±0.0	0.2±0.0	0.4±0.0	nd	nd	nd
1492	3-octen-2-one	Earthy, Mushroom	nd	nd	1.8±0.0	0.5±0.0	0.2±0.0	0.5±0.0	0.3±0.0	nd	nd	nd
	tot		35.0±0.1	43.5±2.8	36.8±0.9	28.3±0.8	25.0±0.0	27.3±1.1	47.9±1.5	11.3±0.4	14.0±1.0	24.4±0.5
	<i>esters</i>											
1015	ethyl butanoate	Sweet, Fruity	nd	nd	nd	1.5±0.1	1.8±0.1	nd	nd	nd	nd	nd
1268	ethyl hexanoate	Sweet, Fruity	5.9±0.1	2.9±0.2	1.6±0.0	12.4±0.3	6.0±0.0	2.2±0.1	0.7±0.0	6.0±0.2	11.6±0.6	2.2±0.1
1295	isoamyl lactate	Sweet	0.5±0.0	0.5±0.0	0.4±0.0	0.4±0.0	1.2±0.0	0.3±0.0	0.7±0.0	nd	0.3±0.0	0.4±0.0
1338	ethyl heptanoate	Fruity	0.5±0.0	0.3±0.0	nd	0.7±0.0	0.3±0.0	0.3±0.0	nd	0.6±0.0	1.2±0.1	0.2±0.0
1501	ethyl octanoate	Fruity, Wine	8.6±0.1	4.4±0.3	0.6±0.0	14.8±0.4	9.4±0.2	1.6±0.1	0.2±0.0	58.2±0.7	7.4±0.4	0.8±0.1
1565	ethyl nonanoate	Fruity, Rose	0.5±0.0	nd	nd	0.4±0.0	0.4±0.0	nd	0.3±0.0	0.8±0.0	0.8±0.1	0.4±0.0
1611	ethyl decanoate	Sweet, Waxy	1.3±0.0	0.6±0.0	nd	2.2±0.0	1.7±0.0	nd	nd	5.9±0.2	0.9±0.1	0.1±0.0
1650	ethyl benzoate	Fruity	0.3±0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd
1660	diethyl succinate	Mild fruity	nd	nd	nd	0.3±0.0	0.2±0.0	nd	nd	1.7±0.1	0.2±0.0	nd
1350	ethyl lactate	Butter, Caramel	6.4±0.1	1.7±0.1	9.9±0.0	3.3±0.1	0.2±0.0	86.8±3.8	4.8±0.2	6.5±0.2	20.7±1.0	12.1±0.2
	tot		23.9±0.1	10.4±0.6	12.5±0.7	35.9±0.8	21.2±0.9	91.2±1.1	6.8±0.1	79.7±0.8	43.0±1.1	16.2±0.8
	<i>acetates</i>											
905	ethyl acetate	Fruity, Sweet	136.5±0.3	70.3±0.3	50.8±0.7	70.8±1.8	31.8±0.0	155.9±6.9	99.2±0.1	152.2±1.6	69.0±3.2	191.6±2.0
1000	isobutyl acetate	Sweet fruity	nd	nd	nd	nd	nd	nd	nd	7.4±0.2	0.8±0.1	nd
1118	2-pentyl-4-methyl acetate	Sweet fruity	20.6±0.0	23.4±1.7	15.3±0.1	23.2±0.6	15.9±0.0	32.5±1.5	6.1±0.0	42.9±0.5	17.2±0.9	24.3±0.3
1130	isoamyl acetate	Sweet fruity	nd	6.0±0.3	3.3±0.1	7.5±0.2	3.1±0.1	19.8±0.8	5.2±0.4	34.2±0.4	12.2±0.7	8.6±0.2
1298	hexyl acetate	Fruity, Green	1.0±0.0	nd	0.7±0.0	0.2±0.0	0.3±0.0	1.4±0.1	0.4±0.0	0.3±0.0	0.3±0.0	0.7±0.0
1780	2-Phenylethyl acetate	Floral, Rose	nd	nd	nd	nd	nd	0.7±0.0	nd	0.9±0.0	nd	nd
	tot		158.1±2.6	99.7±2.7	70.1±1.1	101.7±2.1	51.1±1.1	210.3±3.9	111.0±2.6	237.9±2.2	99.3±3.2	225.2±0.8
	<i>alcohols</i>											
989	ethanol	Strong, Alcohol	235.6±2.4	215.2±13.8	163.8±0.6	305.3±7.5	356.0±2.7	247.4±11.1	227.8±9.9	306.9±3.2	420.5±19.1	223.4±2.3
1120	2-methyl-1-propanol	Ethereal, Winey	nd	2.0±0.2	nd	1.1±0.0	1.8±0.1	0.5±0.0	nd	1.5±0.1	2.8±0.2	nd
1170	1-penten-3-ol	Sour, Green	nd	nd	0.9±0.0	0.3±0.0	nd	nd	nd	nd	nd	nd
1231	isoamyl alcohol	Fruity	35.6±0.3	29.0±1.2	11.9±0.1	70.5±1.7	63.9±1.4	39.3±1.6	11.0±0.2	98.9±1.1	86.6±4.0	17.7±0.3
1260	2-hexanol	Chemical, Wine	2.3±0.1	2.4±0.3	1.5±0.0	1.7±0.0	1.8±0.0	1.4±0.1	3.1±0.1	1.0±0.1	0.9±0.1	1.5±0.1
1273	3-heptanol	Sweet	2.2±0.0	2.0±0.1	1.7±0.0	1.9±0.1	1.5±0.0	1.5±0.1	2.6±0.2	0.8±0.1	0.9±0.1	1.5±0.1
1280	1-pentanol	Oil, Sweet	nd	nd	2.4±0.1	1.2±0.0	nd	nd	nd	nd	0.5±0.0	1.7±0.1
1395	1-hexanol	Green, Fruity	5.9±0.1	2.7±0.3	7.0±0.0	6.7±0.2	2.2±0.0	9.0±0.3	4.0±0.2	1.4±0.1	4.0±0.3	6.9±0.2
1410	3-octanol	Earthy, Mushroom	0.3±0.0	0.2±0.0	nd	0.2±0.0	0.8±0.0	0.3±0.0	nd	nd	nd	nd
1517	1-octen-3-ol	Mushroom	nd	1.1±0.1	nd	2.1±0.1	0.8±0.0	nd	0.8±0.0	0.4±0.0	0.9±0.1	nd
1524	2-ethylhexanol	Citrus, Fresh	0.3±0.0	0.3±0.0	5.0±0.1	0.7±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.1±0.0
1574	2,6-dimethyl-4-heptanol	Mild fresh fermented	nd	nd	nd	nd	nd	13.7±0.6	nd	0.4±0.0	1.5±0.1	0.2±0.0
1587	1-octanol	Waxy, Green	1.1±0.0	0.4±0.0	1.6±0.0	0.5±0.0	0.4±0.0	1.5±0.1	0.6±0.0	0.7±0.0	0.9±0.1	0.9±0.0
1610	(Z)-2-octen-1-ol	Sweet, Floral	0.2±0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd
1665	3-nonen-1-ol (Z)	Floral	0.5±0.0	0.4±0.0	nd	0.2±0.0	0.3±0.0	0.3±0.0	nd	0.6±0.1	0.6±0.1	0.1±0.0
1695	methionol	Sulfurous onion	0.2±0.0	nd	nd	nd	0.2±0.0	0.8±0.1	nd	0.6±0.1	0.4±0.0	0.2±0.0
1889	benzyl alcohol	Floral, Rose	4.0±0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd
1925	2-phenylethanol	Floral, Rose	10.6±0.1	2.9±0.2	3.7±0.1	11.0±0.3	17.4±1.2	10.7±0.5	1.9±0.0	36.5±0.5	25.0±1.2	1.5±0.1
	Tot		299.2±2.1	259.0±16.2	199.6±0.6	403.4±9.9	447.4±5.3	326.7±14.4	253.5±9.7	450.4±5.4	546.5±25.4	256.1±3.3

<i>Acids</i>												
1515	acetic acid	Sharp, Vinegar	33.7±0.4	19.6±2.0	53.5±1.4	11.8±0.3	8.7±0.2	91.6±4.0	28.1±0.3	27.1±0.1	10.6±0.6	59.6±0.7
1570	propanoic acid	Cheesy	nd	nd	0.5±0.1	nd	nd	nd	nd	nd	nd	nd
1590	2-methylpropanoic acid	Sweet, Cheesy	nd	0.4±0.0	nd	1.1±0.0	0.2±0.0	nd	nd	3.0±0.1	nd	nd
1651	2-methylbutanoic acid	Acidic, Cheesy	0.6±0.0	0.4±0.0	nd	0.5±0.0	0.2±0.0	nd	nd	3.6±0.1	0.6±0.1	0.3±0.0
1700	butanoic acid	Acetic, Cheesy	nd	nd	nd	nd	nd	nd	0.2±0.0	nd	nd	nd
1850	hexanoic acid	Sweat, Cheesy	1.4±0.0	0.6±0.0	2.0±0.1	1.0±0.0	0.5±0.0	2.2±0.1	2.1±0.0	0.9±0.0	1.2±0.1	1.7±0.1
1968	heptanoic acid	Cheesy sweat	nd	nd	nd	nd	nd	nd	3.3±0.2	0.3±0.0	nd	nd
2075	octanoic acid	Vegetable, Cheesy	0.4±0.0	nd	0.2±0.0	0.1±0.0	0.6±0.0	0.3±0.0	18.6±0.3	1.5±0.1	nd	nd
2170	nonanoic acid	Cheese, Dairy	nd	nd	nd	nd	0.7±0.0	0.3±0.0	10.0±0.2	0.4±0.0	nd	nd
	Tot		36.1±0.4	21.0±2.2	56.3±1.6	14.6±0.3	10.9±0.2	94.5±4.2	62.3±1.0	36.8±0.9	12.3±0.7	61.6±0.8
<i>terpenes</i>												
1112	betapinene	Fresh Green	1.9±0.0	2.0±0.2	nd	2.5±0.1	nd	1.8±0.1	2.1±0.2	2.3±0.1	2.2±0.2	2.6±0.1
1223	limonene	Citrus	nd	nd	nd	nd	7.4±0.0	0.9±0.0	nd	5.8±0.2	0.6±0.1	nd
1301	alfa terpinolene	Pine citrus	nd	nd	nd	nd	nd	nd	nd	1.3±0.1	nd	nd
	Tot		1.9±0.0	3.1±0.3	nd	3.3±0.1	8.3±0.0	3.3±0.1	2.3±0.2	9.4±0.1	3.9±0.3	3.3±0.1
<i>Others</i>												
1265	2-pentylfuran	Fruity, Green	0.7±0.0	nd	nd	nd	nd	2.3±0.1	0.2±0.0	0.9±0.0	2.2±0.2	1.4±0.1
1290	Styrene	Sweet, Floral	1.1±0.0	1.2±0.1	0.4±0.0	1.4±0.0	0.7±0.0	1.1±0.1	0.4±0.0	1.9±0.1	0.3±0.0	0.5±0.0
	Tot		1.8±0.0	1.2±0.1	0.4±0.0	1.4±0.0	0.7±0.0	3.4±0.1	0.6±0.0	2.8±0.1	2.5±0.0	1.9±0.1

*based on online databases (www.flavomet.org, and www.thegoodscentscompany.com)

Nd =Not detected

RAP=Relative Peak Area (Area Peak Compound/Area Peak Internal Standard)x100 (IS 4-methyl-2-pentanol) (RAP±SD)

RI= Retention Index , identification by comparison with RI database

In order to better understand the differences between the dough samples, a PCA of the 68 volatile compounds recorded in the different doughs was calculated as shown in **Figure 6 A** and **B**. The two PCs explained ca. 49.2% of the total variance of the data.

Seven substances positively loaded on PC1, including high positive loadings for isoamyl alcohol, 2-phenylethanol, ethyl decanoate, 3-nonen-1-ol, ethyl octanoate, 2-methylbutanoic acid, acetaldehyde, diethyl succinate, ethanol, 2-methylpropanoic acid, isobutyl acetate, ethyl heptanoate. Regarding PC2, the main positive contribution was due to 4-heptanone 2-methyl, 2-hexanol, 2,6-dimethyl-4-heptanone. Doughs, as determined by the two PCs (factors), were distributed in different zones of the plane. Regarding the scores plot, most sourdough samples was located in the centre of the plan. Just same samples Z, R, J, I and Q were located more distant respect to the other samples. In particular R and J samples showed negative scores on the PC1 and PC2 and entirely located in the lower left section of the graph, whereas sample Z was located in the lower right section of the graph and samples I and Q in the upper left section of the graph. So, these last samples (Z, R, J, I and Q) resulted very different compared to the most sourdoughs that, although showed some difference in volatile composition, were very similar.

