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Experimental thesis

PERFORMANCES OF WET GRANULATED LACTIC ACID BACTERIA FOR THE DIRECT FERMENTATION OF WHEAT DOUGHS

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1. INTRODUCTION

Sourdough production is one of the oldest biotechnological methods used to produce bakery products. It was known since very old time by Egyptians which had more than 50 types of cakes, unleavened bread and bread and then they made leavened bread with beer foam or sourdoughs (Jacob, 1997).

Traditionally, sourdough bread not used to be made from wheat flour due to the lack of advanced milling technologies and it was made from brown or whole meal flours. At the beginning of the last century and after the introduction of bakery yeast as leaving agent instead of using sourdough or brewing yeast for leavening, the industrial production of whole white bread started. Over decades, many different bread-making processes have been developed, which aimed to improve the overall characteristics of wheat flour bread.

Wheat bread is largely preferable by population and it has many pleasant characteristics such as high volume, soft and elastic crumb structure, good shelf-life and microbiological safety of the product (Cauvain, 2003).Unfortunately, wheat bread is a perishable product and its quality starts to deteriorate immediately after baking. It becomes stale largely because of the physical changes that occur in the starch-protein matrix of the bread crumb.

Textural characteristics are mainly based on the formation of gluten network, which has the ability to extend and keep the gas from yeast fermentation and makes a direct contribution to the formation of a cellular crumb structure.

Many methods have been developed to preserve wheat bread, it had been improved by the addition of additives which were in the form of emulsifiers or enzymes to improve both texture and increase shelf life of bread. These additives are not preferred by the consumers because they are either E-numbered additives or produced with GMO-organisms.(Elhariry *et al*, 2011).Sourdough baking is an alternative to the use of these additives because it is basically contains lactic acid bacteria(LAB) which have been used in food for thousands of years and are "generally regarded as safe", and provide many pleasant properties to bread like its sour taste and aroma as well as improving the shelf life (Elhariry *et al*, 2011).

Furthermore, many studies demonstrate the effectiveness of sourdough fermentation in improving the nutritional value of cereal products due to its high content of dietary fibers and its ability to hydrolyze gluten (Liukkonen *et al*, 2003).

Sourdough fermentation mechanism is a complex and numerous, and not yet fully understood, in which there are many complex biochemical changes during fermentation need to be understood It also has very specific stressful microbial ecosystem characterized by specific adaptations of the micro biota to the variable carbohydrate and nutrient contents, low pH, and variable oxygen tension and redox potential.(De Vuyst *et al.*, 2014; De Vuyst *et al.*, 2016).Furthermore, there are many changes that occur in the matrix of the sourdough can create both desired and undesired consequences in final product. For obtaining good quality final sourdough bread fermentation parameters need to be optimized (Clarke *et al*, 2003).

1.2 Sourdough microbiology and fermentation process

1.1.1. Sourdough microflora

Sourdough is a process in which flour water (and other ingredients) are fermented with microbes originating from preceding sourdough, commercial starter culture, bakery equipment or from the so called natural contaminants of flour.

Spontaneous dough fermentation starts by mixing flour with water without adding a starter culture or portion of a preceding sourdough (mother dough). It depends on the natural contaminant of the flour or any other ingredients and it differ by the origin and storage conditions of the flour, as well as the technological parameters of the fermentation process applied (De Vuyst *et al.*, 2014; De Vuyst *et al.*, 2016). This type of sourdough can be kept for decades by the addition for flour and water at regular time intervals.

Cereals for instance composed of (10^4-10^7CFU/g) bacteria while (10^4–10^6 CFU/g) found in the flour (Stolz 1999) in which (10^2-10^3 CFU/g) belongs to genus Lactobacillus (Salovaara 2004).

The dominating microbes in spontaneously fermented doughs are homofermentative lactobacilli and pediococci, which are found both in wheat and

rye sourdoughs at the level of 3 x10^8 and 3 x10^9 CFU/g. respectively Typical homofermentative lactic acid bacteria (LAB) in spontaneous sourdoughs are *Lactobacillus* (*Lb*.) *casei*, *Lb*. *delbrueckii*, *Lb*. *farciminis*, *Lb*. *plantarum*, *Pediococcus* (*Pd*.) *pentosaceus*. Typical heterefermentative LAB are *Lb*. *brevis*, *Lb*. *buchneri*, and *Lb*. *fermentum* (Stolz, 2003).

Various yeast strains have also been isolated from spontaneous fermentations such as *Saccharomyces* (*S.*) *cerevisiae* and *Pichia satoi* (Beech & Davenport 1971).Universal sourdough yeast appears to be *C. milleri* which is the most prevalent one and recently reclassified as *Kazachstania humilis* or strains closely related to it. *S. cerevisiae* (*S. clade*) is also often reported (Kerrebroeck *et al.*, 2017).

Based on the yeast species diversity sourdough can be classified to four groups depending on the presence of *S. cerevisiae* or *C. humilis*, the presence of both yeast species or none of them in the sour dough. The simultaneous presence of both *S. cerevisiae*, *C. humilis* connected with the presence of *Lb. sanfranciscensis* (Kerrebroeck *et al.*, 2017) As well as the process conditions impact prevailing of yeast diversity in particular with respect to fermentation temperature when the sour dough is fermented at high temperature the dominant yeast strains were *C. glabrata, P. kudriavzevii*, and *W. anomalus* (De Vuyst *et al.*, 2016).

Sourdough microflora is usually composed of stable associations of lactobacilli and yeasts which are often associated in ratio 100:1 (Ottogalli *et al.*, 1996). This is because of they share the same growth requirement in respect to temperature, pH, and organic acids.

However, in some sourdoughs, LAB and yeasts compete for the available substrates; resulting in heterogeneous populations that reflect the media resources and environmental conditions this in turn may change the mother dough completely in a short time in the case of continuous propagation and back-slopping (Ottogalli *et al.*, 1996). Weak microbial associations can occur when LAB present as contaminants in pre-doughs. Or when bakery yeast or starter culture is added this will influence the fermentation and the properties of the final product as compared with spontaneous sourdoughs.

The synergetic positive correlation between yeast and lactobacilli can be represented in the relation between the presence of *Lb. sanfranciscensis* and *C. humilis* which is ascribed to their nutritional mutualistic relationship in that *C. humilis* is maltose-negative and provides fructose and amino acids, while *Lb. sanfranciscensis*is maltose positive and releases acetic acid and glucose (Vogel, 2015).

The presence of *S. cerevisiae* and the absence of *C. humilis* as yeast species correlated positively with the presence of *Lb. paralimentarius* and *Pd. pentosaceus*as LAB species. The presence of *Weissella anomalus* and *C. glabrata* correlated positively with the presence of *Lb*. *brevis*, *Lb*. *fermentum*, *Lb*. *helveticus*, *Lb*. *plantarum*, and *Lb*. *pontis*, and negatively with the presence of *Lb*. *sanfranciscensis*.

No significant correlation occurred between the presence of *Lb*. *sanfranciscensis*as a LAB species and the presence of *C. humilis* in the absence of *S*. *cerevisiae* as yeast species (Kerrebroeck *et al.*, 2017).

In industrial sourdough processes they rely on commercial ready to use starter culture or apportion from a preceding sourdough in which they accelerate the fermentation process more than relying on fortifies flour only. Inoculation of the sourdough with a starter increases the number of lactic acid bacteria to 10^7-10^8 CFU/g, which gives little possibility for growth of contaminating organisms, including those which are imported from flour.

The ability of LAB to dominate over the natural contaminant in the dough is related to many factors LAB are more able to compete for their nutrients and reach a level above the adventitious microbiota, also LAB Lactobacilli rely on fructose and maltose as major source of carbohydrate metabolism which are very enriched in the sour dough environment this reason contribute to the dominance ability of Lactobacilli in the sour dough environment Moreover the temperature and pH conditions during sourdough fermentation match the condition for Lactobacilli growth (Ganzle *et al*., 1998).

Also Lactobacilli possess many mechanisms to overcome exogenous conditions like high/low temperature, high osmolarity or dehydration, oxidation and starvation (Gobbetti, 2001).

All of these factors contribute to the stable presence and dominance of the Lactobacilli in the sourdough.

1.1.2. Type of sourdough fermentation

From a technological point of view, sourdough may be classified into four distinct types (Böcker*et al.*, 1995). For its classification, the production process and the dough consistency are considered. The consistency of the dough is determined by the dough yield (DY), which refers to the proportion of flour and water, and may be calculated using the equation dough yield (DY)(%) = (amount of flour + amount of water) x 100/amount of flour. Taking into account that the microbial adaptation is influenced by the environmental and technological parameters in which it affects the selection and performance of the microbiota (Gobbetti & Gänzle 2013; Cappelle *et al.* 2013; Decock & Cappelle, 2005).

1.1.3. a. *Type I Sourdough Spontaneous Selection of Microorganism*

Type I sourdough known as the traditional method (spontaneous fermentation) initiated by the microbial strains already present in the first dough and carried out at room temperature (20–30°C) (Nionelli *et al.*, 2014). The first dough may be prepared using only flour and water or by the addition of another raw material naturally rich in microorganism (called inocula), such as fruits, yogurt, rumen cuts, and manure in which it will stabilize rapidly the microbiota (Ripari *et al.*, 2016) Daily refreshment of the dough repeated five to ten and the selection of sourdough microbiota will occur spontaneously The number of refreshments occurs daily are dependent on the microorganisms present at the beginning of the process and the desired sensory properties of the final product (Böcker *et al.*, 1995; Hammes & Gänzle, 1998)

Type and count of microorganism is varied during back-slopping in which the variety is the most in the first dough which may contain spore-forming, enteric bacteria and mould that are not suitable for the bread making process (Rocha & Malcata, 2016). Indeed, the population in the first dough reflect the type of microorganism present in the raw material for example: gram-positive (ex.,

Micrococcaceae), gram-negative (ex. *Enterobacteriaceae*), fungi (yeast and mould), LAB, and aerobic bacteria (ex. *Gluconobacter* sp.).

The selection of microorganism started when the redox potential of the dough decreases, the number of facultative anaerobes, such as Enterobacteriaceae and *Micrococcaceae*, LAB, and yeasts are increased. After that the pH values started to decrease during the next refreshment which leads to the lactic acid fermentation, and the inhibition of all the microorganisms sensitive to acidity and to organic acid produced. Afterwards, at the end well adapted LAB species such as *Lb. plantarum*, *Lb. fermentum* and *Lb. brevis* will be dominant (Ercolini *et al.*, 2013).

In particular, *Enterobacteriaceae*, *Bacillus*, or *Staphylococcus* were not detected at any stage of development; adventitious lactic acid bacteria (*Enterococcus* sp.)

Once obtained, the stable sourdough is used as a natural leavening agent, also known as a B sponge or B mother dough in the production of a huge assortment of traditional bread or other bakery products such as San Francisco bread, Panettone, French bread, Pain au levain, and rye bread.

The microbiota found in type I sourdough is composed mainly of heterofermentative and facultative heterofermentative LAB: *Lb.*brevis, *Lb. sanfranciscensis*, *Lb. fermentum*, *Leuconostoc citreum*, *Lb. plantarum*, and *Pd. pentosaceus* (Corsetti, 2012) and yeasts of the species *S. cerevisiae* and *C. humillis* (Minervini *et al.*, 2015)

The use of spontaneous sourdough requires hardworking, time-consuming processes, and skilled labour, but the natural selection of autochthonous microorganisms allow the presence of specific sourdough around the world that harboured distinctive yeast-LAB consortium. Each sourdough is a different natural ecosystem that can produce different quality of bread. Microbial metabolism is strain specific and the remaining topics explain its ability to modify the dough imparting great quality to sourdough-baked goods compared to the conventional bread. Type I sourdough can be maintained for years by continuous propagation using the previous batch as inoculums.

1.1.2.b. Type II Sourdough: use of starter culture

The sourdough type II is produced in a single fermentation step 15_24 h and then of lactic bacteria only or LAB with yeast and then backslopped for one time and so the method called industrial method (De Vuyst *et al.*, 2014). In this type of sourdough, starter cultures are added in a proportion of 100:1 LAB to yeast. In which they are able to dominate and inhibit the growth of natural inhabitants because they are added in high concentration.

The addition of starter culture acidifies the dough faster than the traditional sourdough, so the LAB most often used in this type of dough are acid-tolerant, as *Lb*. *amylovorus*, *Lb. panis*, *Lb. pontis*, and *Lb. reuteri* (Huys*et al.*, 2012).also the fermentation is done using very high temperature more than 30°C. Due to (Corsetti, 2012; Gobbetti, 1998); in order to increase the speed and the value of acidification that can inhibit the growth of natural yeast .And so, in sourdough type II, baker's yeast can be added at the end of the fermentation process (De Vuyst *et al.*, 2016).

In type II sourdough the fermentation temperature has an important impact on the type of microorganism used in the starter culture for this reason this type of sourdough produced in a bioreactor equipped with sensors. An experiment for (Meroth *et al.*, 2003) produced two type-II rye sourdoughs using a commercial starter culture and conducted the fermentation at temperatures of 30 and 40°C. They found that the dough fermented at 30°C the predominant LAB were *Lb. pontis*, *Lb. fermentum* and *S. cerevisiae*. But when the fermentation occur at 40°C, there was an abundant growth in *Lb. crispatus*, *Lb. panis*, and *Lb. frumenti* as well as a total inhibition of the yeasts. In this type the consistency of the dough is liquid (DY above 200) in order to enable the use in industrial bakeries due to its increased facility for pumping and dosing without generating dust (Böcker *et al.*, 1995) After the production, type II sourdough is stabilized by cooling. For starter culture deactivation and interrupting the production of carbon dioxide and organic acids, sodium chloride (NaCl) is added During storage, 1–3 days, part of the mature sourdough maybe used as inoculums for the production of bread, resulting in a final product with greater acidity than the traditional bread, while giving it the traditional sourdough flavors and aromas (Corsetti, 2012).

Type II sourdough presents several advantages over type I sourdough, such as only one fermentation step, better control over fermentation parameters (temperature, pH, acidity), and increased ease in the addition of nutrients, resulting in a better performance and control over microbial metabolism (Mastilovic *et al.*, 2001). Thus, it is possible to reduce the risk of contamination by moulds during the fermentation process due to faster fermentation as well as improve the sensory organoleptic properties and standardize the final products through the selection of starter culture and subsequent induction of the production of relevant metabolites. These qualities make type II sourdough ideal for use in industrial processes.

1.1.2.c. Type III Sourdough: D Type II sourdough

Type III sourdough is produced by dehydrating the stabilized form of type II sourdough. The selection of starter culture is based on their capacity to rapidly acidify the flour-water mixture and/or their ability to produce specific flavours (De Vuyst *et al.*, 2014). Some companies that market type III sourdough do not assure the viability of the sourdough microbiota, so this kind of product is more useful to improve the texture and aroma of the final products, but the addition of baker's yeast to allow the leavening is necessary.

The majority of companies that produce type III sourdoughs ensure a stable starter culture; in this way, it can be used as sponge or leaven in the production of bakery products after rehydration. For the dehydrating treatment of stable starter, sourdough should be considered for the stability of microorganism. Some examples of drying-resistant LAB are *Pd. pentosaceus*, *Lb. plantarum*, and *Lb. brevis* (Böcker *et al.*, 1995).

The most frequently used drying techniques are drum drying and drying using a rotating dryer (Chavan & Chavan, 2011). In drum drying, the water is evaporated while small droplets of sourdough are still in the air and do not come into contact with the sides of the equipment, thus avoiding Maillard's reaction (Decock & Cappelle, 2005). During the process of rotary drying, compounds are formed which give the product taste and aroma of caramelized and toasted malt due to the high

temperatures required for the process. This process also results in a reduction in acetic acid, which is volatilized at 113°C (Chavan & Chavan, 2011)

Furthermore, because it is dehydrated, this product has a smaller volume and may be more easily handled, transported, and stored when compared with type I and type II sourdough. These characteristics together with longer shelf life explain the frequent use of type III sourdough by industrial bakeries and its commercialization in supermarkets. Also, in this case, the critical step is the selection of the starter culture; the strains selected have to be also resistant to drying.

1.1.2.d. Type IV sourdough mixed sourdough

This type of sourdough production is similar to the studies that occur in the laboratory of some artisanal bakeries on the best selection of starter culture (De Vuyst *et al.*, 2014). In this case, sourdough initiated with starter culture consist of commercial yeast strains only, LAB starter culture or combined LAB and yeast starter culture the sourdough then propagated with traditional backslopping way . Then bacteria and yeast may be influenced by dispersal and drift of other, microflora.

The more competitive and well-adapted strains will dominate over the rest of sourdough microbiota. Therefore, a natural selection will occur between species suitable to the development in sourdough ecosystem.

1.2 Biochemical changes during sourdough fermentation

The sourdough process depends on numerous factors including, among other things, the composition of microflora, fermentation and enzymatic activities and flour characteristic, these factors do not act separately but in an interactive way, adding to the complexity of the system. Thus, many factors simultaneously affect the processes involved in sourdough fermentation such as the formation of acidity, the production of volatile compounds and the degradation of carbon and nitrogen compounds (Martínez, 1996). The level and intensity of these modifications during sourdough fermentation determines subsequent bread quality.

1.2.1. Acidification

Sourdough fermentation depends on the production of lactic acid and alcoholic formation which varied with the type of bacteria used in starter culture and on the fermentation conditions. Typical pH and TTA values for wheat flour sourdough range between 3.6.3.8 and 8.13, respectively (Brummer & Lorenz, 1991). Also the average content of acetic acid and lactic acid range between 600-800 mg/100 g 80-160 mg/100 g, respectively (Barber *et al.*, 1992, Hansen & Hansen 1994).

The carbohydrate content in the dough is the main factor that regulates the acidification of the sourdough. However, there presence in wheat flour is very low which range between 1.55-1.84% (sucrose, maltose, glucose, glucose, fructose and oligosaccharides But as the mixing process started the activity of endogenous α-amylase increased in which in turn will increase the initial level of maltose by ten folds. The activity of amylase enzyme depends to the type of flour in which it is very high in whole meal flour especially the bran part (Martínez ,2003).

Each LAB species or even strains use different type of Sugars, but most of the identified lactic acid bacteria in the sourdough are capable of utilizing pentoses, hexoses, sucrose and maltose, with a little variety for example *Lb. sanfransiscensis*, are specific to maltose. Furthermore, *Lb. sanfransiscensis* hydrolyzes maltose and accumulates glucose in the medium in a molar ratio of about 1:1 (Martínez, 2003). Another example is *Lb. plantarum* which in the presence of maltose, glucose and fructose prefers to the first two over the last for rapid growth and it weakly utilize sucrose and this is the case for most of lactic acid bacteria which are fructose negative and grow faster in maltose than in glucose,. Heterofermentative lactobacilli such *Lb. sanfransiscensis*, *Lb. brevis* and *Lb. fermenti* they produce acetic acid by the stimulation of oxygen which shifts the metabolic pathway from ethanol to an acetate route Proton acceptors, such as fructose, have a similar type of effect as they push the metabolism towards the acetate kinase pathway, producing traces of mannitol and an increase in acetic acid. The efficiency of fructose as a proton acceptor depends on concentration, temperature, and dough consistency (Martínez, 2003).

The metabolic activity of a typical sourdough depends on the interaction between yeast and lactic acid bacteria. When *Lb. sanfransiscensis*, *Lb. brevis* or *Lb. plantarum*

are associated with maltose negative yeast such as *S. exiguus*, maltose will be taken up completely by the lactobacilli and so increase in the acidity and the yield. In association with *S. cerevisiae*, a decrease in bacterial metabolism occurs due the faster consumption of maltose and particularly glucose by the yeast, which reduces the availability of glucose when both micro-organisms grow together (Martínez, 2003). The presence of yeast has been reported to diminish acid production (Brummer, 1988). Also the fermentation parameters play an important role in the determination of sourdough acidity fermentation temperature, time and dough yield. Optimal temperatures for the growth of lactobacilli are 30-40°C depending on strain (Stanier *et al.,* 1987) and for yeasts 25-28°C. In general, a higher temperature, a higher water content of sourdough and the utilization of whole meal flour enhances the production of acids in wheat sourdoughs (Brummer & Lorenz, 1991; Lorenz &Brummer, 2003).

1.2.2 Proteolysis

Sourdough consist many proteolytic enzymes which degrade cereal proteins (Thiele *et al.*, 2002) and produces free amino acids, which may act as flavor precursors (Gobbetti *et al.*, 1995).while dough fermentation with yeast reduces the concentration of free amino acids (Thiele *et al.*, 2002).

On the other hand the degradation of gluten protein will lead to the disruption of the rheological properties of wheat doughs due to the alteration in the proper gluten network formation and the result is weak and sticky dough.

The origin of the proteolytic enzyme is contradicted in several studies if the enzyme originates from the cereals or from the LAB. Spicher and Nierle (1988) said that only one third of the proteolytic activity in a rye sourdough originated from cereal enzymes. Other studies have also shown that proteinases from LAB can liberate soluble protein hydrolysates from gluten proteins (Gobbetti *et al.*, 1996; Wehrle *et al.*, 1999). However, recent results indicate that the proteolytic activity of lactobacilli is negligible compared to that of the wheat flour in a wheat sourdough system (Loponen *et al.*, 2004, Thiele *et al.*, 2002, 2003, 2004).

The theory that hypothesized the ability of LAB to hydrolyze gliadinin and glutenin proteins occurs during sourdough fermentation is not correct and the reality is that the low due to pH occur during the fermentation mediate the activation of cereal enzymes which show high activity at pH 3.7, but show no activity at pH 5.5 especially aspartic proteinase which appears to be active in the conditions of wheat sourdough (Thiele *et al.*, 2003; Loponen *et al.*, 2004)

Furthermore, the hydrolysis of wheat proteins is strain specific manner because each bacterial strain able to utilize specific substrate (Di Gagno *et al.*, 2003), for example the liberation of certain amino acids such as ornithine may require specific LAB strain to be utilized(Thiele *et al.*, 2002).

The level of free amino acids in wheat doughs not only depends on the pH of the dough, but also on fermentation time and the type of fermentative microflora that will consume the amino acids (Thiele *et al.*, 2002).if the level of proteolysis exceeds the need of bacteria then amino acids will accumulate in the medium Glutamic acid, isoleucine and valine are essential for the growth of *Lb. brevis* and *Lb. plantarum*. Each individual amino acid, except for lysine, cysteine and histidine, is suitable for the growth of yeasts, which thus expresses a much stronger demand on amino acids and low molecular weights peptides during fermentation.

For this reason the growth of yeast is very slow at the beginning of fermentation due to high pH values and so low proteolytic activity at this stage, yeast follows a log phase of growth that induces a strong demand for nitrogen.

For the same reason in the first four hour of fermentation, LAB have a long lag phase and develop metabolic activities at a slow rate. Thus, the accumulation of amino acids is not observed until the later stages of fermentation (Martínez, 2003).

In wheat sourdoughs, *Lb. brevis linderi*, *Lb. sanfranciscencis*, *Lb. brevis* and *Lb. plantarum* have been reported to increase the levels of aliphatic, dicarboxylic, and hydroxyl amino acids (Collar *et al.*, 1991, Gobbetti *et al.*, 1994). The yeasts *S. cerevisiae* and *S. exiguous* decrease the total level of amino acid in a similar way, the latter being more effective in amino acid removal from the dough (Spicher & Nierle,1984).In typical sourdough when the Lactic acid bacteria combined with yeast there is an intermediate level of amino acids.

Sour doughs that contain *Lb. brevis* or *Lb. plantarum* they have higher estimated content of free amino acids than the estimated content of free peptides because the reactivity of peptides is higher during fermentation in comparison to amino acids, and both of the above-mentioned strains reduce the content of peptides during fermentation, especially if *S. cerevisiae* is associated with these LAB (Mascaros *et al.*, 1994). Furthermore, LAB fermentation has been reported to affect the size distribution of peptides; the presence of lactobacilli decreases the content of larger peptides and increases that of smaller molecules such as dipeptides and amino acids (Thiele *et al.*, 2003).

1.2.3 Production of volatile compounds

Sourdough volatile compounds have been studied for many years and until now 146 compounds have been identified. Including: 43 aldehydes, 35 alcohols, 33 esters, 19ketones, 14 acids, 13 furans, 11 pyrazines, 2 lactones, 2 sulfurs, 21 others and alkanes in which 98 of them were identified in wheat sourdough and 71 volatile compounds in rye sourdoughs (Petel *et al.*, 2017).

In both wheat and rye sourdoughs are hexanal, nonanal, 1-pentanol, 1.4-butanediol, 6-methyl-5-hepten-2-one and octyl acetate. Some volatile compounds are more reported in rye sourdough than in wheat sourdough, such as 2-methylbutanal, 3-methylbutanal, 1-hexanol, 2-pentanone and 2-acetylfuran. One volatile compound is only reported in rye sourdough (1-octanol). In contrast, 13 volatile compounds are more cited in wheat sourdough than in rye sourdough (benzaldehyde, ethanol, 1-propanol, 2- methylpentanol, 2-propen-1-ol, phenol, 2-methylbutanol, 2-octanone, formic acid, octanoic acid, methyl acetate, 2- phenylethyl acetate) while only g-nonalactone is specific to wheat sourdough. Investigating the amount of volatile compounds in rye sourdoughs, Kaseleht *et al.* (2011) taking into account that these results differ my type of normal flora inoculated and the water content studying volatile compounds in bread is more complex due to the presence of lipid oxidation precursors and Maillard reaction.

Odorants are generated in sourdough mainly due to enzymatic and microbial processes during sourdough fermentation. These compounds belong to different

chemical classes such as aldehydes, acids, alcohols, ketones, esters and pyrazines, which are the most cited. However, some compounds are already present in flour. Bread and sourdough volatile compounds originate in the same ways from raw materials or processes. Those brought by raw materials are negligible compared to the total volatile compound content (Kirchoff & Schieberle, 2002) .Other compounds are mainly due to the fermentation process, lipid oxidation and Maillard and caramelization reactions (Prost *et al.*, 2012).

The major route for volatile compound production both in sourdough and bread crumb is fermentation. It produces mainly acids, alcohols, aldehydes, esters and ketones (Pico *et al.*, 2015). Yeast and LAB fermentations can be distinguished. They differ from metabolic and kinetic ways and lead to the formation of specific volatile compounds. Competition between yeast and LAB also has an impact on volatile compound production (Hansen &S chieberle, 2005). Moreover, homofermentative and heterofermentative LAB can be distinguished.

Also spontaneous or inoculated sourdough can be distinguished depending on their volatile profile in which the concentration of alcoholic compounds is higher in spontaneous sourdough (Hansen &Lund, 1987).

LAB produce volatile compounds in a strain-specific manner; each strain has its own profile of volatile compounds. Homofermentative lactobacilli are characterized by the high production of diacetyl, acetaldehyde, hexanal and and2,3-Butanedione, heterofermentative strains are characterized by the production of ethyl acetate, alcohols and aldehydes. Isoalcohols (2-methyl-1-propanol, 2,3-methyl-1- butanol), with their respective aldehydes and ethyl acetate, are characteristic volatile compounds of yeast fermentation (Damiani *et al.*, 1996; Gerekov *et al.*,2011). The largest difference is in 3-methylbutanol content, which varies mainly according to species and strains (Settanni *et al.*, 2013).