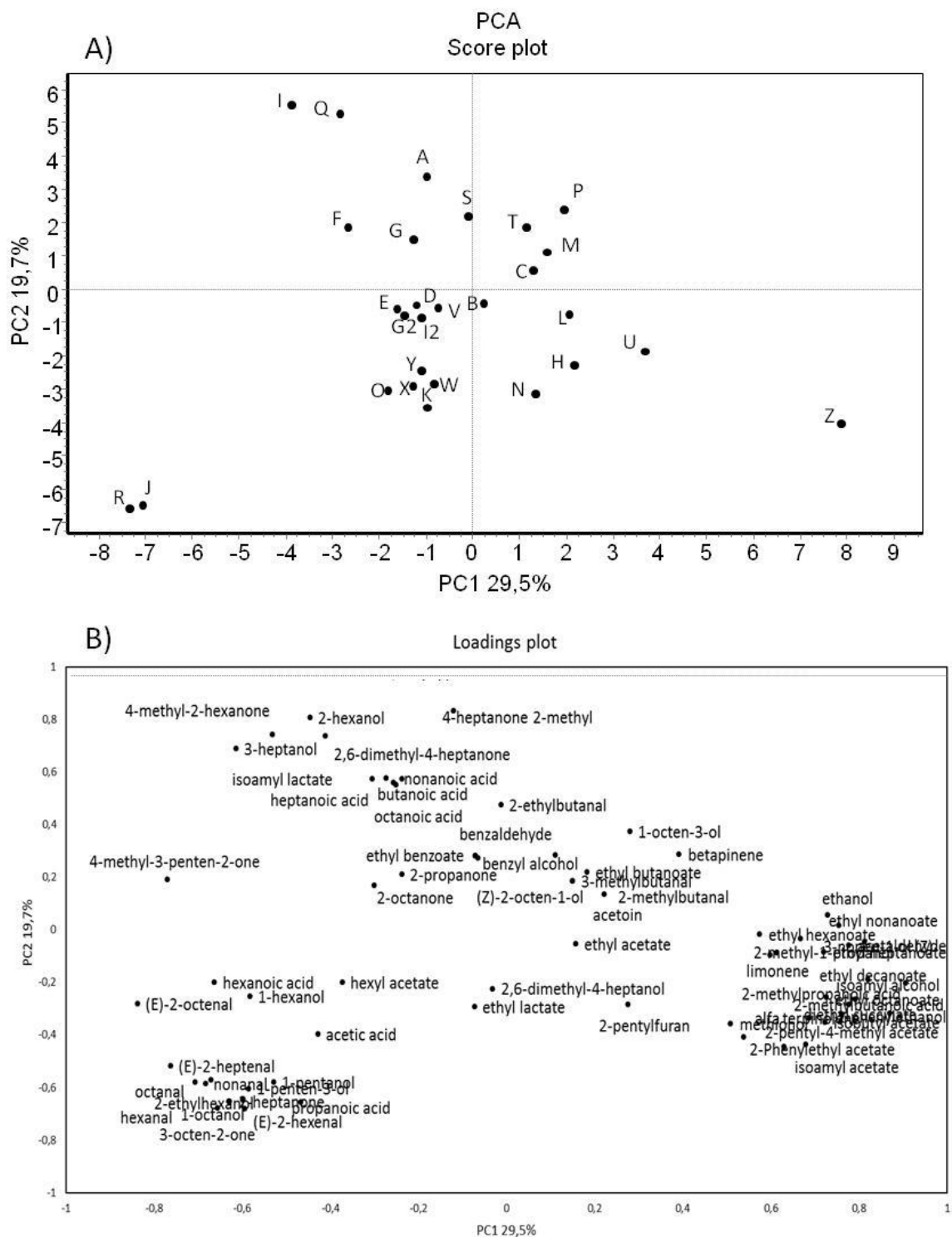


Figure 6. Score plot (A) and loading plot (B) of first and second principal components after PCA based on volatile components that mainly ($P < 0.05$) differentiated 28 sourdoughs samples. Volatile organic compounds used in PCA are listed in Table 2.

3.2.3 Lactic Acid Bacteria Identification

Biomolecular identification allowed to ascertain the presence of numerous species of LAB. Isolation at the highest dilution was used to achieve an approximate estimation of the dominant

sourdough lactobacilli and cocci lab species. **Figures 7 and 8** show dendrograms obtained by similarities among DGGE profiles of lactobacilli and cocci, respectively.

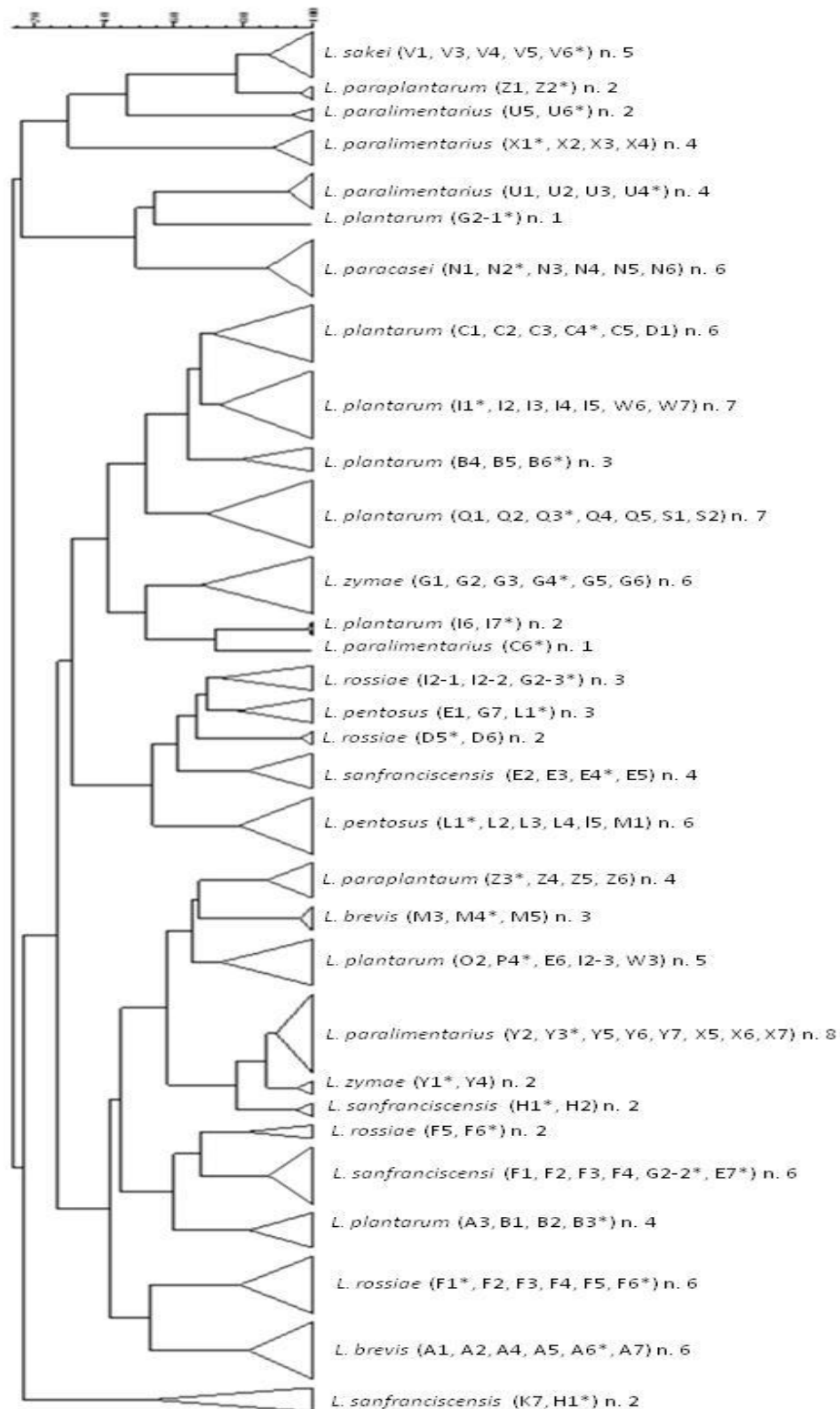


Figure 7. Dendrogram showing the similarity among DGGE profiles of 124 lactobacilli (Asterisks indicate the strains identified by sequencing).

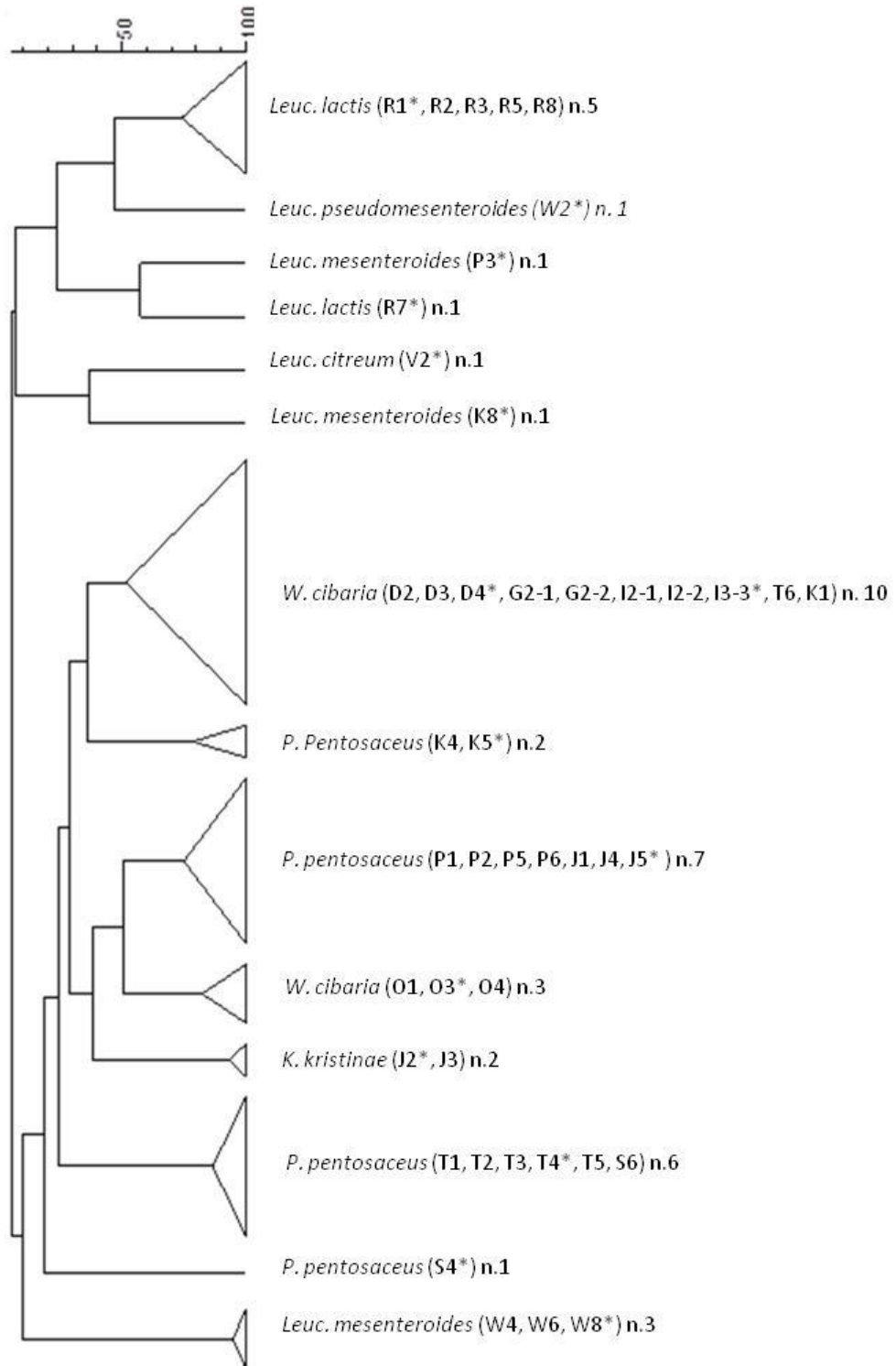


Figure 8. Dendrogram showing the similarity among DGGE profiles of 44 cocci LAB (Asterisks indicate the strains identified by sequencing).

Out of 168 isolates, 124 were lactobacilli and 44 were cocci. Lactobacilli were identified mainly as *L. plantarum* (20.8%), *L. paralimentarius* (11.3%), *L. brevis* (5.4%), *L. pentosus* (5.4%), *L. rossiae* (7.7%), *L. zymae* (4.8%) and *L. sanfranciscensis* (8.3%). A minor presence was registered for *L. paracasei* (3.6%), *L. paraplantarum* (3.6%) and *L. sakei* (2.9%). Lactococci were identified as *P. pentosaceus* (9.5%), and *Lc. lactis* (3.6%), *Leuc. mesenteroides* (3.0%), *Weissella cibaria* (7.7%), *Kocuria kristinae* (1.2%), *Leuc. citreum* (0,6%) and *Leuc. pseudomesenteroides* (0,6%). Genetic identification allowed to evidence a high microbial biodiversity in lactobacilli species usually occurring in typical sourdoughs; *L. plantarum* was found as predominant species in almost all the sourdoughs; *L. sanfranciscensis*, considered a key lab in sourdough, was found only in five sourdoughs; *Lb. sanfranciscensis* was first species reported in the San Francisco sourdough French bread process and it has been widely isolated from rye and wheat sourdoughs of several bread-producing areas. *Lb. sanfranciscensis* is considered a predominant bacteria in traditional production by various stages of continuous propagation (Gobbetti & Corsetti, 1997).