Some volatile compounds are only to some specific LAB such as benzaldehyde (almond-like), which is only reported for *Lb. helveticus* (Kaselehte*t al.*, 2011), or 2-heptenal (sour), which is produced by *Lb. plantarum* and *Lb.f arciminis* (Damiani *et al.*, 1996). In contrast, the same volatile compounds can be produced by homo and heterofermentative LAB, but their content differs. For example, the reduction of

aldehydes occurs more in hetero-than in homofermentative species (Kaseleht *et al.*, 2011).

Yeasts produce ethanol, aldehydes, iso-alcohols and ethyl acetate and are therefore mainly responsible for ethanol (alcohol), 2- methyl-1-propanol (ethereal), 3-methylbutanol (alcohol), 2- phenylethanol (rose), 2-methylbutanol (roasted), aldehyde (acetaldehyde, hexanal, octanal, nonanal), 3-hydroxy-2-butanone(sweet), benzyl alcohol (floral) or octanoic acid (fatty) production (Hansen &Schieberle, 2005) Some other volatile compounds are due to amino acid conversion by yeast such as benzaldehyde (almond-like) and 2-phenylethanol (rose) (from phenylalanine), methional (potato)(from methionine), 2-methylpropanal (aldehydic) (from valine), 2- methyl butanal (musty) and 2-methylbutanoic acid (acidic) (from isoleucine), 3-methylbutanal (ethereal) and 2-methylbutanoic acid (acidic) (from leucine), 2,3-butanedione (from asparagines) or acetic acid (vinegar) (from asparagines, glycine, serine or alanine)(Maga, 1974).

Association of lactic acid bacteria and yeast result in a synenergistic increase of alcohols in comparison to fermentation with yeast alone. The main bacterial volatile compounds, ethyl acetate and carbonyls are significantly decreased (Martínez 2003). For example, the association of *Lb. brevis ssp linderi*, or *Lb. plantarum*, and *S. cerevisiae* increases the formation of yeast fermentation products such as 1-propanol, 2-methyl-propanol and 3-methylpropanol and the number of aroma compounds detected (Gobbetti *et al.*, 1995).

LAB fermentation requires more than 12 h to produce a sufficient amount of volatiles, while yeast can produce them in few hours (Chavan & Chavan, 2011; Hansen & Schieberle, 2005).

The other portion of volatile compounds is produced as a result of lipid oxidation which begins during mixing in presence of active enzymes and oxygen (Maire *et al.*, 2013). Moreover, lipid oxidation can be influenced by the addition of fatty ingredients and the kind of fatty matter in the recipe. It is due to lipoxygenase action during kneading and storage. This enzyme oxidizes polyunsaturated fatty acids into free radicals, peroxides and hydroperoxides (Galey *et al.*, 1994), which are then converted into volatile compounds during baking. In sourdough and bread crumb,

lipid oxidation mainly leads to aldehydes ((E)-2-octenal, hexanal, heptanal, octanal, nonanal, 2-heptenal…), ketones (2-octanone…), alcohols and esters depending on the initial fatty acid (Maire *et al.*, 2013).

Lipid oxidation is significantly reduced during sourdough fermentation (Ganzle *et al.*, 2007) and some LAB can convert some lipid oxidation compounds into their corresponding alcohols (Vermeulen, 2006). For example, 3- methyl butanoic acid is derived from the oxidation of 3-methybutanal by aldehyde dehydrogenase (Guerzoni *et al.*, 2007). Lipid oxidation is not specific to the sourdough or bread process, but it modifies volatile compound formation by changing the amount of precursors and generating new volatile compounds. Thus, it is necessary to control lipid oxidation through the ingredients, mixing parameters and storage conditions to monitor the volatile profiles of sourdough and SD-bread.

On the other hand a small portion of volatile compounds is produced due to Maillard reaction which is depends directly on the dough precursors: sugar and amino acids.

Only 3 volatile compounds are found in wheat and rye sourdoughs (2- ethylbutanal, 3-methylbutanal and 2,3-butanedione) (Heinio *et al.*, 2003)

LAB activity also has an impact on the Maillard reaction as they liberate amino acids as precursors for this reaction and decrease dough pH (El Dash, 1971). However, in sourdough, the drying process is the most efficient way to create more Maillard compounds. Yet, dried sourdough is inactive and cannot confer the “sourdough bread” denomination.

Type and quantity of organic compounds present in the sour dough is affected by the type of dominant LAB for instance *Lb. plantarum* produce *the* highest amount of lactic acid when it compared *with Lb. brevis, Lb. fermentum* while *Lb. brevis* produce the highest amount of acetic acid compared with the other two strains (Saeed *et al.*, 2017)

Temperature, time and the number of backs-sloppings influence the yeast and lactic acid bacteria (LAB) fermentations and lipid oxidation and hence the volatile profile of sourdough.

It is possible to control the formation of volatile compounds, besides choosing the appropriate starter culture, by adjusting fermentation conditions such as time, in

which longer processing (fermentation) times enhance the flavor of the final product. (Gobbetti *et al.*, 1995) reported an increase in the levels of volatile compounds in acidic sourdoughs when the fermentation time was extended from 3 to 9 hours. However, after 24 hours of fermentation, the amount of volatile compounds had reduced, probably due to the evaporation of volatile compounds from the dough, secondly is the fermentation temperature, strains. In sourdough fermented with mixed cultures, raising the temperature from 25 to 35°C increased yeast fermentation. At 25°C, ethyl acetate, acetic acid and lactic acid were formed; whereas at 30°C, ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol were typical products. However, increasing the temperature further to 35 ºC did not modify the flavour profile (Gobbetti *et al.*, 1995).

Third the extraction rate of flour for example a high ash content of flour has been reported to increase the amount of volatile compounds in mixed fermentations (Hansen & Hansen 1994, Czerny & Schieberle 2002) and finally dough consistency. In which in firm dough, volatile compounds are formed by lactobacilli, whereas in soft doughs, ethanol and ethyl acetate are predominant with high levels of isoalcohols in dough containing heterofermentative.

1.2.4. Exopolysaccharides formation

Lactic acid bacteria are able to produce exopolysaccharides, which have a positive effect on bread volume and shelf-life (Korakli *et al.,* 2001; Tieking *et al.*, 2003). Beside its main function which is the protection of bacteria from drying and other stress factors (Salkinoja & Lounatmaa, 2002).

In order to induce a positive change on the texture of the dough it should contain for example, xanthan and dextran between 0.1-2%.

It is generally agreed that EPS influence product texture, mainly due to their ability to influence viscosity of the product. Dextran, xanthan and levan are one of the most important EPS produced by bacteria also fructans and glucans, which exhibit positive impact on the shelf life volume and the texture of the bread (Tieking *et al.*, 2003). The production of these compounds in sufficient amounts during sourdough

fermentation would create the possibility to replace hydrocolloids in baking. Hydrocolloids have been reported to improve bread quality (Rosell *et al.*, 2001).

1.3 Commercial method to preserve starter culture of sourdough

In the 1920s, the first sourdough came to market, was a non-fermented mixture of pre-gelled flour added with lactic acid known as BIreks Fertig Sauer (Benedickt & Meyer, 2003). Since this sourdough was not well-received by the public in 1970, the development of a dehydrated sourdough began. It could be used by bakeries and large production centres (Brandt, 2006). Currently, the type III sourdough is also being commercialized in local markets for domestic use. Dehydrated dough has a longer shelf life as compared to fresh doughs due to their lower susceptibility to contamination by microorganisms and their greater physical chemical stability. These characteristics have facilitated commercialization of sourdough and led to the development of different varieties of type III sourdough applied as starters in sponge dough. A few examples are starters sourdough developed for use with products made by rye flour and whole-wheat flour (rye sourdough starter and whole wheat sourdough starter), gluten-free sourdough, pancakes, waffles, and even traditional San Francisco sourdough many methods have been developed to produce dehydrated ready to use sourdough.

1.3.1. Drum drying

Drum dryers were developed in early 1900s. They were used in drying almost all liquid food materials before spray drying came into use. Nowadays, drum dryers are used in the food industry for drying a variety of products, such as milk product, baby foods, breakfast cereal, fruit and vegetable pulp, mashed potatoes, cooked starch, and spent yeast. 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. A

drum-dried Type III sourdough will not only add a sourdough flavor to the end product, but at the same time also some malted, caramelized flavor notes up to a toasted aroma (Rupesh & Shraddha, 2011)

1.3.2. Freeze drying

Freeze-drying is widely used as a long-term preservation technique for bacteria and yeast, where they need to be previously frozen and water is removed by sublimation without passing through liquid phase (Santo *et al.*, 2013). Low temperatures, especially below freezing point, may cause severe damage to micro- organisms due to intracellular ice crystals formation (Momose *et al.*, 2010).Freeze drying can be performed on fresh microbial cells culture either single strain or mixed strain cultures, and active sourdough which contains fresh starter cultures (Cossignan *et al.,* 1996). Many experiments revealed that there is no significant difference in the rheological and fermentative characteristics of both freeze dried mixture starters and freeze dried wheat sourdough. On the other hand they produce less volatile compounds and less fermentative performance if compared with fresh cell starters (Cossignani *et al.,* 1995).

1.3.3. Spray drying

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. (Rupesh & Shraddha, 2011).Preservation of micro-organisms by spray drying is a convenient, fast and widely used method for producing large quantities of bacterial probiotic cultures (Silva *et al.*, 2011).

For preserving the function of micro-organisms in the dry state, sucrose and trehalose have been utilized the most (Ying *et al.*, 2012), although other sugars (e.g. maltose and fructose) and sugar alcohols (e.g. sorbitol and inositol) may offer protective effects as well (Ying *et al.*, 2012).

Spray dried sourdough add many chemical and rheological properties to the wheat flour dough it led to a decrease in wet gluten and sedimentation value. A significant increase in water absorption and degree of softening and a remarkable decrease in stability were also observed in all doughs containing spray dried sourdough. In addition, dough extensibility was decreased and resistance to extension was increased in the blend doughs. This indicated major changes in the dough structure which were caused by low pH (Golshan *et al.*, 2013).

AIM OF THE STUDY

Sourdough fermentation has been intensively studied during recent decades, but it is still not a well-understood process due to its complicated nature. As matter of fact, designed sourdough processes are not easily achieved.

The aim of this study was to obtain a mixed starter culture for the fermentation management. For this purpose, several LAB strains were isolated from mature sourdoughs. Cultures were characterized and identified. The fermentation abilities of the cultures were assessed and more promising strains were evaluated in combination.

Selected strains were combined to prepare defined multi-species ready-to-use sourdough starters which were evaluated in model systems.

An understanding of sourdough preservation conditions is still a crucial challenge in the related industries. It is necessary to maintain its activities over a period of time in order to prolong its shelf life. During thermal drying, yeast and LAB may undergo numerous changes such as destruction of cell membranes, denaturation of proteins or enzymes, or even death. Therefore, optimal operational conditions in the drying process are required in order to minimize such adverse effects of thermal drying. Different industrial dryers are available in industries; but operative conditions needs to be defined case by case. To attempt a stabilization of the sourdough starters, two methods were tested in this work freeze-drying of active sourdough and wet granulation of fresh microbial cells.

2. MATERIAL AND METHODS

2.1. Microorganisms and culture conditions

A total of 43 LAB cultures were isolated by mature sourdoughs during a previous survey. Strains were identified and genotipically characterized (Aponte *et al.*, 2014). All cultures were stored at −25°C in liquid MRS (Oxoid, Basingstoke, Hampshire, UK) after addition of 20% sterile glycerol (Sigma-Aldrich S.r.l. Milan, Italy) and propagated twice in modified MRS (mMRS) (maltose and fresh yeast extract added at 1% and 5%, respectively, and the final pH was 5.60), before experimental use.

2.2. Strains features of technological interest

Strains were screened for urease activity according to Mora *et al.* (2002). The ability to hydrolyze starch was evaluated on starch agar plates drop-inoculated. After incubation at 30°C up to colonies visible growth, plates were covered with Lugol solution for one minute. After washing, strains were considered positive if a clear halo around colonies could be detected. Screening for phytase activity was performed according to Anastasio *et al.* (2009) in modified Chalmers medium.

Exopolysaccharide (EPS) production was tested in APT agar (Difco) supplemented with filter-sterilized solutions of different carbohydrates (1% vol/vol) and ruthenium red (0.08 g/L). Glucose, maltose, starch, saccharose and fructose were added one by one. Acid fermentation by maltose, fructose, xylose, arabinose, and fructose plus maltose was evaluated by growth in MRS medium (pH 7.2-7.8) without glucose and Lab Lemco powder, but supplemented with phenol red (0.17 g/L) and filter sterilized sugar solutions (0.5%). β-glucosidase activity was assayed using 4-nitrophenyl β-D glucopyranoside (Fluka, Milan, Italy) according to the method proposed by Fia *et al.* (2005).

Fermentative abilities of the strains were quantified in terms of acids and gas development. Briefly, (62 g) of rice or wheat flour, were mixed with cell suspensions

(about 37.5 mL) of LAB grown for 24 h at 28°C in mMRS broth. In detail, cells were collected by centrifugation (14.000 rpm, 10 min.), washed in sterile potassium phosphate buffer (50 mmol/L, pH 7.00) and resuspended in the same volume of sterile tap water. Pure bakery yeast was added at 1%. Initial LAB load, as well as volume increase, pH and Total Titratable Acidity (TTA) after 24 h of incubation at room temperature were evaluated.