Species *L. zymae* and *L. rossiae* have been found in 1 and 4 sourdoughs, respectively. They are lab species not frequently isolated in sourdough, but important for the definition of typical sensorial characteristics of sourdoughs.

Observing the dendrogram in **Figure 8** is possible to evidence a high biodiversity also in cocci LAB species. *Weissella cibaria* and *Pediococcus pentosaceus* resulted the predominant species in 6 and 5 samples, respectively. Also *Leuconostoc* spp. was found as predominant species in different samples. In detail, *Leuc. lactis*, *Leuc. pseudomesenteroides*, *Leuc. mesenteroides*, *Leuc. citreum* were found in 1, 1, 3, and 1 sample, respectively. In one sample (J) was found also *Kocuria kristinae*. The presence of *Weissella* and *Leuconostoc* spp., obligately heterofermentative species, is important, because these genus has been traditionally considered one of the most important microorganisms able to synthesize EPS.

During sourdough fermentation, a selection of microbial population with specific nutrient requirements and growth conditions occurs; lactobacilli, among all the bacteria inhabitant of sourdoughs, are highly adapted to the environmental conditions (temperature, pH, acidity, antimicrobial products, etc.) of sourdough (Vera et al. 2009). For this reason, lactobacilli represent the dominant microbial group and commonly they occur with the highest concentration, especially in mature sourdoughs (Reale et al. 2011).

Table 4 - Microbial MAP of 28 sourdoughs

Geographical areas of sourdoughs	Samples	Microbial species																
		Lactobacilli										Cocci						
		<i>L. brevis</i>	<i>L. sanfranciscensis</i>	<i>L. paralimentarius</i>	<i>L. zymae</i>	<i>L. sakei</i>	<i>L. paracasei</i>	<i>L. paraplantarum</i>	<i>L. plantarum</i>	<i>L. rossiae</i>	<i>L. pentosus</i>	<i>Weissella cibaria</i>	<i>Pc. pentosaceus</i>	<i>Kokuria kristinae</i>	<i>Leuc. lactis</i>	<i>Leuc. pseudomesent.</i>	<i>Leuc. mesenteroides</i>	<i>Leuc. citreum</i>
Montella	A	■																
Avellino	B									■								
Serino	C			■														
Avellino	D									■								
Avellino	E		■															
Luogosano	F		■															
Volturara	G				■													
Avellino	G2		■															
Monteforte	H		■															
Avellino	I									■								
Avellino	I2																	
Sperone	J																	
Avella	K		■														■	
Grottaminarda	L																	
Grottaminarda	M	■																
CastelBaronia	N									■								
Mugnano del C.	O																	
Sant'Angelo dei L.	P																■	
Frigento	Q																	
Sperone	R																	
Mirabella Eclano	S																	
Montemiletto	T																	
Calitri	U			■														
Avellino	V																	■
Montecalvo	Z									■								
Montemiletto	W																	
Avella	X			■														
Avella	Y			■	■													

Table 4 shows that 10 samples (B, H, I, L, N, Q, R, U, Z, X) were characterized by the predominance of a single microbial species, 10 (A, C, F, J, M, O, S, T, V, Y) by two microbial species, 7 samples (D, E, G, G2, I2, P, W) by three species and in only one samples (K) occurred four lactic acid bacteria species.

Some authors found that *L. plantarum*, *L. paralimentarius*, *L. sanfranciscensis*, *L. rossiae* and *L. brevis* are the main species dominating sourdough fermentation processes that are characterised by low incubation temperatures and continuous backslopping (De Vuyst *et al.*, 2009). In our study, the main bacterial species isolated from the 28 sourdoughs were *L. plantarum*, detected in thirteen samples, *L. paralimentarius*, *L. rossiae* and *L. pentosus* detected in four samples, and *L. sanfranciscensis* detected in five samples (**Table 3**). These results are in agreement with other studies on the bacterial population in Sicilian (Randazzo *et al.*, 2005), Sardinian (Catzeddu *et al.* 2006), Apulian (Ricciardi *et al.*, 2005) Campanian and Molise (Coppola *et al.*, 1996; Reale *et al.*

2011) traditional sourdoughs, which also confirmed that *L. plantarum* has a high prevalence. Also Iacumin *et al.* (2009) found that *L. plantarum* and *L. brevis* are the dominant species occurring in traditional Italian sourdoughs and that *L. plantarum* was isolated singly or often in association with *L. brevis*. In a study on sourdoughs used for the production of Altamura bread Ricciardi *et al.* (2005) highlighted that sourdoughs had a similar composition in lactobacilli species; in fact 88% of isolates were identified as *L. plantarum*, *L. casei* and *L. paracasei* ssp. *paracasei*, 12% as *L. brevis* and *Leuc. mesenteroides*. Moreover *L. sanfranciscensis*, described as the dominant bacterial species in many traditional Italian baked products (Gobbetti *et al.*, 1994; Vogel *et al.*, 2002; Foschino *et al.*, 2001) was found in five samples.

3.3 Conclusion

The study provides an overall characterization of sourdoughs used to manufacture typical irpinian breads, characterized by distinctive nutritional and sensorial features. Results evidenced that Southern Italian sourdoughs are characterized by a major acidification respect to other Italian mature sourdoughs studied by other authors (Minervini *et al.*, 2012; Lattanzi *et al.*, 2013). Moreover, as reported in literature, in spontaneous sourdoughs, the LAB:yeast ratio is generally 100:1 (Minervini *et al.* 2012). In this study, this proportion was registered only for eight samples. Many sourdoughs, showed LAB:yeast ratio ranged from 10:1 to 1:1. Sourdoughs resulted populated by a mix of different and harmonious species of lactic acid bacteria evidencing a high biodiversity in the microbial community and the importance of LAB cocci in the sourdough ecosystem, along with lactobacilli. The recorded wide variety of volatile metabolites, jointly with the microbial profile, may be considered a reliable marker for bread tipicity.

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CHAPTER 4

**BIOTYPIZATION AND TECHNOLOGICAL CHARACTERIZATION OF LACTIC
ACID BACTERIA**

Due to increasing consumer demands for more natural, tasty and healthy food, the traditional process of sourdough bread production has been enjoyed renewed success in recent years, (Lopez *et al.*, 2003). Sourdough was traditionally used as leavening agents until it was replaced by baker's yeast in the 19th century (Corsetti & Setanni, 2007). Today, sourdough is employed in the manufacture of a number of products, such as breads, cakes and crackers (De Vuyst & Gänzle, 2005). Some of these products have strictly regional and artisanal character and some of these are widely distributed on the world market (De Vuyst & Neysens, 2005).

Many sourdough exist in the world and they differ in the type of flour, the added ingredients, the applied technology and the environmental conditions for bread production. These aspects cause the selection of a characteristic microflora that generally contains a complex mixture of lactic acid bacteria (LAB), in association with yeasts.

Aim of this chapter was the biotyping and the technological characterization of lactic acid bacteria isolated from typical southern Italian sourdoughs, previously collected. The objective was to select biotypes as putative starter cultures for bread production.

4.1 Materials and methods

Lactobacilli and cocci LAB strains previously identified were subjected to biotypization by RAPD-PCR and to technological characterization. Following the procedure utilized.

4.1.1 RAPD-PCR

Amplification reactions were performed in a 25 µl reaction volume containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 200 mmol/L of each dATP, dGTP, dCTP and dTTP, 1.5 mmol/L MgCl₂, 1 mmol/L primer, 80 ng DNA and 1.25 U Taq-DNA polymerase (Finnzymes, Finland). Amplifications were performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following primers and amplification conditions: (a) M13: 5' GAGGGTGGCGGTTCT 3' (Huey & Hall 1989); the amplification was carried out for 35 cycles of 94°C for 1 min, 40°C

for 20 s, ramp to 72°C at 0.5°C/s, 72°C for 2 min; (b) D8635: 5'GAGCGGCCAAAGGGAGCAGAC 3' (Akopyanz et al. 1992); after an initial step of 94°C for 2 min the amplification was performed for 35 cycles of 94°C for 1 min, 42°C for 1 min, 72°C for 1 min and 30 s, and a final step at 72°C for 10 min.

The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel (Sigma-Aldrich, Steinheim, Germany) in 0.59 TBE buffer and then subjected to Gel Red 1x staining. RAPD-PCR gels were digitally captured and analysed as previously described for DGGE analysis.

4.1.2 Technological characterization of LAB Strains

EPS production. EPS synthesis was determined on four different sugars: glucose, fructose, glucose, sucrose. EPS synthesis was preliminarily determined in triplicate by growing cell colonies on MRS agar plates modified with the different sugars (50 g/L). Cell suspension (OD₆₀₀= 0,3) was prepared in MRS broth by growing the isolates for 24 h at 30°C. Cells from 0,5 ml of culture were harvested by centrifugation (3,260 g, 5 min), washed with 1 ml of sterile water and resuspended in 0,2 ml of sterile water. Inoculation was performed by spotting 2µl of bacterial suspension (about 5x10⁷) on MRS (which contain glucose) and MRS–sucrose agar media. After incubation at 30°C for 24-48 h the isolates which produced slimy colonies were recorded as capable of producing EPS and classified according to visual appearance (compact, creamy or liquid slime). The diameter of the zone of the bacterial growth was also measured (Bounaix *et al.*, 2009). LAB were inoculated on MRS agar plate modified with Ruthenium Red and incubated at 28°C for 48h. The isolates, which produced white colored colonies were recorded as ropy (Ruas-Madiedo *et al.*, 2005).

Acidifying activity The acidifying activity of the strains was determined on MRS. The strains were revived in MRS broth at 28°C for 24 h. The microbial culture was inoculated (1% v/v) in MRS broth and incubated at 28°C for 24h. pH was measured after 6 and 24h of incubation. Results were expressed as ΔpH, i.e. the difference between the pH after 6 or 24h of incubation and the pH at time zero.

4.2 Results and Discussion

4.2.1 Biotyping of Lactic Acid Bacteria

In order to identify intraspecific differences between the different strains of lactic acid bacteria under study it was performed RAPD-PCR analysis by the use of D8635 and M13 primers. The

use of D8635 and M13 primer has allowed to obtain representative RAPD profiles of each block and can provide a good discrimination of the strains. These electrophoretic patterns were then processed using the GEL COMPARE software in order to identify the percentage of similarity between the different strains. The results obtained are reported in the dendrograms of **Figure 1, 2** (LAB of rod shaped) and **3** (LAB of coccica form).

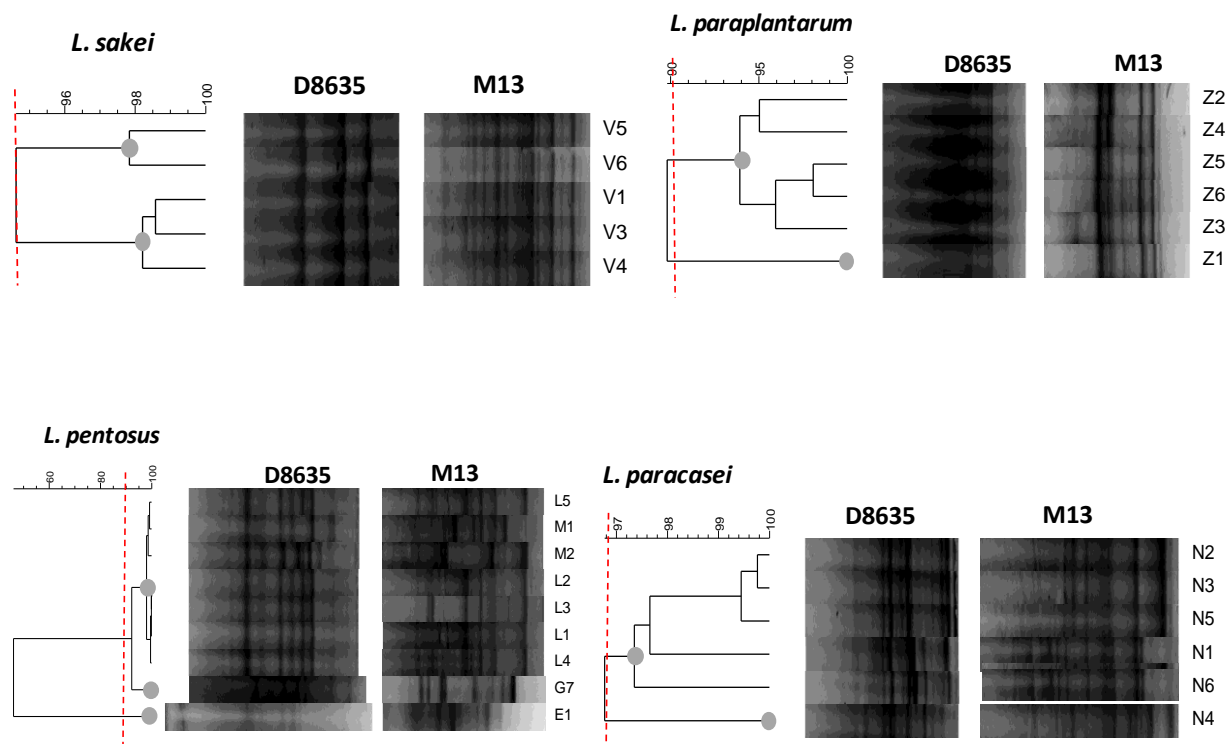
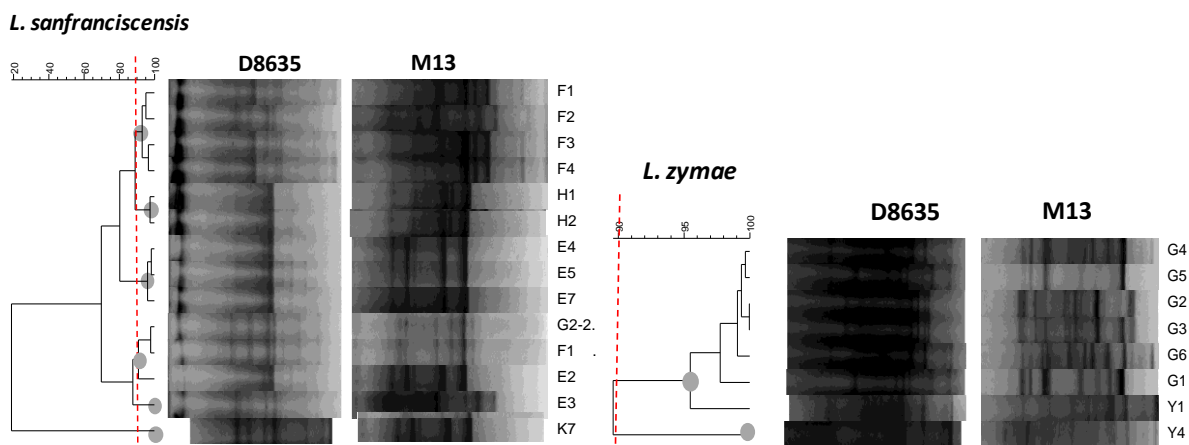