2.2.1. LAB and yeast counts

LAB were counted before and after fermentation by drop method (Collins, 1989) onto agar plates of differential MRS medium modified (dMRS) according to Ricciardi *et al.* (2015) supplemented with Cycloheximide (0.1 g/L). The number of yeasts was estimated at 28°C for 48 h on Malt extract Agar medium supplemented with chloramphenicol 0.2g/L. Dough samples were diluted up to 10^{-9}. 12 µL aliquots were spotted onto agar plates. After incubation for 24h at 30°C colonies in each countable drop were counted.

2.2.2.pH and TTA determination

Samples of dough at time 0 and after 16 h of fermentation were collected for pH and TTA assessment. pH values were determined by a pH meter. TTA was measured on 10 g of sample, which was homogenized with 90 mL of Ringer solution and expressed as the amount (mL) of 0.1 M NaOH needed to achieve the pH of 8.5.

2.3. Fermentation ability of sourdough starters on a lab scale

MRS cultures in exponential phase of growth were mixed in ratio 2:1. Specifically 8 mL of each *Lactobacillus* strains (alternatively E73, E75 or C710) were mixed with 4 mL of the *Pediococcus* strain B72. 1.2 mL of bakery yeast were added as pure culture in Malt extract broth (after incubation at 28°C for 24 h). After centrifugation for 15 min at 6500 rpm, cells were washed with ringer solution and resuspended in an

equal volume (13.2 mL).The cell suspensions were then mixed with 20 g of wheat flour (Barilla S.p.A., Italy) under aseptic conditions to get the dough. After 16 h of incubation at 30°C, the height (cm) reached by the dough in a graduated tube was recorded. Sourdough samples were submitted to pH and TTA evaluation. The set of trials was repeated by using the same amount of flour but with microbial population levels ten- or one hundred-fold lower (0.8/0.4/0.12 and 0.08/0.04/0.012 mL).

2.4. Bread production

Doughs were prepared by using the previously described microbial combinations with an additional control sample which consisted of yeast only. Experiments were carried on by using a presumptive initial LAB load of about 7 Log CFU/g, namely the intermediate concentration within the three evaluated in the previous set of trials. Four hundred g of wheat flour were mixed with 215 mL of Ringer solution by using an electronic mixer. Doughs were then incubated at 28°C for 16 h. Samples were collected before and after 16 h of fermentation for bacteria and yeast counts. pH and TTA evaluation were carried on as previously detailed. Doughs samples were even subject to VOCs analysis.

Volatiles analysis was performed using an Agilent Technologies(Santa Clara, CA) 7890A gas chromatograph coupled to an Agilent Technologies 5975 mass spectrometer equipped with a30 m x 0.25 mm ID, film thickness 0.25 lm capillary column(HP-INNOWAX, Agilent Technologies). Gas carrier was helium (flow 1.5 mL/min) and SPME injections were split less (straight glass line, 0.75 mm ID) 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. Mass spectrometer operated in scan mode from m/z 33–300 (2 s/scan) at an ionization potential of 70 eV.

Identification of volatiles was achieved by comparing mass spectra with the Wiley library (Wiley7, NIST 05). The volatile compounds were identified by matching the

retention indices (RI) calculated according to the equation of Van den Dool and Kratz (1963) and based on a series of alkanes. Blank experiments were conducted in two different modalities: blank of the fibre and blank of the empty vial. These types of control were carried out after every 20 analyses. All the analyses were performed in triplicate.

2.5. Fermentation monitoring by gas development

Four doughs were made using 200 g of flour and the three previously described bacterial combinations plus one control sample which contains yeast. Doughs were stuffed into tightly sealed bottles to avoid any gas escape. Lids were equipped with a manometer for the measurements of the gas development. The initial pressure inside the bottle at the onset of fermentation was recorded. The value was subtracted from that obtained at the end of the incubation at 30°C for 16 h.

2.6. Freeze-drying of mature sourdoughs

For freeze drying, mature sourdoughs prepared by using the E75-B72 combination were submitted to desiccation by freeze-drying after 16 h of fermentation at 28°C. Doughs were frozen at -80°C as they were, and after dilution 1/10 with sterile Ringer solution. In both cases, doughs were distributed on trays in a thin layer. After 24 h, doughs were subject to freeze drying process in an integrated plant consisting of a drying chamber, and cooling and heating systems, as well as a control and measuring system with an interface. The drying chamber allows for the process to be conducted using one or several (up to a maximum of 5) hot plates. The components of the vacuum system are a single-phase engine with 160 W of power, and a rotary vacuum pump connected to the drying chamber by a flexible pipe through an electro-magnetic

valve. The freeze-out system consists of a spiral evaporator (ice condensator) located outside the drying chamber. Other elements of the cooling system are integrated within the plant. The work of the cooling system is regulated automatically; hence, the temperature in the ice condensator cannot be regulated separately (and it usually equals -55°C). The heating system consists of five hot plates located on a support stand. The heat required for the phase transition is supplied to the material by direct contact. Both the measurement and the regulation of the hot plate temperature are conducted using a temperature sensor located inside one of the lyophilisator plates. The process of drying was continued until the mass of the sample reached constant moisture (3% w.b.) (Roozyło *et al.,* 2015).pH, TTA and microbial counts were determined as previously described.

The ability of the freeze dried sourdoughs to make bread was tested by mixing at powders at10% (wt/wt) with flour (18 g) and ringer solution (12 mL).The same experiment was repeated by using viable cells of the E75-B72 combination as control. pH, TTA, Microbial loads, as well as the height reached by doughs after 16 h of fermentation at 28°C, were evaluated.

2.7. Wet granulationof sourdough starters

Granulation is one of the most significant unit operations in the production of pharmaceutical dosage forms, allowing to yield free-flowing agglomerated particles that can be more easily and uniformly distributed inside capsules as compared to disaggregated, and often cohesive, powders (Shanmugam, 2015). First attempts have been made to improve the survival of *Lb. plantarum* through wet granulation with poor success (Woraharn *et al.*, 2010). More recently, enteric coating of granules containing *Lb. Plantarum* has been proposed to protect the bacteria from the acidic gastric environment and enhance their bioactivity (Aponte *et al.*, 2018).

2.7.1. Microorganisms and culture conditions

Granules containing each type of the bacterial/yeast combinations were produced by wet granulation. Eighty mL of MRS broth cultures of each *Lactobacillus* strains were added to 40 mL of the *Pediococcus* B72 and of 12 mL of ME broth culture of the bakery yeast. Cells were collected by centrifugation, washed with Ringer solution and weighed. Alive cells loads were assessed before the granulation process by drop counts onto MRS and ME agar plates.

2.7.2. Preparation of the wet granules

For the preparation of the granules, pharmaceutical grade corn starch (CS), microcrystalline cellulose (MC) and lactose monohydrate (LM) at different weight ratios were used as diluents after sieving through a 50-mesh sieve (300 μm). When needed, aqueous polyvinylpyrrolidone (PVP) (2% w/w) was employed as binder. About 7 mL of aqueous PVP were mixed with3.2 g of CS, 3.2 g of MC and 3.8 g of LM. Then, bacteria cell suspensions (12.6, 11.8, 12.8, and 9.3 CFU/g for E75-B72, E73-B72, C710-B72, and Yeast, respectively) were gradually added to the excipients and mixed in the mortar until the formation of a coherent mass. The obtained paste was passed through a 12-mesh sieve (1.7 mm) and dried in an oven at 30°C for 12 h. After drying, all granules were screened through an 18-mesh sieve (1 mm) and stored at 4°C until use. To prevent any microbial contamination, filtered ultrapure water (Millex® 0.22 μm sterile syringe filters, Millipore, USA) was employed throughout the experiment. All critical stages in the manufacture were carried out in a Grade a laminar flow hood (Aponte *et al.*, 2018)

2.7.3. Determination microbial cells viability after wet granulation process

Bacterial viability per gram was assessed using in parallel: direct counting at microscope in Petroff-Hausser chamber (total cells), live/dead BacLight™ Bacterial Viability Kit(Molecular Probes, Eugene, OR) followed by epifluorescence microscope observation, and plating onto dMRS agar after incubation at 37°C for 48 h (living cells).For the fluorescence microscopy test, cells were dyed using two

fluorochromes: SYTO 9 (green dye: undamaged cells) and propidium iodide (red dye: dead or injured cells). After staining followed by incubation in the dark for 15 min at room temperature, samples were observed by using a Nikon EclipseE400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a UV lamp and a 9 100 magnification objective. Images were captured by a Nikon Coolpix 4500Digital Camera equipped with a microscope adapter.

2.7.4. Doughs preparation by dried cells

Fermentation abilities of granulates were tested by mixing 1 g of each microbial combination with 10 g of flour. The test was performed by using fresh cells as well. Doughs were incubated at 18C° for 16 h. Samples were taken at time 0 and after 16 h of fermentation for bacteria and yeast count and for the evaluation of organic acids and reducing sugars by HPLC analysis. The height in (cm) reached by each combination was recorded too.

Samples were analysed on a Gilson 307 Series HPLC system equipped with a refractive index detector (RID 133, Gilson) and using an MetCarb68H column (6.5 300 mm, Varian). In which sample was prepared by mixing 0.5 g of dough with 4.5 mL of water to have 1:10 dilution then mixed with 2.71 μL of 0.1 N sulfuric acid solution, the mixture was then homogenized by a Mixer for 5 min and centrifuged for 15 min then filtered through a 0.22 mm membrane filter. Samples were analyzed according to the following conditions. The flow rate was 0.4 mL/min and the mobile phase was 0.01 N H_2SO_4. The injection volume of mixed standards was 20 mL. The temperature of the column was set at 65°C. Identification was carried out by comparing retention times with those of standards under the same HPLC conditions.

2.8. Sensory analysis of bread

Four bread samples were made using sourdough from the best combination (E75-B72- B sample), bakery yeast alone as negative control(A sample), sourdough produced by using wet dried starter culture of the best combination (E75-B7 - D

sample) and positive control(C sample) which was manufactured by using a natural sourdough collected from an artisanal bakery located in Meta (Naples). The final breads were subjected to the sensory analysis performed by a descriptive panel consisting of 8tasters familiar with the sensory analysis of foods, but not specifically trained in the evaluation of sourdough breads.to evaluate 23 descriptors chosen among those reported by Comendador *et al.* (2012) including crust color, crust thickness, crumb color, alveolation, alveolation uniformity, odor intensity, bread odor, yeast odor, sourdough odor, unpleasant odor, aroma intensity bread aroma, yeast aroma, sourdough aroma, unpleasant aroma, salty, acid, bitter, taste persistency, crispness and the overall assessment. The tasters expressed the intensity of each attribute with a mark on a 6-point hedonic scale (5 = extremely high; 0 = extremely low).The samples were presented to the assessors coded and in random order. The questionnaire is reported here below.

6-point hedonic scale

0	1	2	3	4	5

Extremely low ➲ Extremely high

Descriptor		A						B						C						D					
		0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
Crust																									
	Colour																								
	Thickness																								
	Elasticity																								
	Crispness																								
Crumb																									
	Gummy																								
	Colour																								
	Alveolation																								
	Alveolation uniformity																								
Odor																									
	Intensity																								
	Bread																								
	Yeast																								
	Sourdough																								
	Unpleasant																								
Aroma																									
	Intensity																								
	Bread																								
	Yeast																								
	Sourdough																								
	Unpleasant																								
Taste																									
	Salty																								
	Acid																								
	Bitter																								
	Persistency																								
Overall assessment																									

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Differences between the means were established using one-way analysis of variance (ANOVA) Differences at the 5% level ($p< 0.05$) were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Cultures technological characterization

Forty-three strains were isolated from mature sourdoughs and genotipically characterized by means of RAPD PCR (Aponte *et al.*, 2014). One or more representative of each RAPD-type were identified by 16S rDNA partial sequence followed by BLAST comparison of the obtained sequences with the nucleotide database of the NCBI. All strains were analysed for biochemical features of technological interest (Table 1).

Only strains referable to the species *Pd. lolii* expressed ureolytic activity. Such results confirmed evidences obtained by Zotta *et al.* (2007) for lactobacilli isolated from sourdoughs used for the production of the "Cornetto di Matera". According to authors, no strain out of 41 was able to hydrolyze urea (Zotta *et al.*, 2007). During sourdough fermentation, the production of lactic and acetic acids by LAB promotes a drop in pH values which may affect the metabolic activities of LAB and decrease their performance. The responses of bacteria to acid damage differ. Among the stress response mechanisms, urease activity seems to play a role in protecting some microorganisms from the harmful effect of acid conditions, by increasing the environmental pH as a result of the conversion of urea into ammonia and carbon dioxide (Guchte *et al.*, 2002). The relationship of urease activity to pH modulating capacity has been demonstrated in *Helicobacter pylori* (Toledo *et al.*, 2002) and *Streptococcus thermophilus* (Blaiotta *et al.*, 2011).

Table1: Biochemical characterization of LAB strains isolated by Aponte and co-workers (2013).

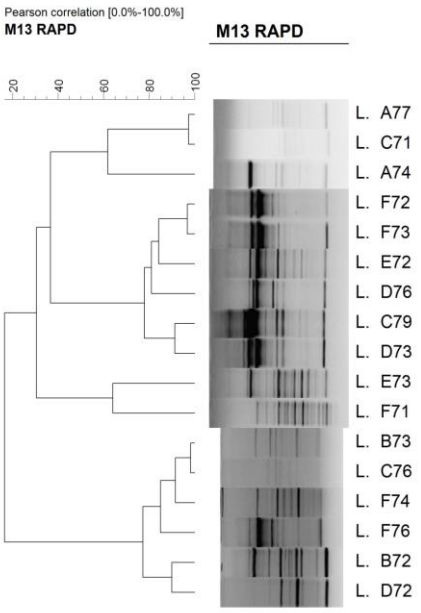
	Activity			EPS production from						Acid from			
Strain	Phytasic	Starch hydrolisis	Ureolytic	Glucose	Maltose	Starch	Sucrose	Fructose	Maltosio	Fructose	Maltose+ Fructose	Arabinose	Xylose
A71	+	-	-	+	+	+	+	+	+	+	+	-	-
A72	-	-	-	+	+	-	+	+	+	+	+	-	-
A73	-	-	-	+	+	-	+	+	+	+	+	-	-
A74	-	+	-	+	+	-	+	+	+	+	+	-	-
A75	-	+	-	+	+	-	+	+	+	+	+	-	-
A76	-	-	-	+	+	-	+	+	+	+	+	-	-
A77	-	+	-	+	+	-	+	+	+	+	+	-	-
A78	-	-	-	+	+	-	+	+	+	+	+	-	-
A79	-	+	-	+	+	-	+	+	+	+	+	-	-
A710	-	+	-	+	+	-	+	+	+	+	+	-	-
B71	+	-	-	+	+	+	+	+	+	+	+	-	-
B72	-	-	+	+	+	+	+	+	-	+	+	-	+
B73	+	-	-	+	+	+	+	+	+	+	+	-	-
B74	-	-	-	+	+	-	+	+	+	+	+	-	-
B75	+	-	-	+	+	+	+	+	+	+	+	-	-
B76	+	-	-	+	+	+	+	+	+	+	+	-	-
C71	-	-	-	+	+	-	+	+	+	+	+	-	-
C72	-	-	-	+	+	-	+	+	+	+	+	-	-
C73	-	-	-	+	+	-	+	+	+	+	+	-	-
C74	-	-	-	+	+	-	+	+	+	+	+	-	-
C75	-	+	-	+	+	-	+	+	+	+	+	-	-
C76	-	+	-	+	+	-	+	+	+	+	+	-	-
C77	-	-	-	+	+	-	+	+	+	+	+	-	-
C78	-	-	-	+	+	-	+	+	+	+	+	-	-
C79	-	-	-	+	+	-	+	+	+	+	+	-	-
C710	-	-	-	+	+	-	+	+	+	+	+	-	-
D71	-	-	+	+	+	+	+	+	+	+	+	-	+
D72	-	-	+	+	+	+	+	+	-	+	+	-	+
D73	+	-	-	+	+	-	+	+	+	+	+	-	-
D74	+	-	-	+	+	-	+	+	+	+	+	-	+
D75	+	-	-	+	+	-	+	+	+	+	+	-	-
D76	+	-	-	+	+	-	-	+	+	+	+	-	-
E71	+	-	-	+	+	+	+	+	+	+	+	-	-
E72	+	-	-	+	+	+	+	+	+	+	+	-	+
E73	-	+	-	+	+	-	+	+	+	+	+	+	-
E74	+	-	-	+	+	-	+	+	+	+	+	-	-
E75	+	-	-	+	+	+	+	+	+	+	+	-	-
F71	+	-	-	+	+	+	+	+	+	+	+	-	-
F72	+	-	-	+	+	+	+	+	+	+	+	-	-
F73	+	-	-	+	+	+	+	+	+	+	+	-	+
F74	+	-	-	+	+	+	+	+	+	+	+	-	-
F75	+	-	-	+	+	+	+	+	+	+	+	-	-
F76	+	-	-	+	+	+	+	+	+	+	+	-	-

Moreover, none of the tested strains expressed β-glucosidasic activity with precursor used for testing. Actually, the properties of bread may be improved by the hydrolysis of structural and storage polysaccharides. β-glucosidases, a major group of glycosyl hydrolase enzymes, catalyse the selective cleavage of β-1,4-glycosidic linkages of various disaccharides, oligosaccharides, and alkyl-and aryl-β-D-glucosides (Yan *et al.*, 1998). Hydrolysis of glycosidic bonds plays an important role in several biological pathways. β-glucosidases are responsible for the hydrolysis of cello-oligosaccharides and cellobiose in the terminal step in the degradation of cellulose, an important fibre source in cereal foods. In addition, these enzymes hydrolyse toxic and/or bitter glucosides, release aromatic compounds and synthesize various oligosaccharides, glycoconjugates, alkyland amino-glucosides (Bhatia *et al.*, 2002). Results obtained during this survey did not fit the large differences in production of intracellular β-glucosidase found within strains of the *Lb. plantarum* group recorded by Zotta *et al.* (2007). According to authors, several strains of Lb. plantarum exhibited high levels of p-NPG degradation. With reference to phytasic activity, almost 44% of the tested cultures were able to degrade the phytic acid. In cereal grains, most of the total phosphorus is present as phytic acid (myo-inositol hexaphosphate), which is located mainly in the outer layers of the grain. Phytic acid is often regarded as an anti-nutritional compound since it chelates proteins, amino acids and divalent cations such as Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, preventing their absorption by the intestinal mucosa (De Angelis *et al.*, 2003). Because of this, the degradation of phytate by fermentation before the product is baked is desirable. During fermentation phytate-degrading enzymes, which are present in yeasts and LAB isolated from sourdoughs (Lopez *et al.* 2000), catalyse the stepwise hydrolysis of phytic acid to myo-inositol via penta- to monophosphates or orthophosphates (Reale*et al.,* 2004). In addition, the low pH resulting from acid production by LAB promotes the activation of endogenous flour phytases, and so contributes to further phytate degradation (Lopez *et al.*, 2001). The 19% of the strains, all belonging to the Lb. plantarum group, were able to hydrolyze starch and about the 40% to produce EPS in presence of this compound. On the other hand, all tested strains were able to produce EPS in presence of glucose, maltose, saccharose and fructose. With

reference to the use of pentose as the sole carbon source, it must be pointed out that just one strain, *Lb. plantarum* E73, was able to ferment arabinose and six strains, namely B72, D71, D72, D74, E72 and F73, were able to use xylose. All strains were capable to grow in presence of fructose and fructose and maltose, while just two strains, B72 and D73, both belonging to the species *Pd. lolii*, were unable to metabolize maltose.

3.2. Comparison between strains' genotypical and biochemical features

By strains grouping according to biochemical profile new rearrangements appeared. In order to compare all acquired info, data regarding strain typing at both molecular and phenotypical levels were combined (Figure 1).



	Attività			Produzione di EPS						Fermentazione				
	Ptasica	Amilasica	Ureasica	Glucosio	Maltosio	Amido	Saccarosio	Fruttosio	Maltose	Fruttosio	Maltosio +Fruttosio	Arabinosio	Xilosio
A77	-	+	-	+	+	-	+	+	+	+	+	-	-
A710	-	+	-	+	+	-	+	+	+	+	+	-	-
C71,C73, A72	-	-	-	+	+	-	+	+	+	+	+	-	-
A74, A75, A79	-	+	-	+	+	-	+	+	+	+	+	-	-
A71, B76	+	-	-	+	+	+	+	+	+	+	+	-	-
A73, A76, A78, C72 B74	-	-	-	+	+	-	+	+	+	+	+	-	-
F72, B71, B75	+	-	-	+	+	+	+	+	+	+	+	-	-
F73, F75	+	-	-	+	+	+	+	+	+	+	+	-	-
E72	+	-	-	+	+	+	+	+	+	+	+	-	+
D76	+	-	-	+	+	-	-	+	+	+	+	-	-
C75	-	+	-	+	+	-	+	+	+	+	+	-	-
C77, C74, C79	-	-	-	+	+	-	+	+	+	+	+	-	-
D73, D75	+	-	-	+	+	-	+	+	+	+	+	-	-
E73	-	+	-	+	+	-	+	+	+	+	+	+	-
F71	+	-	-	+	+	+	+	+	+	+	+	-	-
B73	+	-	-	+	+	+	+	+	+	+	+	-	-
C76	-	+	-	+	+	-	+	+	+	+	+	-	-
C78, C710	-	-	-	+	+	-	+	+	+	+	+	-	-
F74, D74	+	-	-	+	+	-	+	+	+	+	+	-	+
F76 E75	+	-	-	+	+	+	+	+	+	+	+	-	-
B72	-	-	+	+	+	+	+	+	-	+	+	-	+
D71	-	-	+	+	+	+	+	+	+	+	+	-	+
D72	-	-	+	+	+	+	+	+	-	+	+	-	+

Figure 1. Combined analysis of biochemical and molecular evidences

As clearly evident from figure 1, a sub-stratification could be highlighted. In some cases, i.e. strain E73, the pattern uniqueness is at both levels. In other cases, strains coming from different sourdoughs but characterized by the same DNA

fingerprinting even presented the same biochemical profile. This is for instance the case of *Lb. plantarum* strains F74 and D74 or F72, B71 and B75. On the other hand, for strains isolated from wheat containing sourdoughs a higher variability at biochemical level could be recorded. As matter of fact, seven strains with the same RAPD pattern could be discriminated into three different biochemical profiles.

All strains were then evaluated for the fermentation abilities in a system able to mimic conditions prevailing during sourdough fermentation. According to outcomes, strains were grouped as good (11), fair (13) and bad (19) fermenters (Data not shown). TTA, pH and counts on MRS for the best cultures are reported in figure 2.

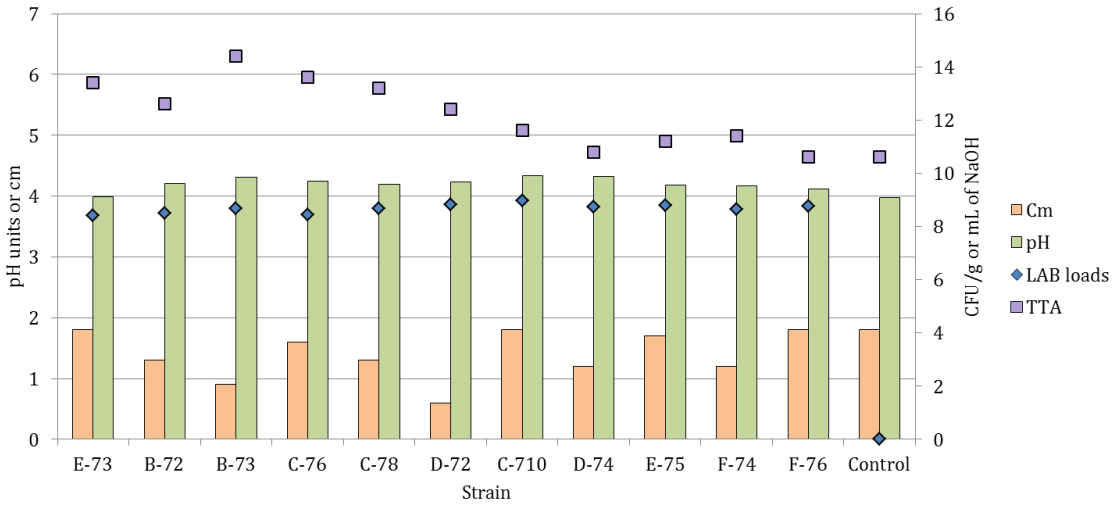


Figure 2: Height, pH, TTA, values and bacterial count for the isolated strains

Values recorded for pH and for LAB loads did not significantly differ within strains, while TTA, and above all the height reached by the doughs, were quite variable. In detail, strains E-73, C-76, C.710, E-75 and F-76 allowed to reached the same height obtained for the control (Figure 2).

Within the best cultures, three lactobacilli were selected on the basis of evidences retrieved by combining biochemical features with molecular strain typing. In detail, *Pd. lolii* B72, a strain unable to ferment maltose was combined with *Lb.*

*plantarum*E75, with phytase activity, or *Lb. plantarum* C710 unable to produce EPS by starch, or with *Lb. plantarum* E73, a strain capable to ferment xylose.

3.3. Fermentation abilities of selected sourdoughs starters

Three sourdough starters were obtained by combining *Pd. Lolii* B72 with *Lb. plantarum* E75 or C710 or E72.In the first set of trials, the three selected combinations were evaluated for the ability to ferment wheat flour. Three levels of inoculums were considered: around 8, 7 and 6 Log CFU/g, respectively. In figure 3 it is reported the initial loads of LAB, as well as of lactobacilli and pediococci separately counted on the differential MRS medium. The height reached by the dough, the pH and the TTA after 16 h of fermentation at 30°C were reported too.