Figure 1. Dendrogram showing the similarity among RAPD-PCR profiles of lactobacilli belonging to the same species. In detail, are reported results related to *L. sakei*, *L. paraplantarum*, *L. pentosus*, *L. paracasei* species.



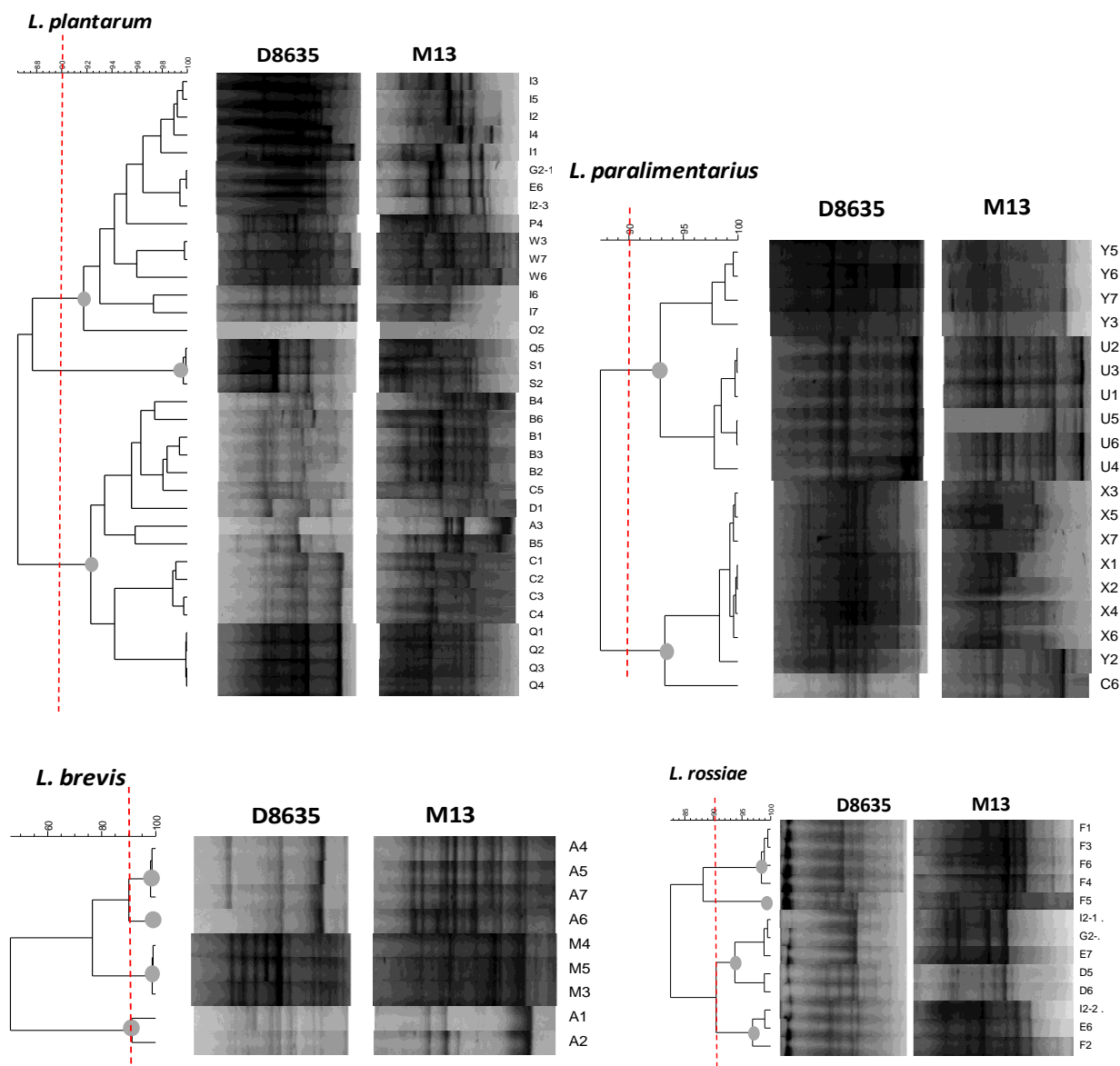


Figure 2. Dendrogram showing the similarity among RAPD-PCR profiles of lactobacilli belonging to the same species. In detail, are reported results related to *L. sanfranciscensis*, *L. zymae*, *L. plantarum*, *L. paralimentarius*, *L. brevis*, *L. rossiae* species.

RAPD-PCR technique revealed, as reported also by other Authors (Andrighetto et al. 2004; Catzeddu et al. 2006), to be a good tool for typing lactic acid bacteria and for obtaining information on genetic diversity.

For each species, distinct clusters emerged at the similarity level of <90%. In detail, we found a good biodiversity for all the species. However, a stronger biodiversity was observed between *L. sanfranciscensis*, *L. brevis* and *L. rossiae* strains compared to the other species of lactobacilli. In fact, considering the profiles of bands and results given by the cluster analysis, at the least 6, 4 and 4 different biotypes were individuated, respectively. Also, some strains seemed to be

genetically related even if they were isolated from sourdoughs produced in different bakeries and this fact was more evident for *L. rossiae*, *L. paralimentarius* and *L. plantarum* strains. As reported also by Reale *et al.* 2011, a great number of variables can differently affect the microbiota of mature sourdoughs, first of all specific technological parameters of production. So, presumably it can be assumed that strains genetically related, even if isolated from different doughs, could derive from the same flour probably used by a certain number of bakeries which were studied. In fact, as reported by De Vuyst *et al.* (2009) the type and the quality of the cereal flour used is indeed the source of autochthonous LAB and plays a key role in establishing stable microbial consortia within a short time. In addition it has been shown that previous introduction of flour into the bakery environment helps to build up a house microbiota that may serve as an important inoculum for subsequent sourdough fermentation, as sourdough and bakery environment isolates are genetically indistinguishable (Reale *et al.*, 2011).

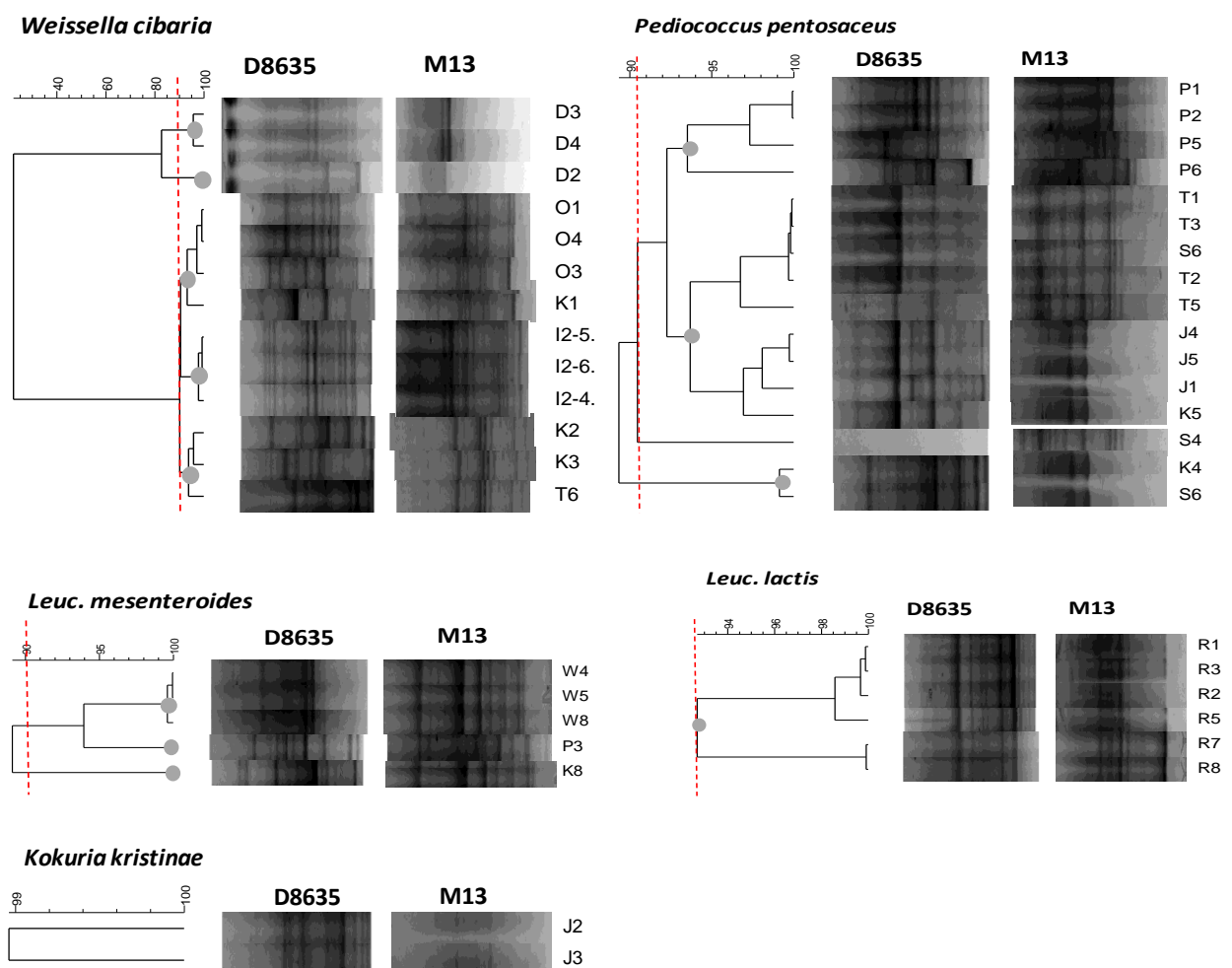


Figure 3. Dendrogram showing the similarity among RAPD-PCR profiles of cocci LAB belonging to the same species. In detail, are reported results related to *Weissella cibaria*, *Pediococcus pentosaceus*, *Kokuria kristinae*, *Leuconostoc mesenteroides*, *Leuconostoc lactis* species.

Regarding cocci LAB, we found a good biodiversity only for *Weissella cibaria*, *Leuc. mesenteroides* and *P. pentosaceus*. In fact, five and three different biotypes were found, respectively. A lower biodiversity was found for *Leuc. lactis*.

4.2.2 EPS Production

The LAB strains expressed different ability to produce EPS from different sugars in solid media (Table 1).