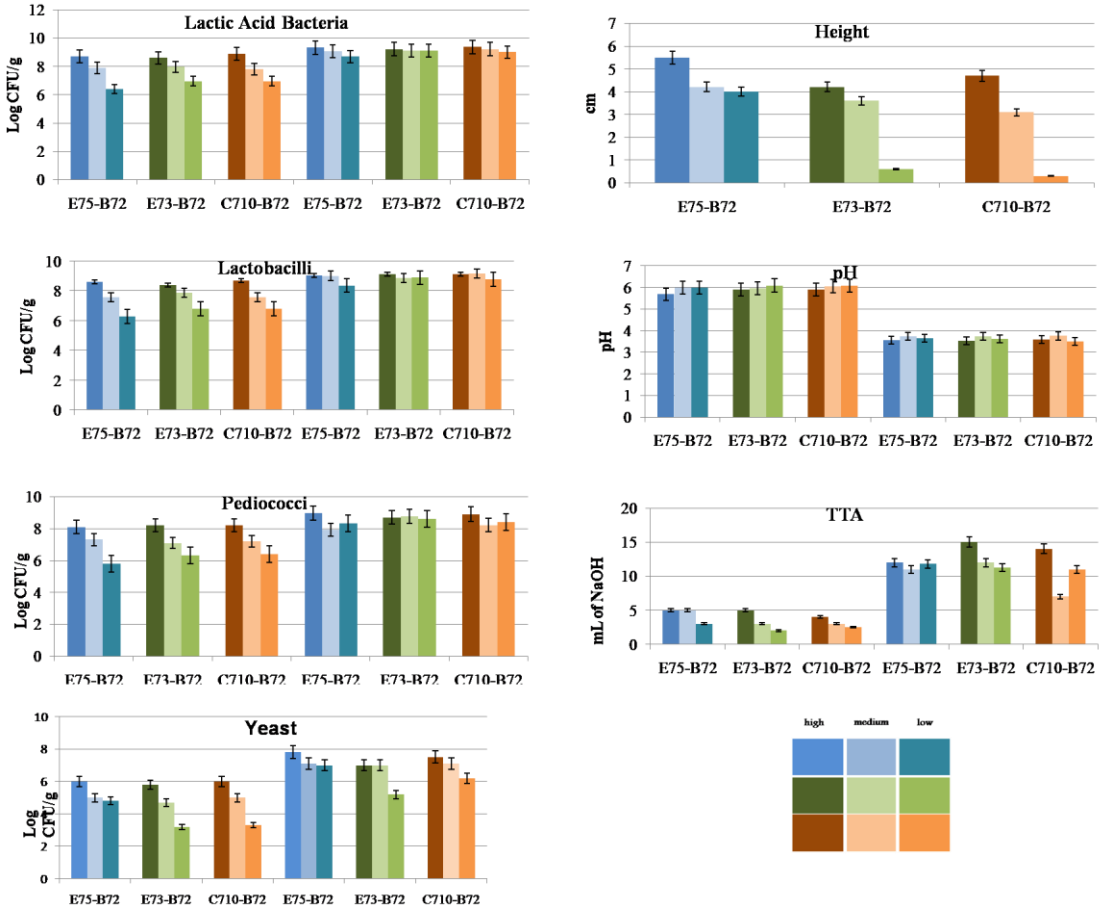


Figure 3. Evaluation of LAB, yeasts, pH and TTAafter fermentation at 30°C for 16 h. Experiments were carried on by using three different initial population level: high (~8 Log CFU/g), medium (~7 Log CFU/g) and low (~6 Log CFU/g).

Bacteria and yeast counts was evaluated before and after 16 h of fermentation on the previously described medium. In spite of the three initial loads level (8, 7and 6Log CFU/g), respectively, all doughs were able to reach around 9 Log CFU/g after 16 h of fermentation. The yeast population grew by nearly 2 logs for all combinations in the three different loads

According to data the height reached by sourdough starters after 16h of incubation was different from one combination to the other. The highest height was recorded in the sourdough containing combinationE75-B72which was 5.5cm higher from the beginning volume by using the highest load(H).No significant difference in leavening height could be observed by using the 10 folds lower (M) or 100 folds lower (L)bacterial load(*p* value=0.35 >0.05), which was nearly 4 cm higher in both loads. On the other hand the height reached by E73-B72 and C710-B72 combinations proved to be variable with the initial bacterial load: height reached by the dough was around 4 cm when high initial population level was applied, around 3 cm in the case of a mean value and less than 1 cm with the lowest load

At time zero, pH values were similar in all combination ranging from 5.68-6.06. A considerable drop in pH were reported after 16 h of fermentation which was range from 3.54-3.76 (p value=0.03, <0.05). Similar results were reported by sae Bom *et al* (2017) in which the final pH of LAB starters decreased to 3.5–3.9 using different microbial starters (*Leuconostoc citreum, Lb. brevis,* and *Lb. lactis*).This variation was also recorded for TTA values that starting from 3 reached about 12 mL of NaOH after fermentation. According to results ten folds lower loads (medium) were adopted for the subsequent experiments.

3.4. Fermentation rate during time

The three sourdough combinations were monitored up to 8 h of fermentation at 30°C. Results are reported in figure4. As clearly evident the maximum height was reached at the end of monitoring by the combination E75-B72, which was the more

effective even in the first set of experiments. On the other hand, the rate of leavening appeared higher for the combination E73-B72.

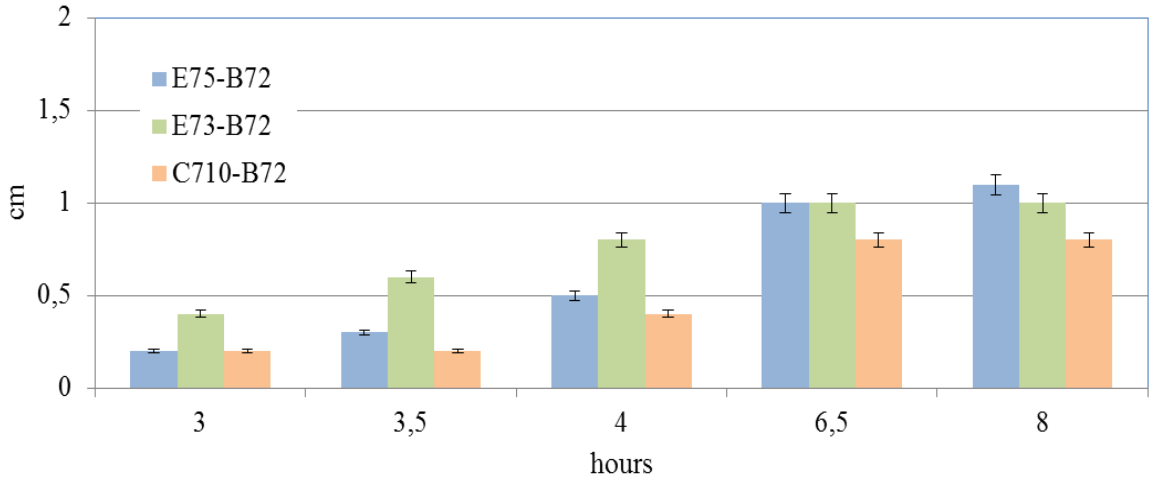


Figure 4.Fermentation rate during time for the three starter sourdoughs

3.5. Bread production

Sourdough combinations were used to prepare bread by using 400 g of wheat flour. The three microbial combinations were evaluated. Bakery yeast alone served as control. Initial bacterial loads were those corresponding to M in the first set of experiments. In all cases, starting from 7.5-7.6 logs, the LAB population level in the dough reached about 8.4 Log CFU/g after 16 hours of fermentation (figure 5).

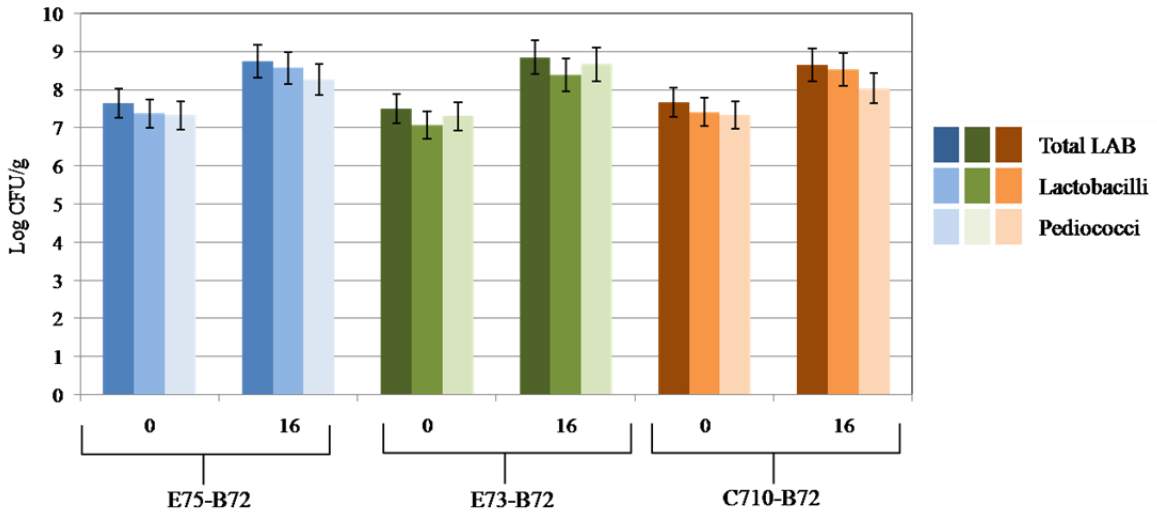


Figure 5.Bacterial LAB loads recoded in doughs of the three combinations before and after 16 h of fermentation at 28°C**.**

Yeast counts were assessed before and after fermentation, by counting on ME medium. As expected, the initial yeast loads were almost identical for all combinations including the control (yeast alone) at about 5 Log CFU/g. Yeast populations increased of about 2 logs at the end of fermentation (Figure 6).

pHlevel varied from 5.80 to6.14 which is very near from the control value 6.23.After 16h,pH level dropped to be around 3.7. TTA values were high in all combinations at time 16h as shown in figure 6.

The significant reduction in pH values and the elevation in TTA values are due to the production of lactic acid by the bacteria after fermentation. Similar results were obtained by (Lefebvre *et al.,* 2001) by using an heterofermentative strain of *Leuconostoc mesenteroides:* pH values decreased from 6.2 to 3.9 after 22 h of fermentation.

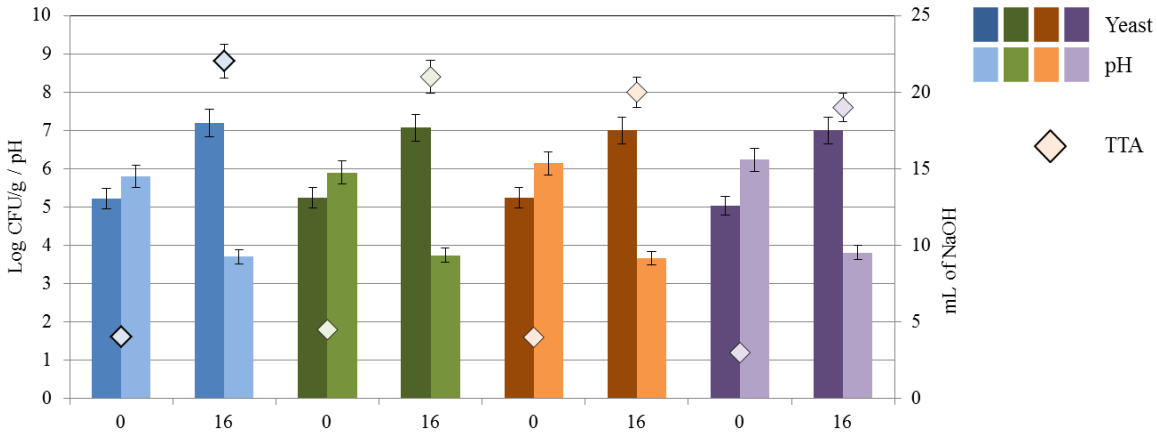


Figure 6: Yeast count, pH and TTA at time 0 and after 16h of fermentation

The highest height was reached by combination E75-B72, with an increase of about 7.5cm, while it was 3.6 cm and4.8 cm for E73-B72 and C710-B72, respectively. Apparently E75-B72 combination appeared to be the most promising (Figure 7).

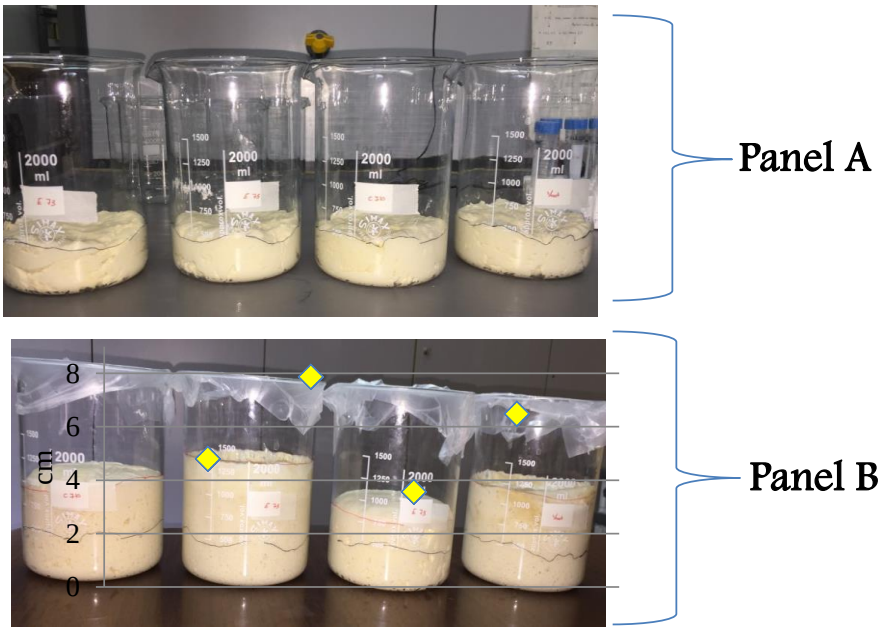


Figure 7. Height reached by the doughs at time 0 (Panel A) and after 16 h of fermentation (Panel B). The yellow dot indicated the average value (in cm) reached by the doughs.

No instrumental methods were used to evaluate bread characteristics. However the best appearance among bread samples was observed for combination E75-B72 and for control samples (Image 1).

Image 1: Bread made from three bacteria combination and control

Although the main part of the aroma is produced upon cooking, many aroma precursors are formed during the fermentation process. The volatile compounds of sourdough bread are produce by biological and biochemical actions during fermentation and contribute to flavour (Hansen & Schieberle, 2005).

To describe VOCs profile, three sourdoughs, samples were analyzed at time zero, after 16 of fermentation and upon cooking by means of SPME-GC/MS analysis.

Table 2 lists all identified VOCs. VOCs of sourdoughs may derive from microbial metabolism (e.g., alcohols, 2,3-butanedione, 3,esters and acids), enzymatic oxidation or autoxidation of flour lipids (e.g., aldehydes, ketones and 2-pentylfuran), caramelisation under drying conditions and rearrangement of carbohydrates via Maillard reaction (e.g., furans, pyrazines, phenolic compounds) and genetic and environmental factors (e.g., terpenes and lactones) (Aponte *et al.*, 2013).

However, the origin of most other aliphatic aldehydes and alcohols, as well as of aliphatic hydrocarbons and 2-pentylfuran, is the oxidative and/or thermal

degradation of unsaturated lipids (Grosch, 1982).3-methyl-1-butanol (isoamyl alcohol) is reported as the most important flavour-active compound produced by yeast fermentation. According to results, 3-methyl-1-butanol is present in fermented sourdough by three combinations and in bread made from yeast only. Similar results were obtained by Hansen *et al.*(1994, 1996).2-methyl-1-propanol and 1-butanol were also found in sourdough bread and in control dough after 16 h of fermentation, respectively, while ethanol remained undetectable.

The development of alcohols can be attributed to the activities of high levels of LAB occurring during steeping, as well as to the yeasts' activity (Rehman *et al.*, 2006). Aldehydes, such as hexanal, could result from the degradative oxidation of unsaturated fatty acids. According to data, it was present in sourdough after 16 h of fermentation and in bread (Table 2). These results were also found in many studies (Ravyst &De Vuyst, 2016; Ripari *et al.*, 2016; Settanni *et al.*, 2013; Damiani *et al.*, 1996). Also 3-methylbutanal, found in both sourdough and bread, is likely produced by Strecker degradation of amino acids. It is a common aroma compound, recorded in corn and corn products (Buttery & Ling, 1998) and rice cakes (Buttery *et al*., 1999). Ethyl acetate was found in doughs before and after fermentation. It has also been reported in spontaneously fermented maize dough (Annan *et al*., 2003) and whole and ground buckwheat grains (Prosen *et al.*, 2010).

In their study, Saeed *et al*. (2017) used three types of LAB - *Lb. brevis*, *Lb. fermentum*, and *Lb. plantarum*–for wheat flour sourdough fermentation. They found that the content 1-hexanol and hexanal by using the three different single strains as starters varied from 2.9 to 9.4% and from to 2.5 to 16% after 18 h of fermentation. They also found that 2-methyl-1-propanol was only produced by the control yeast culture, but not by LAB. In detail it was very high for *Lb. brevis* and *Lb. fermentum*, and low in the third with *Lb. plantarum* as well as in control samples after 18 h of fermentation. (Saeed *et al.*, 2017).

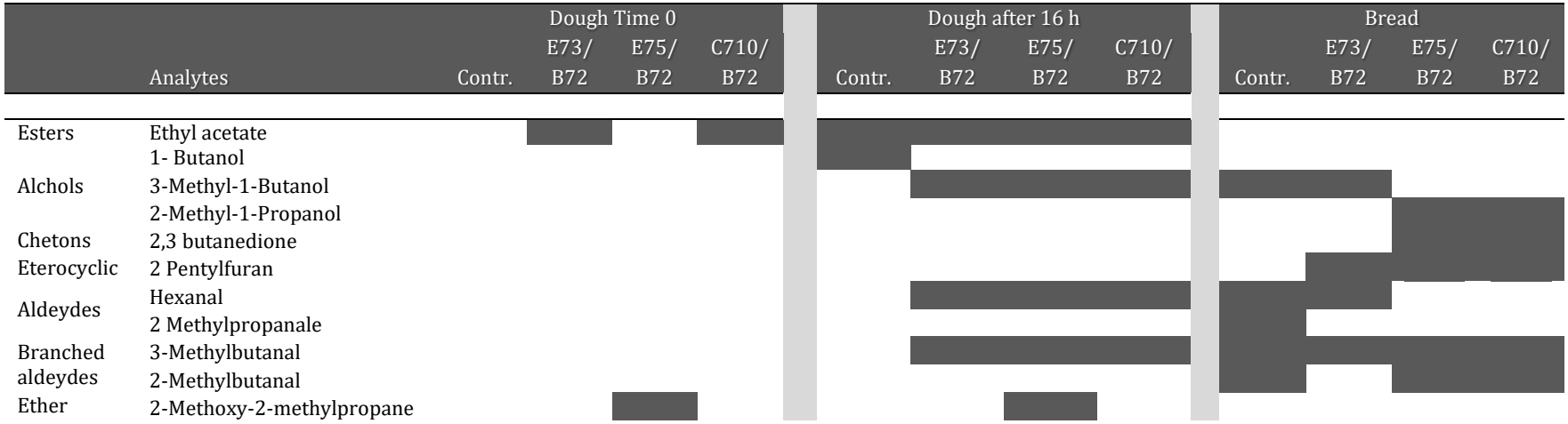


Table 2: VOCs detected in doughs at time zero, sourdough samples after 16 h of fermentation, as well as in bread samples.

Regarding to the effect of baking process on volatile compounds, similar results were also obtained by Gobbetti *et al*. (1995).In sourdough made by combining lactobacilli and *S. exiguous*, 2-methyl1-propanal appeared after baking, but only in sourdoughs started with association between LAB and *S. exiguous*. 2-methyl-1-propanal and 2- and 3-methyl-l-butanal were mainly present in sourdough breads with LAB and *S. exiguous* (Gobbetti *et al.,* 1995)

2,3-butadione and acetoin which are supposed to be very important fermentation indexes were not retrieved. Beside acetic acid, the most abundant volatile acid produced during sourdough microbial fermentation by heterofermentative LAB, was not detectable (Table 2).

3.6. Dough's gas development

The measurement of gas released by bacteria and yeast during fermentation was evaluated by using an apparatus designed *ad hoc*. Results are reported in figure 8.

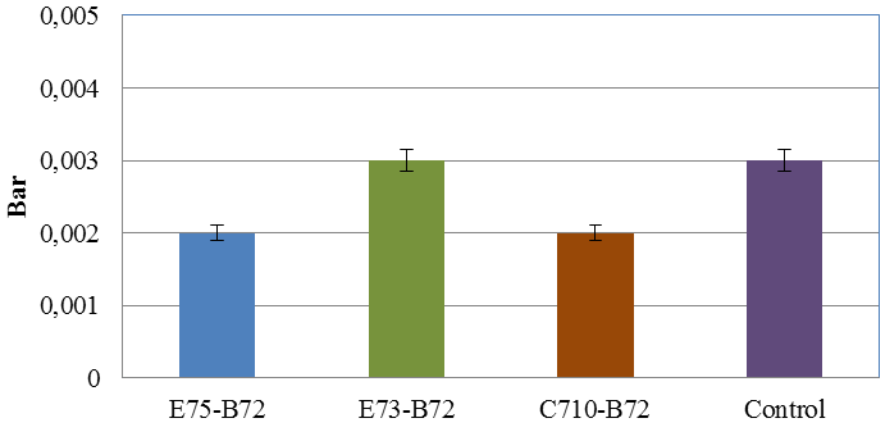


Figure 8: Gas development during sourdoughs fermentation

According to histogram in figure 6, no significant difference in gas pressure produced by microbial combinations and control (yeast alone) can be observed (*p* value =0.553- > 0.05)

3.7. Granulation process

The three bacterial combinations plus the yeast alone were subject to wet-granulation. In table 3 is reported the wet weight of the cells and the weight of the four size fractions which were obtained after granulation (<0.710 mm, 0.710-1mm; 1-1.4 mm and >1.4 mm).

Microbial combination	Cell weigh (g)	
	before granulation	after granulation
E73/B72	1.69	3.206
E75/B72	1.09	2.024
C710/B74	0.92	2.165
Control	0.34	0.593

Table 3: Weight for microbial cells before and after granulation process

In figures 9 is reported the survival of LAB and yeast after granulation compared with the initial load in the cultures used to collect the cells.

In each panel LAB and yeast counts in granules of each size is compared with the values assessed in the broth cultures before drying. For all combinations, bacterial loads varied from 9.9-10.6 Log CFU/mL before granulation and yeast count was similar in all combinations 7.6-8 logs. With regard to the size of the granules, even if differences proved to be not significant (p value =0.9> 0.05)the cells survival appeared to be directly correlated to the size of the granulates: as bigger granules were in size, as higher was the cell survival, likely due to the ability of the matrix to entrap more microorganisms (Figure 9).

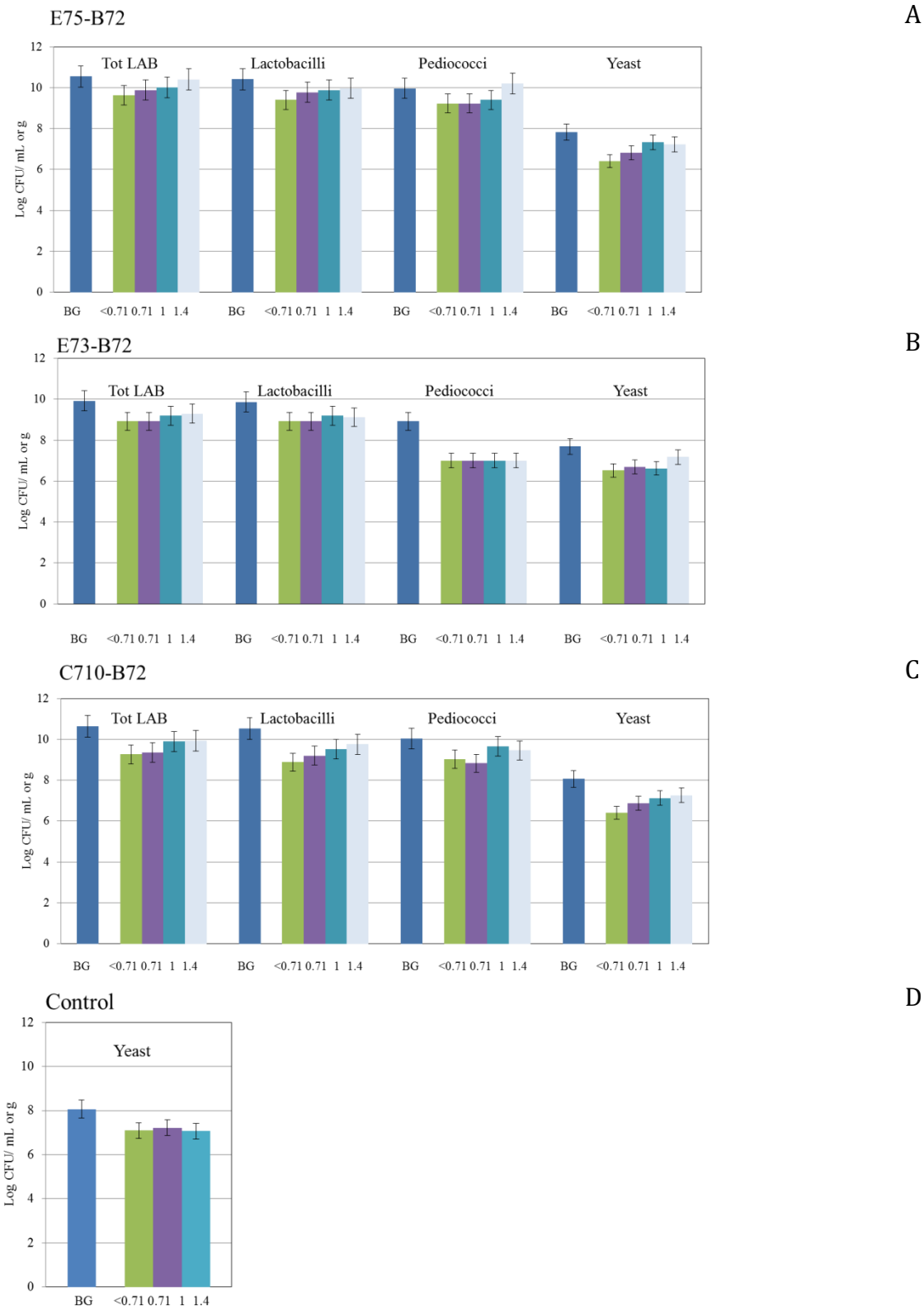


Figure9. Effect of granulation on the microbial counts for the three combinations plus the control.

3.7.1. *Cells viability after wet granulation*

To get the most reliable quantification of the cells not injured by the granulation process a parallel approach was adopted. In detail, total cells were obtained by direct counting Petroff-Hausser chamber, while alive and injured or dead cells were assessed by counting at fluorescence microscope after BacLight Kit dying. Alive cells were quantified by counting on MRS agar plates and data was compared with undamaged cells as green after Live/Dead BacLight staining. The sum of damaged and dead cells was obtained by BacLight Kit by counting red cells (Figure 10).