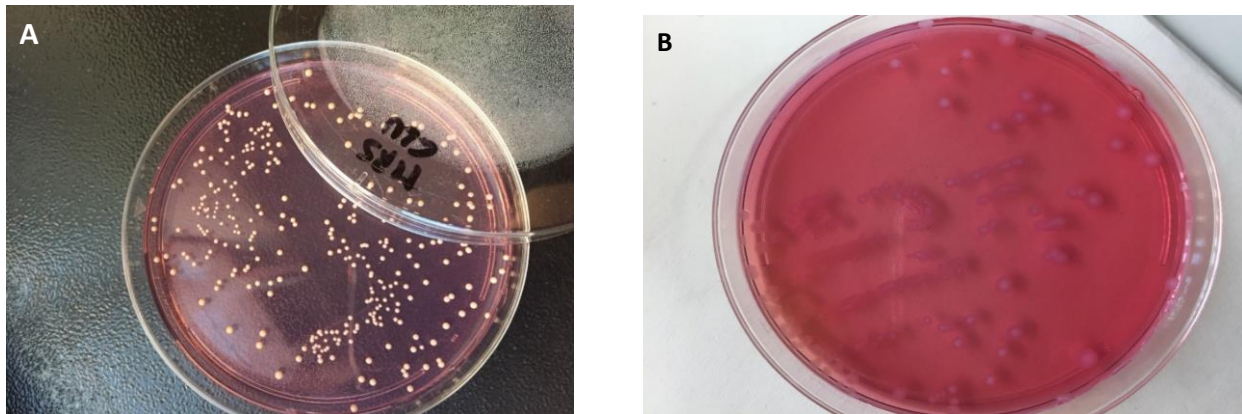


Figure 4 – Ruthenium red plate assay showing white colonies (A), EPS-producing strain from glucose, and red colonies, no EPS-producing strain from glucose (B).

Numerous strains were able to produce esopolysaccharides. Among lactobacilli, *L. plantarum*, *L. rossiae*, *L. zymae*, *L. paralimentarius*, *L. sanfranciscensis* and *L. pentosus* evidenced the highest ability to produce EPS from different sugars.

L. sanfranciscensis and *L. pentosus* strains produced EPS only from glucose, maltose and sucrose, whereas *L. plantarum*, *L. rossiae*, *L. zymae* and *L. paralimentarius* strains were able to produce EPS from all the sugars used. Few strains of *L. paracasei* (Figure 4) produced EPS from glucose and sucrose, whereas 1 strain of *L. paraplantarum* produced EPS only from glucose. None of the strains of *L. brevis* and *L. sakei* produced EPS.

Among cocci LAB, *Weissella cibaria* and *Leuc. mesenteroides* evidenced the highest ability to produce EPS. In detail, about *Weissella cibaria* specie, 6 strains were EPS producer from glucose, 1 from maltose and 4 from sucrose. Two strains of *Leuc. mesenteroides* were able to produce EPS from glucose and fructose. *Kokuria kristinae* and *Leuconostoc citreum* species produced any EPS. Only two strains of *Pediococcus pentosaceus* produced EPS from glucose, one strain of *Leuc. lactis* EPS from fructose and 1 *Leuc. pseudomesenteroides* produced EPS from glucose, fructose and maltose.

Table 1 - Number of strains of each isolated species able to produce EPS from different sugars

Species		EPS from			
		Glucose	Fructose	Maltose	Sucrose
lactobacilli	<i>L. zymae</i> (8)	3	4	3	2
	<i>L. plantarum</i> (35)	10	10	10	7
	<i>L. paralimentarius</i> (19)	4	6	4	4
	<i>L. paracasei</i> (6)	4	---	---	2
	<i>L. brevis</i> (9)	---	---	---	---
	<i>L. sanfranciscensis</i> (14)	6	---	6	6
	<i>L. pentosus</i> (9)	2	---	6	5
	<i>L. rossiae</i> (13)	10	2	10	6
	<i>L. sakei</i> (5)	---	---	---	---
	<i>L. paraplantarum</i> (6)	1	---	---	---
cocci	<i>W. cibaria</i> (13)	6	---	1	4
	<i>P. pentosaceus</i> (16)	2	---	---	---
	<i>K. kristinae</i> (2)	---	---	---	---
	<i>Leuc. lactis</i> (6)	---	1	---	---
	<i>Leuc. mesenteroides</i> (5)	2	2	---	---
	<i>Leuc. citreum</i> (1)	---	---	---	---
	<i>Leuc. pseudomesenteroides</i> (1)	1	1	1	---

The ability to produce EPS can be considered an important feature to select culture starter for breadmaking because numerous scientific studies ascertained that EPS favourably influence host of technological properties of dough and bread by facilitating water absorption, softening the gluten content of the dough, improving the dough rheology and machinability, increasing specific loaf volume, retarding bread staling and prolonging shelf-life (Korakly *et al.*, 2003; Patel *et al.*, 2012; Zannini *et al.*, 2015).

4.2.3 Acidifying capacity

All the strains of LAB showed a good acidifying ability and high variability among strains of the same species was highlighted.

In **Figures 5** and **6** for the acidifying capacity of 168 strains under study were shows the results. In particular, the acidifying capacity was expressed as Δ pH, obtained from the difference in pH between the initial values of the substrate (immediately after inoculation) and the final (after 6 and 24 hours of incubation). In detail, after 6h of incubation at 28°C Δ pH ranged between 0,1

and 1 unit (**Figure 5**); after 24h of incubation ΔpH values ranged between 0,7 and 2 unit (**Figure 6**).

This activity allowed to select strains with the best acidifying activity.

After six hours of incubation (**Figure 5**) all strains belonging to the species *Lactobacillus sakei*, *Lactobacillus brevis* and *Lactobacillus paracasei* exhibited low acidifying activity with pH values comprised between 0 and 0.5. Most of the strains belonging to the species *Lactobacillus paralimentarius* (80) and *Leuconostoc lactis* (75%) recorded ΔpH values comprised between 0 and 0.5 units.

On the contrary a good ability acidifying ($0.5 < \Delta\text{pH} < 1$), within the first six hours of incubation, was recorded for the whole of the strains belonging to the species *Lactobacillus rossiae*, *Lactobacillus paraplantarum*, *Weissella cibaria* and *Leuconostoc mesenteroides*. Almost all the strains belonging to the species *Lactobacillus plantarum* (85%), *Lactobacillus pentosus* (90%), *Lactobacillus sanfranciscensis* (75%) and *Lactobacillus pentosaceus* (72%) were recorded of ΔpH values between 0.5 and 1.

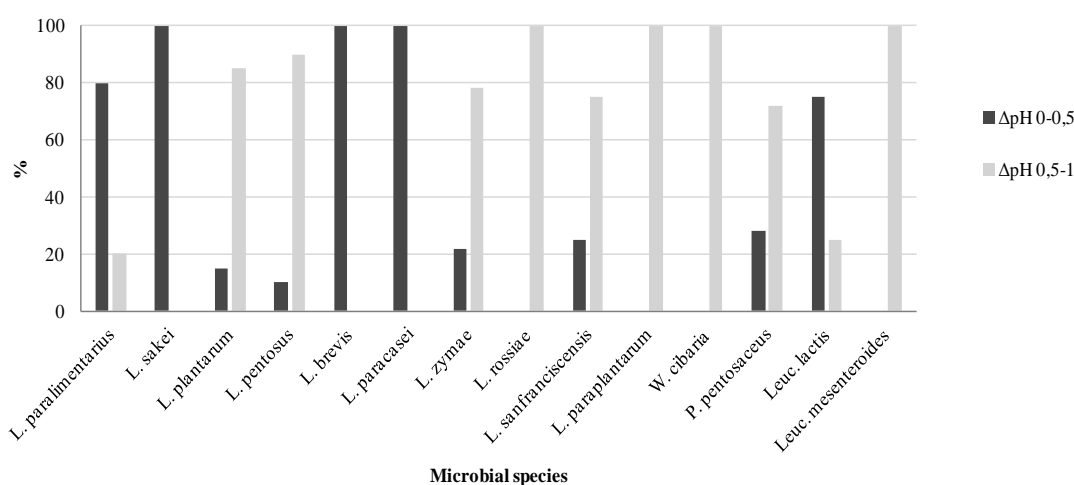


Figure 5 – Acidifying ability of 168 strains of LAB after six hours of incubation at 28 °C.

On the contrary a good ability acidifying ($0.5 < \Delta\text{pH} < 1$), within the first six hours of incubation, was recorded for the whole of the strains belonging to the species *Lactobacillus rossiae*, *Lactobacillus paraplantarum*, *Weissella cibaria* and *Leuconostoc mesenteroides*. Although almost all the strains belonging to the species *Lactobacillus plantarum* (85%), *Lactobacillus pentosus* (90%), *Lactobacillus sanfranciscensis* (75%) and *Lactobacillus pentosaceus* (72%) were recorded of ΔpH values between 0.5 and 1.

After 24 hours of fermentation (**Figure 6**) of the strains was good record of ΔpH values between 1 and 2. The strains belonging to the species *Weissella cibaria* and *Lactobacillus plantarum* group (which includes the species *Lactobacillus plantarum*, *Lb. paraplantarum* and *Lb. pentosus*) exhibited a similar decrease in pH.

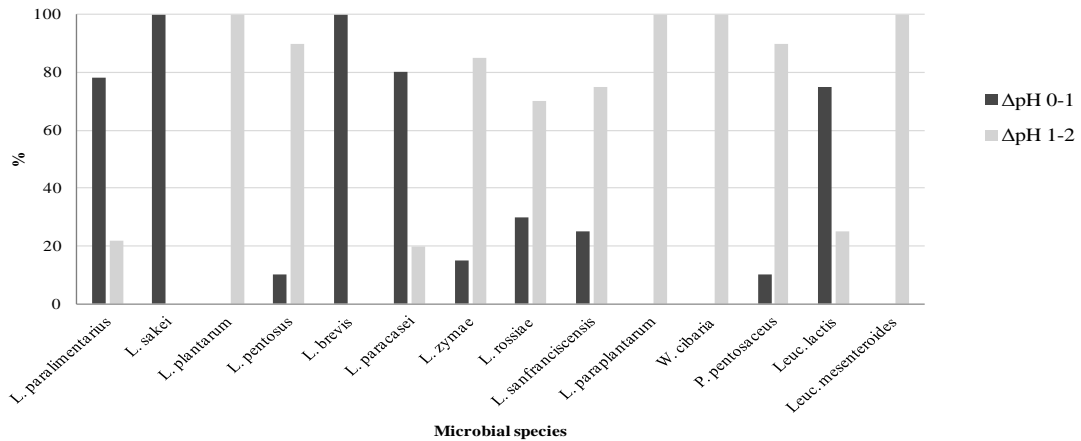


Figure 6 – Acidifying capacity of 168 strains of LAB after 24 hours of incubation at 28 °C.

Almost all the strains belonging to the species *Lb. zymae*, *Lb. rossiae*, *Lb. sanfranciscensis* and *P. pentosaceus* showed, after fermentation, low pH resulting in a ΔpH comprised between 1 and 2. All the strains belonging to the species *Leuconostoc mesenteroides* had the highest levels of acidifying capacity.

The acidification of the dough, due to the production of lactic acid by homofermentative lactic acid bacteria strains and lactic and acetic acids by heterofermentative ones, is an important activity for the definition of sensorial features of bread.

The traditional sourdough fermentation in bread making allows natural acidification that is able to control rope spoilage. Studies have shown that starting wheat bread with increasing sourdough contents enhances thermal inactivation of *B. subtilis* spores. However, although there are many advantages associated with sourdough fermentation in bread making, this process is quite complex and time-consuming compared to the simpler straight dough process.

4.3 Selection of sourdough

After microbiological and technological characterization of the 28 typical sourdoughs, one was selected to be used in the subsequent experimentation of stabilization by drying, freezing, freeze drying, spray drying methods. Firstly, the parameter used to select the sourdough were reported

in Tab.2: a) low pH (<4,0); b) high TTA (>10); c) high LAB (>7 log cfu/g) and Yeast (>6 log cfu/g) count.

Table 2 - First screening for the selection of sourdough				
Sourdough Samples	pH<4	TTA>10	LAB>7 log cfu/g	YEAST>6log cfu/g
A				
B				
C				
D				
E				
F				
G				
G2				
H				
I				
I2				
J				
K				
L				
M				
N				
O				
P				
Q				
R				
S				
T				
U				
V				
W				
X				
Y				
Z				

*Grey colour means positive data; Black colour means negative data

After the first screening, samples B, E, G2, H, I2 and Y, positive to all the parameter considered, were selected for biodiversity in lactic acid bacteria (number of predominant species found in

sourdough), PCA position in the PCA score plot, presence of strain producing EPS and acidifying ability of strains (**Table 3**).

Table 3 – Second screening for the selection of sourdough				
Sourdough	Number of predominant LAB	PCA plane	Acid production	EPS production
B	1 species (<i>L. plantarum</i>)	IV quadrant	+	+
E	3 species (<i>L. plantarum</i> , <i>L. sanfranciscensis</i> , <i>L. pentosus</i>)	III quadrant	+ '-'	+,+,-
G2	3 species (<i>L. sanfranciscensis</i> , <i>L. rossiae</i> , <i>W. cibaria</i>)	III quadrant	+ '+'	+,+,-
H	1 species (<i>L. sanfranciscensis</i>)	IV quadrant		+
I2	3 species (<i>L. plantarum</i> , <i>L. rossiae</i> , <i>W. cibaria</i>)	III quadrant	+ '+'	+,+,+
Y	2 species (<i>L. paralimentarius</i> , <i>L. zymae</i>)	III quadrant		+

Samples E, G2, I2, were selected because they were characterized for a major number of sourdough predominant species (3 species) compared to samples B and H, characterized for only one predominant species, and Y sample, having two predominant species.

Respect to the volatile composition samples E, G2 and I2 were very similar, in fact, they had the same position (III quadrant) in the plane of PCA score plot.

At last I2 sample, composed by *L. plantarum*, *L. rossiae*, *W. cibaria* species, was selected because all the predominant strains were characterized for ability to produce EPS and for good acidifying activity.

4.4 Conclusion

In conclusion, the present work confirms previous findings, showing that *L. plantarum*, occurring singly or in association with other lactic acid bacteria, are the main lactobacilli found in the type I sourdoughs used in traditional southern Italy. If DGGE analysis and 16S rRNA gene sequencing result a suitable multiple approach to identify lactobacilli from sourdough, RAPD-PCR analysis represents an important tool in order to individuate genotypic differences between strains from the same species. In fact, RAPD-PCR highlighted a good biodiversity among strains belonging to the same species and isolated from the same dough. This evidence should be

considered as a feature which positively influences the characteristics of natural sourdoughs. In this study were also selected the strains with the best acidifying and EPS production activities.

Microbiological and technological characterization of sourdough and lactic acid bacteria allow to select a representative sourdough type I to be used in subsequent experiment of sourdough preservation.

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PRESERVATION OF SOURDOUGHS: COMPARISON OF FREEZING, FREEZE DRYING, DRYING AND SPRAY DRYING TECHNIQUES

Starter cultures provide a basis in the production of fermented foods. Commercial starter cultures were initially supplied in liquid form prior to the production of concentrated starter cultures.

Progress in biotechnology later led to the application of concentrated starter cultures in frozen and freeze dried forms for direct incorporation into the food formulation.

Application of frozen or freeze-dried starter cultures eliminates in-plant sub-culturing, reduces the costs associated with bulk culture preparation and lowers the risk of bacteriophage infection (Desmod *et al.*, 2002). Very low transportation and storage temperatures are the main commercial disadvantages of frozen starter cultures (Ghandi *et al.*, 2012). Besides the risk of thawing, high transportation costs may limit the use of frozen starter cultures in distant areas or countries.

Starters cultures are usually preserved by freeze thawing and lyophilization. In spite of being efficient methods, freezing and freeze drying have high manufacturing costs and energy consumption. For this reason, increasing attention has been paid on alternative drying processes such as spray drying, fluidized bed drying and vacuum drying.

Spray drying is considered a good long-term preservation method for starter cultures. The speed of drying and continuous production capability are very useful for drying large amounts of starter cultures. Since then, much research has been reported on the spray drying of bacteria without loss of cell activity in order to overcome the difficulties involved in handling and maintaining liquid stock cultures.

Spray drying is a common industrial and economic process for the preservation of microorganisms and for the preparation of starter cultures that are used to prepare lactic fermented products. Aim of this chapter was, therefore, to compare different techniques of preservation in order to identify the one that could better preserve the microbial characteristics of the sourdough.

To this end, a sourdough, previously characterized by chemical-physical and microbiological conditions, has been subjected to different storage modes: freezing, drying, freeze-drying and spray drying. Microbial vitality and chemical-physical properties of the doughs after the preservation treatments were assessed.

5.1 Materials and Methods

5.1.1 Sourdough Sample

A selected fresh sourdough, previously characterized for pH, TTA, lactic acid bacteria and yeast counts (chapter 4), was used to compare four preservation techniques: freezing, drying, freeze drying and spray drying. The procedures were following described.

5.1.2 Preservation Techniques

1) Freezing

Fresh sourdough was frozen overnight at -18°C for 24 h in sterile air-tight-capped containers (Sarstedt, Germany) and stored at -18°C for 6 months. Two replicates were carried out for each experiment.

2) Drying

Fresh sourdough was delivered into sterile Petri dishes and dried in a dryer at 40°C for 48h in under vacuum condition. The dried samples were packed in sterile air-tight-capped containers and stored at room temperature for 6 months. Two replicates were carried out for each experiment.

3) Freeze Drying

Fresh sourdough was frozen at -40°C and then desiccated under vacuum at $150\ \mu\text{Pa}$ in a freeze-drier Genesis 25ES for 48 h (the condenser was cooled at -40°C and the shelves were heated at $+20^{\circ}\text{C}$). The resulting freeze-dried powders were packed in sterile air-tight-capped containers and stored at room temperatures for 6 months. Two replicates were carried out for each experiment.

4) Spray Drying

a) Optimization of Spray Drying Condition

Spray drying experiments were carried out in a Büchi mini B-290 spray dryer equipped with a two-fluid nozzle (Buchi Labortechnik AG, Switzerland) (**Figure 1**). To optimize spray drying conditions, different batches of sourdough feed solution at different concentrations (sourdough: distilled water or physiological solution) were spray dried. Spray drying experiments were performed at varying inlet air temperatures, different % aspiration and flow rate. Each

experiment was carried out in duplicate. Lactic acid bacteria and yeast count and drying yield were estimated to evaluate the optimal spray drying conditions.



Figure 1. Büchi mini B-290 spray dryer.

b) Optimal Spray Drying Condition

The optimal spray drying condition was obtained using fresh sourdough dissolved in sterile distilled water (1:5) and spray-dried with inlet and outlet air temperatures of 130°C and 54°C, respectively. The optimal spray drying condition was compared with the other techniques above described.

c) Optimization of Rehydration Procedures

Rehydration is considered to be a critical step in the recovery of spray-dried starter cultures. The solution used for rehydration and the rehydration conditions may affect the survival of dried microbial cultures. For this reason sprayed sourdough was suspended in different rehydration media: a) skim milk (10%) in distilled water, SM; b) physiological solution, PHI; c) skim milk

(10%) plus sucrose (1%) in distilled water, SMSUCDW; d) skim milk (10%) plus sucrose (1%) in physiological solution, SMSUCPHI; e) flour plus skim milk (10%) in distilled water, FSMDW; f) flour plus skim milk (10%) in physiological solution, FSMPHI; g) flour plus sucrose (10%) in distilled water, FSUCDW; h) flour plus sucrose (10%) in physiological solution, FSUCPHI. Spray dried sourdoughs were dissolved in the media described above and rested for 30 min at 28°C. Lactic acid bacteria and yeast counts were carried out to evaluate the microbial survival.

5.1.3 pH and Total Titratable Acidity (TTA) Assessment

The pH values were determined with a pHmeter Medidor PH Basic 20 (CRISON, Spain). TTA was measured on 10g of sample (fresh or freeze/dried/freeze dried/sprayed samples), homogenized with 90 mL of distilled water for 1 min in a Stomacher laboratory blender (Bag Mizer 400, Interscience, France) and was expressed as the amount (mL) of 0.1 N NaOH necessary to achieve pH 8.3.

5.1.4 Water Activity

The water activity of the powders was measured using a water activity meter (Aqualab, Decagon Devices, USA) at a constant temperature of 23 ± 1 °C. Two readings were made for each sample.

5.1.5 Drying Yield for Spray Dried Powders

After spray drying, the drying yield (%) was determined as the % of the powder weight collected from the receiver to the initial amount of solids contained in the solution feed.

5.1.6 LAB and Yeast Viability

For LAB and yeast counts, samples were prepared according to the following procedure: 10 g of each sample was aseptically transferred into a sterile stomacher bag and diluted with 90 mL of physiological solution (9 g/L NaCl). After 1 min of agitation in Stomacher, the samples were serially diluted and plated in duplicate. LAB were counted using MRS (Oxoid, Milan, Italy) agar medium supplemented with 4 mg/100 mL cycloheximide (SIGMA Aldrich, Germany) after incubation at 30°C for 72 h in anaerobic conditions (Gas Pack AnaeroGen™, Oxoid). Yeasts were counted using YPD Agar (20.0 g/L dextrose, 20.0 g/L bacteriological peptone, 20.0 g/L agar, 10.0 g/L yeast extract) after incubation at 28°C for 72h. Plates with 30-300 colonies were selected and the colonies were counted and the cfu/mL calculated. Microbial counts were

transformed to logarithmic reduction using the equation: $\log(N/N_0)$, where N is the microbial cell count in the fresh sourdough and N_0 is the microbial cell count immediately after treatments.

5.2 Results and Discussion

5.2.1 Optimization of spray drying condition

Before to compare the different techniques to stabilize sourdough, the spray dry conditions were optimized. For this purpose three different experiments were carried out, changing inlet air temperatures, % aspiration and flow rate as reported in **Table 1**. Moisture, dried matter, ash, a_w , pH, TTA, drying yield, LAB and yeast counts were assessed to evaluate the optimal spray dry condition.

Table 1 - Spray dry conditions

	A	B	C
Temperature inlet	120 °C	130°C	130°C
Temperature outlet	70°C	60°C	54°C
Aspiration	100%	95%	95%
Pump	15%	20%	30%

Table 2 shows the results obtained in the three spray drying experiments. All the experiments allowed to produce a sourdough powder with low moisture content ranged from 7,56 % (A) to 6,23 % (B) and low a_w values ranged from $0,202 \pm 0,02$ (A) to $0,199 \pm 0,04$ (B). Very similar resulted the values of pH and TTA, whereas ash values differed mainly in the sample A-SDS (4,493 % d.m.) respect to the other samples. Sourdough powder A resulted in the highest ash value because the sourdough was originally diluted in physiological solution (1:2) containing salt. Experiment A was not considered as the optimal condition because high amount of salt in the sourdough powder could negatively compromise cell viability during storage. Moreover this condition produced a low drying yield (36.3%). In the experiment B sourdough was diluted in distilled water (1:2) allowing to record low ash value (0.814%) but low drying yield (35.5%), too. Experiment C, instead, produced the highest drying yield (56%) thanks to a higher % aspiration and % pump value. Moreover in the experiment C sourdough was diluted in distilled water 1:5 to facilitate the atomization.

Table 2 - Optimization of spray drying condition

Experiment			Moisture (%)	DM (%)	Ash (% d.m.)	aw	pH	TTA	Drying yield (%)
A	Fresh Sourdough	A-FS	41.00 ± 0.21	59.01	0.871	0.995±0.01	4.0±0.1	6.0±0.1	---
	Spry dried sourdough diluted in physiological 1:2	A-SDS	7.56 ± 0.07	92.44	4.493	0.202±0.02	4.0±0.2	6.2±0.0	36.3
B	Fresh Sourdough	B-FS	42.6 ± 0.05	57.4	0.559	0.989± 0.03	4.1±0.1	5.8±0.1	---
	Spry dried sourdough diluted in water 1:2	B-SDS	6.23 ± 0.08	93.77	0.814	0.199± 0.04	3.9 ±0.0	6.5±0.1	35.5
C	Fresh Sourdough	C-FS	43.0 ± 0.14	57.0	0.732	0.991± 0.02	4.3±0.0	6.1±0.1	---
	Spry dried sourdough diluted in water 1:5	C-SDS	7.09 ± 0.31	92.91	0.866	0.200± 0.01	3.9 ±0.1	6.7±0.2	56.0

Furthermore, the experimental condition C made it possible to spray dry at a low outlet air temperature (54°C) respect to experimental conditions A and B that had a higher outlet air temperature of 70°C and 60°C, respectively. This fact, strongly affected the survival of LAB and yeasts. Sourdough powder obtained in the Experiment C, in fact, after spray dry, showed a reduction of only 1 logarithmic cycle respect to those obtained in the Experimental A and B that showed a reduction of 2 logarithmic cycle both for yeasts and LAB (**Figure 1**).

As reported by Peighambardoust *et al.* (2011) the outlet air temperature is believed to be the major drying parameter affecting the viability of spray-dried starter cultures. This parameter depends on the inlet air temperature, air flow rate, product feed rate and medium composition, and atomized droplet size.

So, the optimal spray drying condition was obtained using fresh sourdough dissolved in sterile distilled water (1:5) and spray-dried with inlet temperatures of 130°C, 95% aspiration and 30% flow rate. This spray drying condition was compared with the other techniques.

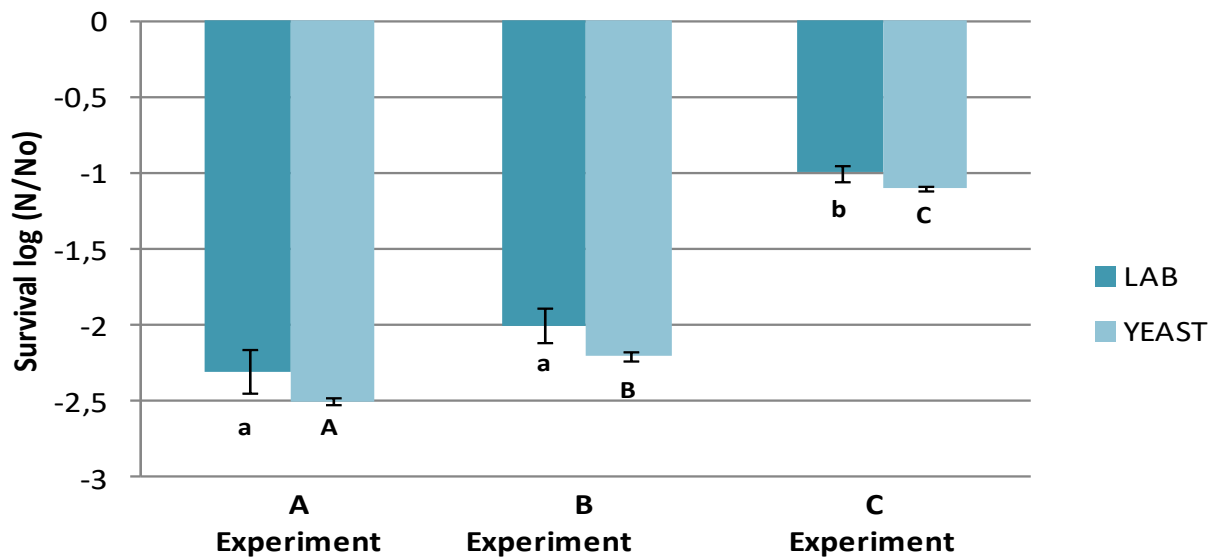


Figure 1. Survival of LAB and yeast in sourdoughs in different spray dry conditions. Survival was expressed as reduction of $\log(N/N_0)$ cycles, where N_0 and N are the number of viable cells, respectively, before and after sprayzation. Letters on plot bars indicate significant differences (Tukey's HSD, $p < 0.005$) in survival within lactic acid bacteria (a, b) and yeast (A, B, C) grown in the different conditions. Standard error bars are shown.

Figure 2 shows that spray drying let to the highest survival both of yeasts and lactic acid bacteria respect to the other techniques. Freezing strongly affected the microbial survival reaching a reduction of about 4 logarithmic cycles both for LAB and yeasts. Drying and freeze drying determined a reduction of almost 3 logarithmic cycles.

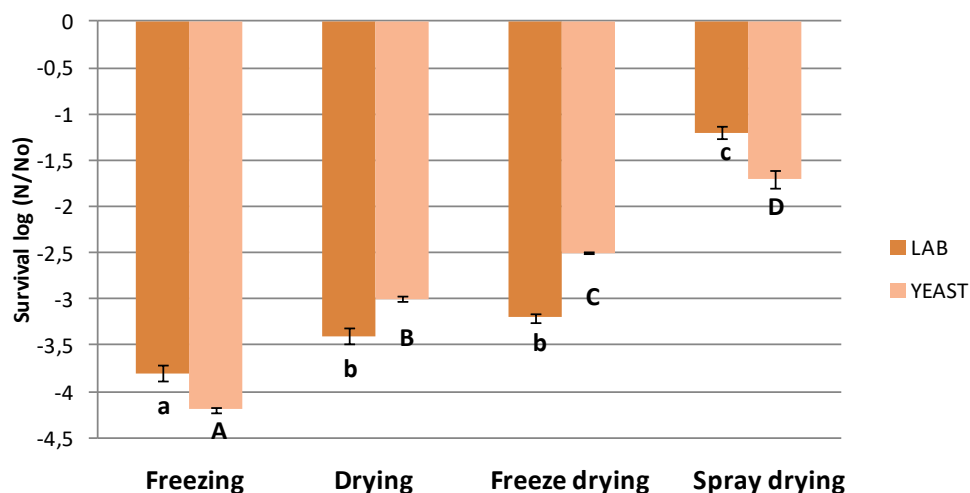


Figure 2. Survival of lactic acid bacteria and yeast in sourdoughs after different storage techniques. Survival was expressed as reduction of log (N/N₀) cycles, where N₀ and N are the number of viable cells, respectively, before and after exposure to the different conditions. Letters on plot bars indicate significant differences (Tukey's HSD, $p < 0.005$) in survival within lactic acid bacteria (a, b, c) and yeast (A, B, C, D) grown in the different conditions. Standard error bars are shown.

Then, the sourdough powders were used in dough leavening experiments (data not shown). Results showed that both freeze sourdough and dried/freeze-dried/spray-dried sourdoughs had relatively a long lag phase before onset of growth, determining a delay in leavening of the dough. Our experiments highlighted, as also showed by Boza *et al.* (2004), that the dried/freeze-dried/spray-dried starter cultures cannot be used for direct inoculation in breadmaking fermentations.

For this reason in the breadmaking experiment a refreshment of the injured sourdoughs was required and a microbial rehydration procedure was optimized. The best rehydration procedure consisted in mixing the treated sourdoughs with equivalent part of wheat flour plus sucrose (10%) in physiological solution, FSUCPHI (data not shown).

5.3 Conclusion

The results showed that among the different storage techniques tested, spray drying showed the highest survival both of yeasts and lactic acid bacteria compared to the other techniques.

5.4 References

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BREAD PRODUCTION BY STABILIZED SOURDOUGH STARTER

The use of sourdough is one of the oldest processes in the production of breads. Sourdough is a heterogeneous population of yeasts and LAB resulting from a fermentation of a flour dough and water (Aponte *et al.*, 2013, Moroni *et al.*, 2011). These LAB are the main responsibilities for producing amino acids that contribute to the flavor of the product (Thiele *et al.*, 2002, Guerzoni *et al.*, 2007). The bread flavour is considered as the most important attribute consumer acceptability (Heenan *et al.*, 2002). Non volatile components provide basic tastes such as sweet, sour, salty, bitter, etc. While volatile compounds, despite being in very low concentrations, contribute to the overall flavour of the bread (Plessas *et al.*, 2011, Choi *et al.*, 2012).

Fermentation performed with sourdough is slower and more acidic than that produced with commercial yeast (Barber *et al.*, 1991, Meignen *et al.*, 2001). The formations of different acids have different effects on the fermentation and baking process, depending on the concentration in which they are.

The advantages of using sourdough with respect to the commercial baker's yeast are: a) better preservation of the product due to the greater acidity of the mass that retards the development of fungi; b) the flavour and aroma accentuated by the formation of volatile organic compounds and aromatic products which are formed during cooking between amino acids and sugars and c) the consumption of bread made with natural yeast brings a nutritional benefit because of the acidic mass makes decrease postprandial glucose response and increase the bioavailability of minerals of bread (Choi *et al.*, 2012, Lappi *et al.*, 2010).

However, sourdough ecosystem can easily undergo modification due to factors affecting the management and preservation of the dough itself, such as modification of the ingredients used and the type of flour, the change in the storage temperature, the number of refreshments made, the hygienic conditions of the processing environment and the operator. Furthermore, the maintaining of a fresh sourdough starter requires a little extra time and effort because daily or weekly refreshments are necessary to guarantee its good vitality.

For these reasons, numerous industrial bakeries use sourdough of type III, that is the most convenient way to introduce authentic bread taste into nowadays high-tech bakery industry. Sourdough of type III consist mainly in different preparations of dried or lyophilized sourdoughs

which are often characterized by reduced microbial vitality and which almost always include the addition of "*Saccharomyces cerevisiae*" yeast, which is presented as an "activator" but which actually is responsible only for the leaven, often causing flattening of the aromatic characteristics of the finished products.

Aim of this chapter was the assessment of the effect of stabilized sourdough starters on sensorial characteristic of bread. To this end, the stabilized sourdough starter were used in breadmaking manufacture to assess the ability to leaven and ferment the doughs. The breads obtained were subjected to sensorial analysis and digital image analysis. Positive and encouraging results were obtained mainly in the spray-dried sourdough.

6.1 Material and Methods

6.1.1 Bread Production by Stabilized Sourdough

Six different breads were produced: fresh selected sourdough (FS); freeze sourdough (FZS), refreshed freeze sourdough (RFZS), dried sourdough (DS), freeze dried sourdough (FZDS), sprayed sourdough (SS) samples were used in breadmaking experiments.

Before use, freeze refreshed sourdough (FZRS), dried sourdough (DS), freeze dried sourdough (FZDS), sprayed sourdough (SS) were refreshed with equivalent part of flour, 10% sucrose and 60% physiological solution, and incubated at 28°C for 20h. After refreshment bread doughs were prepared. The basic ingredients were: wheat flour, sourdough (20%), salt (1.5%), and water (60%). The dough was kneaded in a mixer for 20 min, after which the dough was rested for about 10 min in a proofer, after that it was kneaded for others 10 min. Doughs were cut, formed and placed in the molds (250 gr). Then followed leavening for about 4-5h at 28°C. In the production laboratory breads were baked, cooled and evaluated. Baking was carried out at 225 ± 5 ° C for 60 min in a deck oven. Loaves were allowed to cool for 120 min at room temperature.

6.1.2 Sensory analysis

A sensory evaluation test was conducted for all breads. The samples were evaluated by 20 evaluators, aged 30-50 years, with food expertise, at Dep. AAA, University of Study of Molise. The samples were sliced into equally sized pieces and served as coded randomized duplicates. Bread produced with fresh sourdough was used as a standard. The comparative preferences were rated on a 7-point hedonic scale (7= excellent, 6= very good, 5=good, 4=satisfactory, 3=unsatisfactory, 2=bad, 1 = very bad) for appearance, flavor, texture and overall quality.

6.1.3 Image Acquisition and Digital Images Analysis

Bread crumb structure was determined by digital image analysis. The image acquisition of bread slices took place after 24h from baking. For each bread sample, three loaves were sliced transversely using an electric slicer to obtain 15 mm thick slices. They were scanned in colour and in black and white images using a flatbed scanner (HP ScanJet 8300, Hewlett Packard Co., CA, USA) with 300 dpi of resolution and the following settings: highlight 70, shadows 58, and midtones 0.5. The images were saved in TIFF format.

Images were analyzed by Software Image-Pro Plus 4.5 (Media Cybernetics, Georgia, USA, Windows 98), to evaluate the distribution of aveoli and determine the ratio between alveolar

cross section and slice areas. The number of alveoli and pore circularity values were categorized in different class.

6.2 Result and Discussions

6.2.1 Sensory analysis

So, all the sourdough samples were rehydrated and were rested at 28°C for 20h; after that they were used as starter for breadmaking. The doughs took about 4-5 h at 28°C to leaven.

In **Figure 1** the images of the slices of breads obtained using the different sourdough powders are showed. A fresh sourdough was used as references. Samples SS (sprayed sourdough) and RFZS (refreshed freeze sourdough) showed good overall quality, very similar to the FS-control. FZS, DS and FZDS, instead, showed reduced volume.

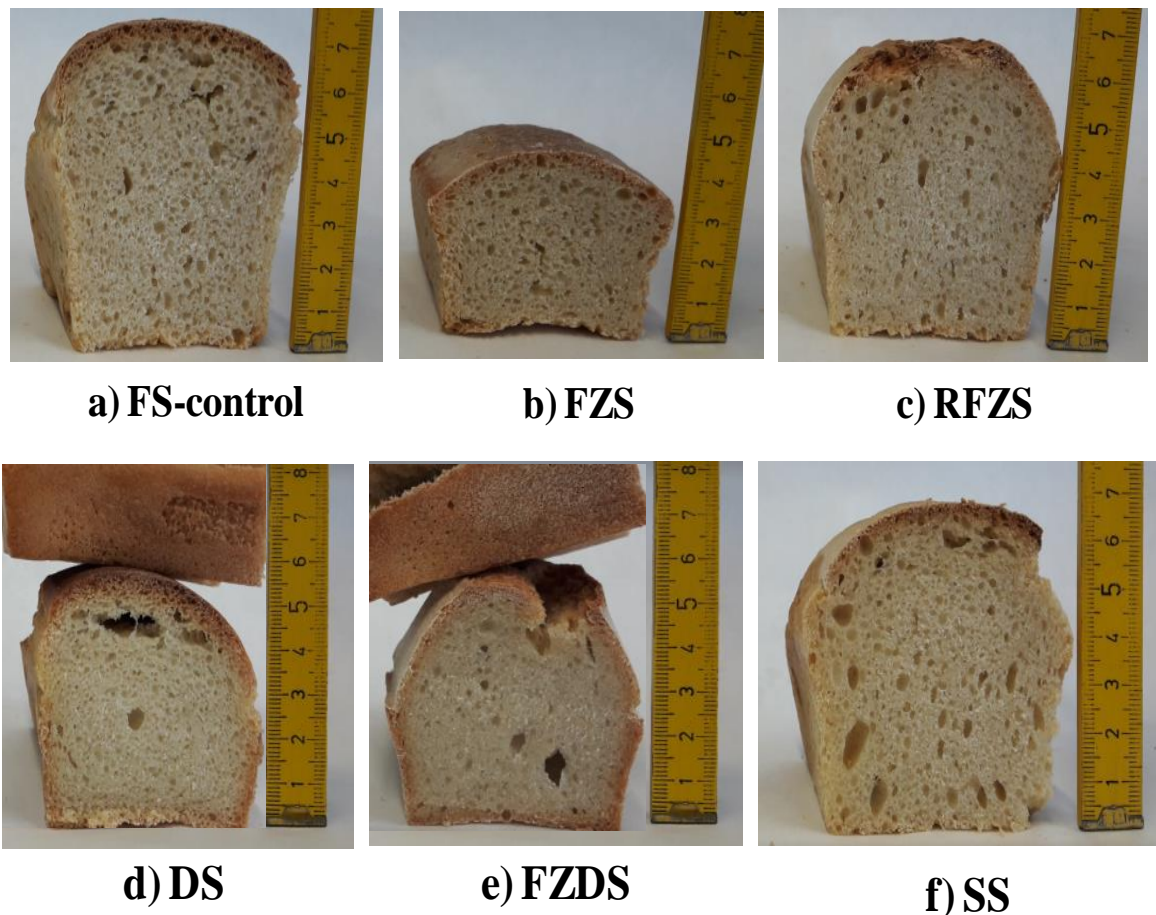


Figure 1. Images of slices of different bread samples: a) bread produced with fresh selected sourdough (FS-control); b) bread produced with freeze sourdough (FZS); c) bread produced with refreshed freeze sourdough (RFZS); d) bread produced with dried sourdough (DS); e) bread produced with freeze-dried sourdough (FZDS); f) bread produced with spray-dried sourdough (SS) samples.

The sensory scores showed that not all the bread samples were acceptable (**Figure 2**).

In particular FS-control, FZS and FZDS samples had lower values on texture and appearance, depending by a reduced development of the dough and by a low leavening ability of the sourdough starter.

Sensory evaluation of the breads revealed significant differences regarding flavour, texture and appearance between FS-control and FZS, DS and FZDS breads. Instead, not significant differences were observed between FS-control and SS and RFZS samples respect to overall quality.

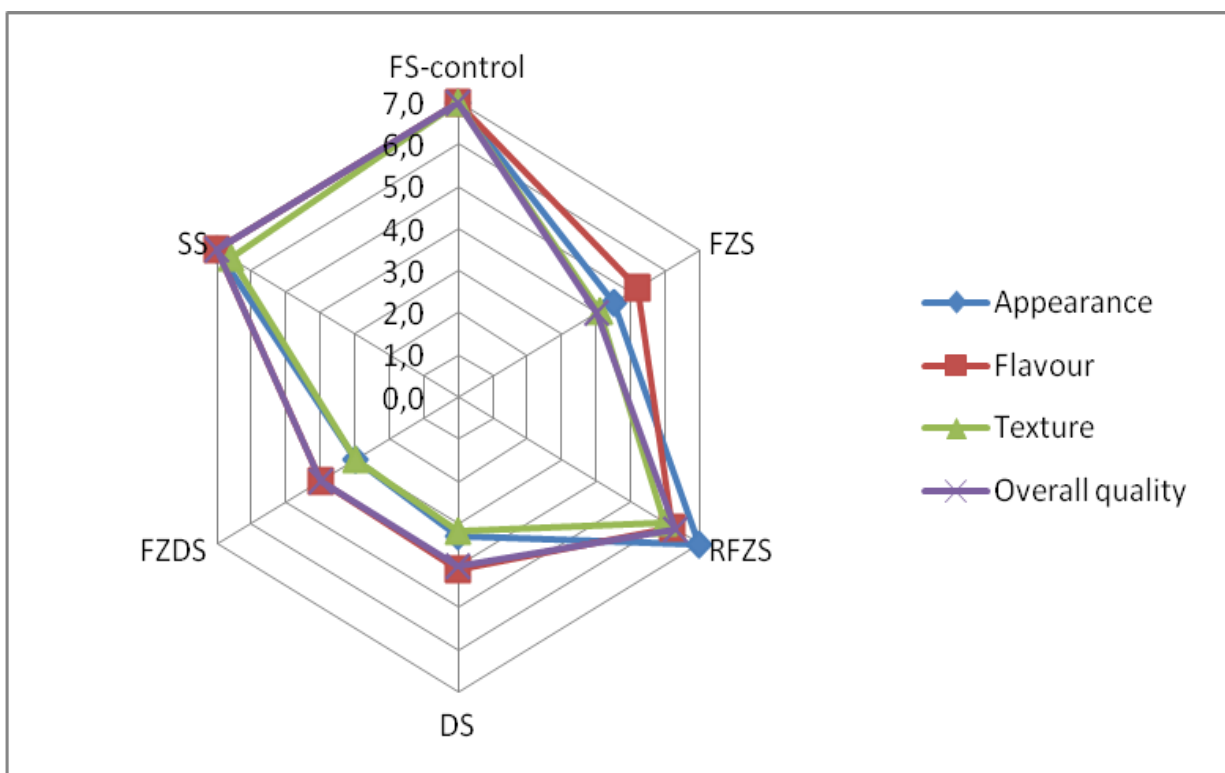


Figure 2 – Sensorial analysis of breads obtained with sourdoughs stabilized with different techniques. Bread produced with fresh selected sourdough (FS-control); bread produced with freeze sourdough (FZS); bread produced with refreshed freeze sourdough (RFZS); bread produced with dried sourdough (DS); bread produced with freeze-dried sourdough (FZDS); f) bread produced with spray-dried sourdough (SS) samples.

6.2.2 Image acquisition and digital analysis images

Eighteen typical bread crumb images of different bread samples (Three images for each sample) are shown in **Figure 3**.



Figure 3. Bread slices used for digital image analysis (FS-control); b) bread produced with freeze sourdough (FZS); c) bread produced with refreshed freeze sourdough (RFZS); d) bread produced with dried sourdough (DS); e) bread produced with freeze-dried sourdough (FZDS); f) bread produced with spray-dried sourdough (SS) samples.

Samples FS-control, SS and RFSS show homogeneous textures with sample RFSS being slightly finer than other two samples.

Sample FZDS and DS are an example of a non-uniform open crumb structure, evidencing a more reduced ability of sourdough starter to leaven the dough.

To analyze the porosity and distribution of the alveolar structure of bread slices, different characteristics of alveoli (number, size, shape, colour) were determined. To classify the objects, 10 different classes were arbitrarily defined. In detail, **Table 1** shows the min and max range of area (mmq) of alveoli for each class.

Table 1 – Classification of the objects (alveoli)

Class	Range of Area (mmq)	
	min	max
1	0.1	14.0
2	14.0	28.0
3	28.0	41.9
4	41.9	55.8
5	55.8	69.7
6	69.7	83.7
7	83.7	97.6
8	97.6	111.5
9	111.5	125.4
10	125.4	139.3

For each class, digital image analysis allowed to record the parameters (number of objects, % objects, Total Area, % Area, Mean area) reported in **Figure 4**.

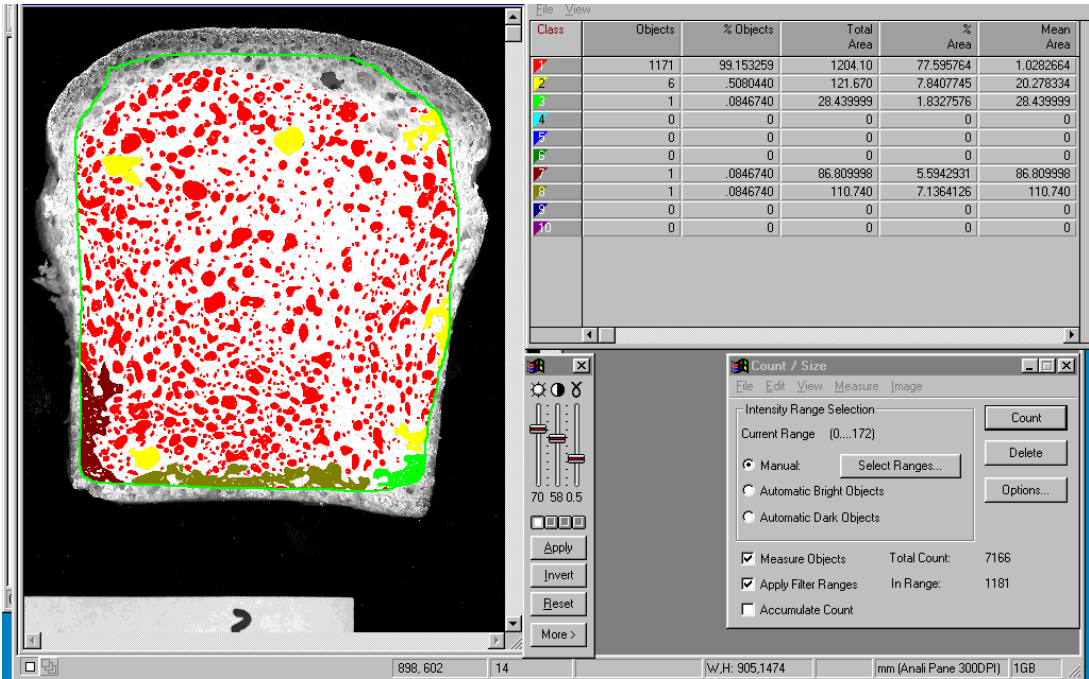


Figure 4. Digital image analysis of a sliced bread.

Following are reported results relative to two more representative parameters of digital image analysis: the number of alveoli per classes and the %Area of each class.

Table 2 show the number of objects (alveoli) for class of each sample. Sample FS-control, SS and RFZS had the highest number of object in Class 1 evidencing a same structure. Instead, sample FZS, FZDS and DS showed a reduced number of objects in Class 1, showing a little leavened dough. All samples showed low number of objects in the other classes 2, 3, etc..

Table 2 – Number of objects for class for each sample						
OBJECTS						
Class	SS	FZS	FZDS	FS-control	DS	RFZS
1	1042.7±4.7	694.0±5.7	566.0±3.6	1106.0±6.3	516.0±5.7	1022.3±7.7
2	9.0±1.7	2.0±1.2	3.7±1.7	5.0±2.2	2.0±1.1	5.7±1.2
3	0.7±0.5	1.7±0.7	1.0±0.0	1.0±0.7	0.0	4.0±1.3
4	0.3±1.1	0.3±0.3	0.0	0.0	0.0	1.0±0.5
5	0.0	0.7±0.4	0.7±0.1	0.3±0.1	0.0	0.7±0.3
6	0.3±0.1	0.3±0.1	0.0	0.0	0.0	0.0
7	0.0	0.0	0.3±0.1	0.0	0.0	0.0
8	0.0	0.0	0.3±0.1	0.0	0.0	0.3±0.0
9	0.0	0.0	0.0	0.0	0.0	0.3±0.1
10	0.0	0.0	0.3±0.2	0.0	4.0	0.3±0.1

*data are mean±DS of three determination obtained on three different slice of the same bread

In **Table 3** is reported %AREA of objects of each class for each sample. For almost all samples % total Area was represented by the sum of area of alveoli of class 1, 2, 3, and 4. Some samples (FZDS and RFZS) showed a high %Area also in class 7, 8, 9 and 10 evidencing non-uniform open crumb structure. Sample DS had 90,1% AREA in Class 1 and 9% Area in Class 10 highlighting, as also evident in **Table 3** a fault structure.

Table 3 – % AREA of objects for class for each sample						
% AREA						
Class	SS	FZS	FZDS	FS-control	DS	RFZS
1	80.7	75.4	67.8	80.5	90.1	60.8
2	15.5	6.4	8.3	7.2	0.9	10.1
3	2.2	5.5	3.8	2.4	0.0	11.2
4	1.3	2.0	0.0	0.0	0.0	4.1
5	0.0	4.8	5.8	1.2	0.0	3.7
6	0.3	2.9	0.0	0.0	0.0	0.0
7	0.0	0.0	3.9	1.4	0.0	0.0
8	0.0	0.0	4.5	1.2	0.0	2.7
9	0.0	0.0	0.0	0.0	0.0	3.8
10	0.0	0.0	5.9	0.0	9.0	3.7

*data are mean±DS of three determination obtained on three different slice of the same bread

6.3 Conclusion

The results suggested that spray-dried sourdough, opportunely refreshed, can be successfully used for breadmaking, leading to bread with sensorial characteristics comparable to those produced using fresh sourdoughs.

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In this PhD thesis the comparison among different sourdough storage methods highlighted advantages and several limits for each techniques used.

Traditional techniques, such as freezing, drying and freeze-drying, are actually used to preserve sourdough over time although the vitality of yeast and lactic acid bacteria are compromised and almost always sourdough starter need the addition of "*Saccharomyces cerevisiae*" yeast, which is presented as an "activator" but which actually is responsible only for the leaven, often causing flattening of the aromatic characteristics of the finished products.

Spray drying technique, highly used to storage pure culture starter, was used in this study to preserve sourdough. This application is very promising and interesting, and its use allowed the obtainment of sourdough characterized by a major vitality of both yeast and lab respect to the traditional techniques.

Breadmaking experiments with the sourdough starter stored in the different way, highlighted that spray-dried sourdough, opportunely refreshed, can be successfully used for breadmaking, leading to bread with sensorial characteristics comparable to those produced using fresh sourdoughs.

In future trends it would be interesting to investigate on the effect of spray drying on the survival of the single microbial species occurring in sourdough, selecting those with the highest resistance to spray drying conditions.