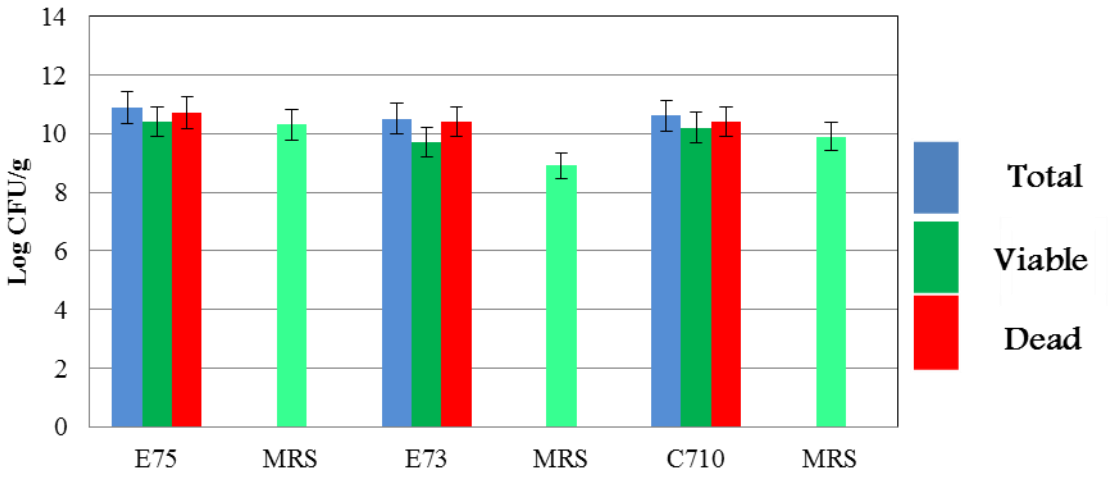


Figure 10. Viability and count of microbial combination after wet granulation.

According to data, bacterial combinations did not significantly differ in their ability to survive after granulation process(p = 0.6 > 0.05).First of all, the maximum population level of all strains appeared almost one log higher if compared with the growth exhibited in MRS medium. The count of green viable bacterial cells was around 10 Logs CFU/g which is nearly same count obtained from direct count on MRS medium while the count of red injured cell was slightly higher than the viable cells. Indeed, it could be noted that, during observation at the microscope, several cells which were green at the beginning turned into red as long as the time passed.

This may be likely due to the toxic effect of the dying on the cells viability. Images of cells entrapped into granules are reported in the following frame (Image 2).

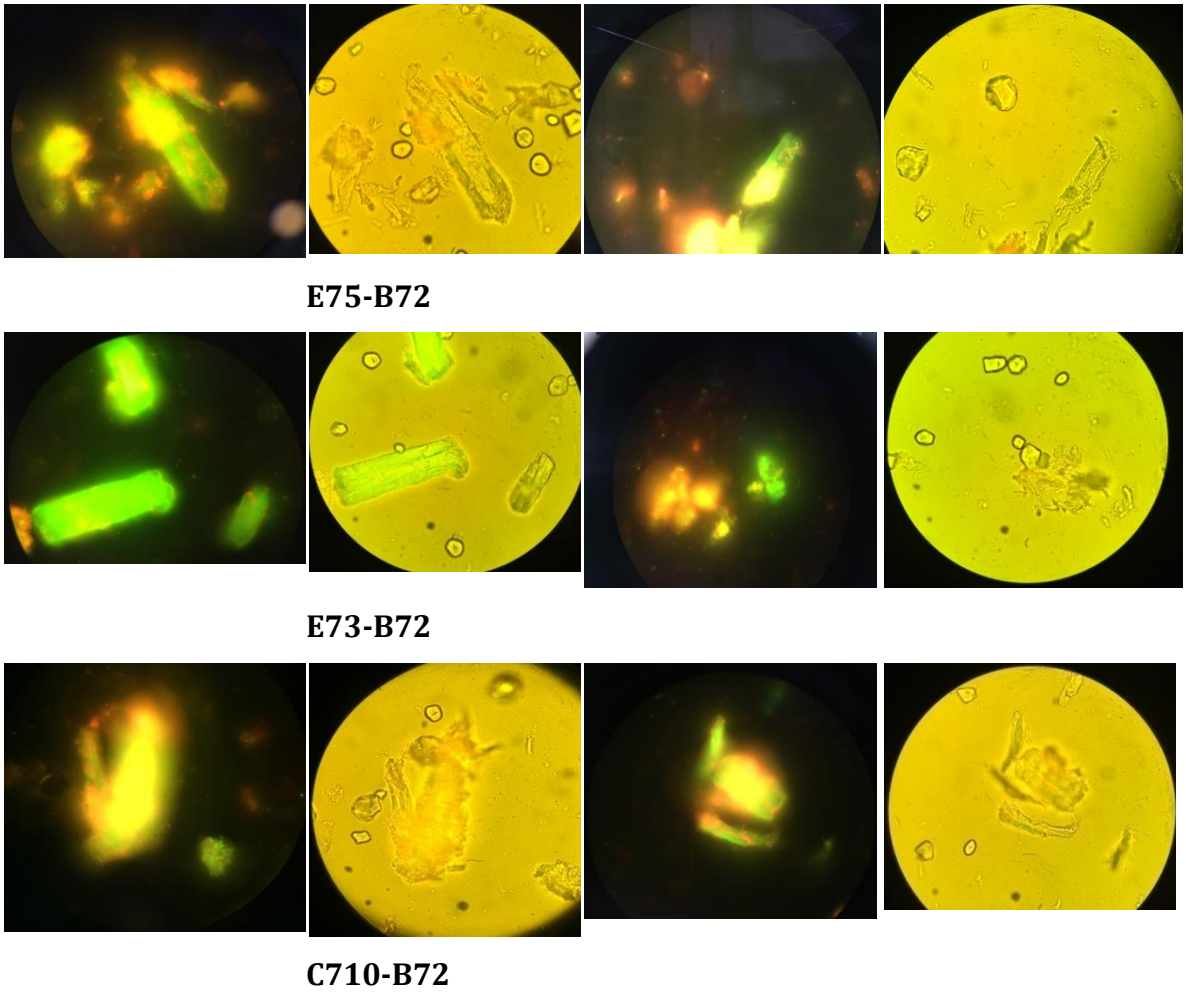


Image 2. Appearance of wet granulated cells under epifluorescence microscope.

3.7.2. Fermentation abilities of the granulated sourdough starters

The three combinations were tested as fresh cells and as granules according to the protocol previously detailed. The amount of one g of granules was calculated in order to reach almost the same concentration of alive cells which were incorporated in the dough at the beginning of the fermentation. Except that for combination C710-B72, significantly higher amount of LAB was counted in the granules if compared to the fresh counterpart. The initial cell loads were almost identical for LAB as well as for yeast (p value =0.8 >0.05).

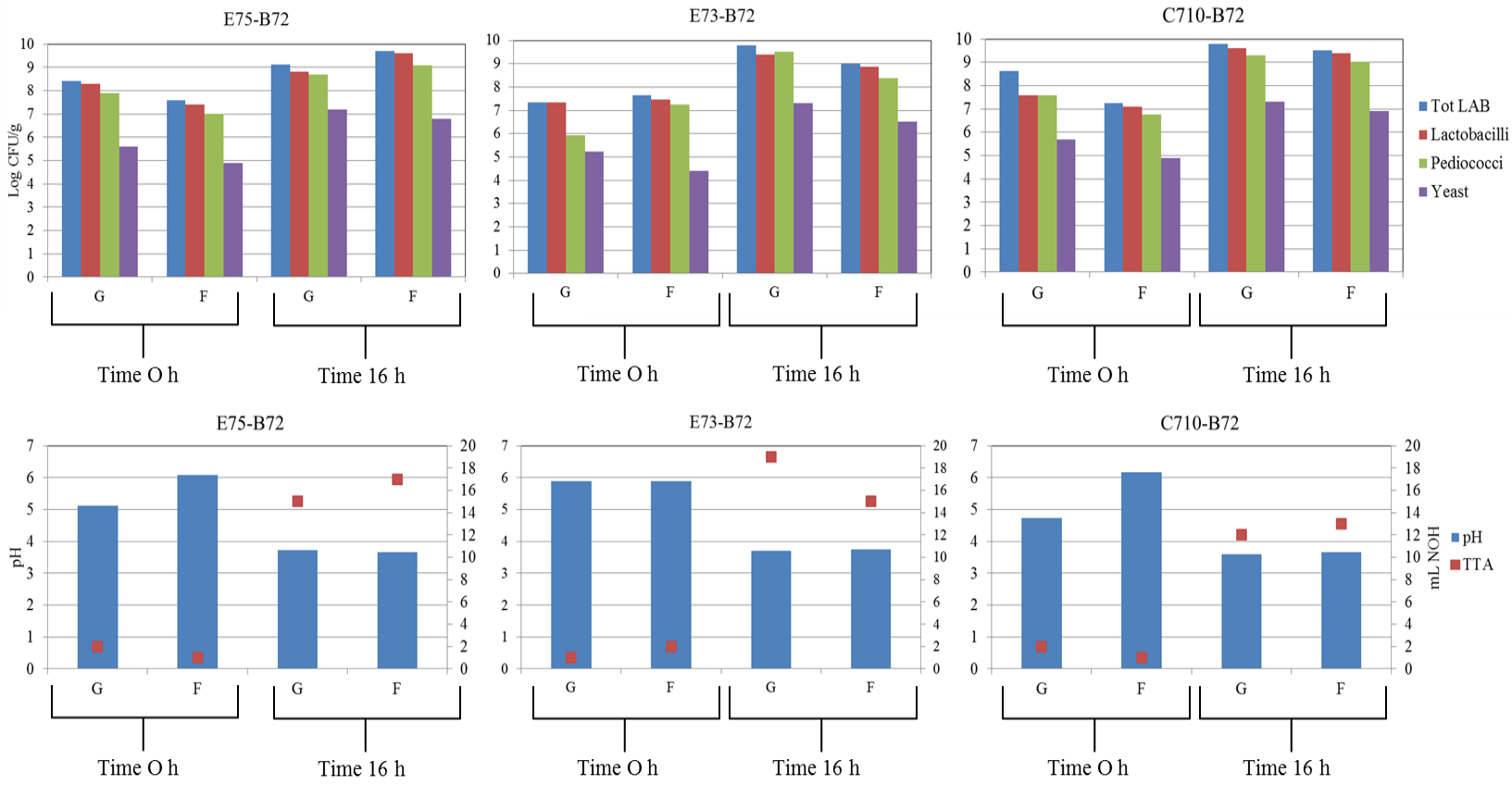


Figure 11. Microbial counts, pH and TTA values at time 0 and after 16 h of fermentation for sourdough made by using four wet granulated microbial combinations

After 16 of fermentation the population level for fresh cells and granules was comparable for the combination C710-B72. For the other two combinations a contradictory behaviour could be noticed (Figure11).

Such apparent inconsistency is fully reflected by the recorded TTA values which, at the end of fermentation, were the same for the sole combination C710-B72. In the other two trials, the acidity was higher in the option characterized by a greater LAB population level (Figure 11).

The height reached by the different mature sourdough is reported in figure 12.

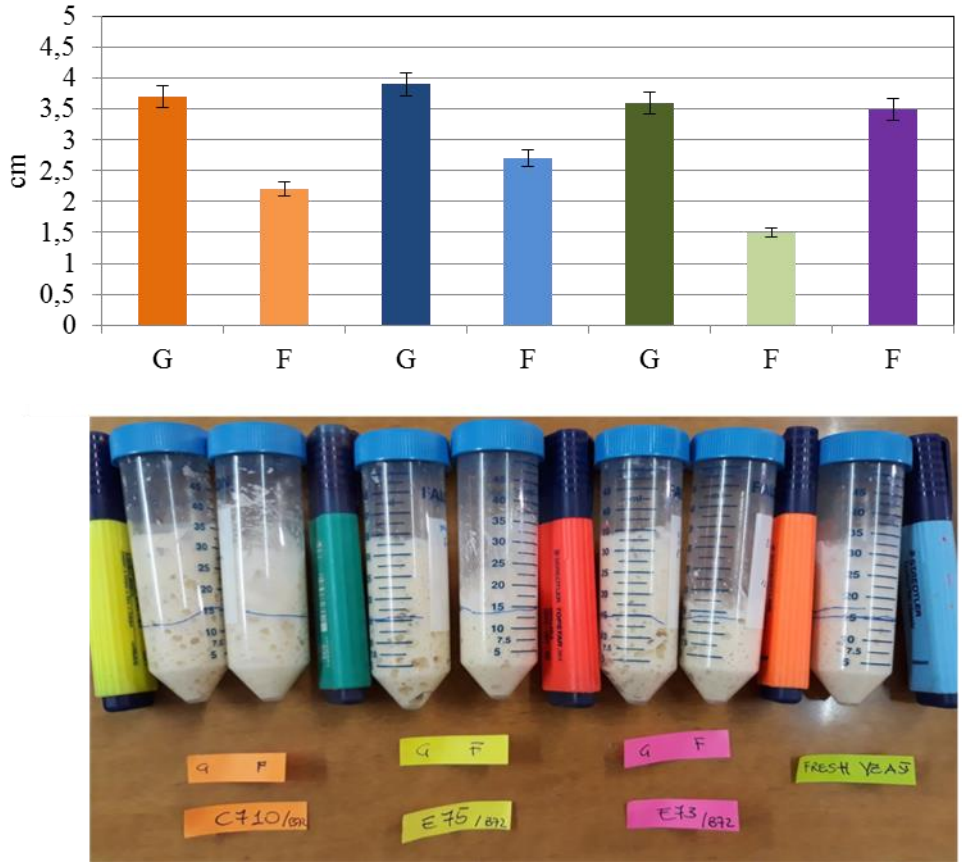


Figure12. Height reached by both wet granulated and fresh cells.

3.7.3. HPLC analysis of sourdough obtained by both granulated and fresh cells

Reducing sugars and organic acids variations during sourdough fermentations were assessed by HPLC analyses. Samples to be analysed were collected before and after 16 h of fermentation The results of ethanol, lactic acid and sugars content in sourdoughs prepared by using both wet granulated and fresh cells are illustrated in figure 13.

Glucose contents ranged by 2-3g/L in all doughs at the onset of fermentation and, as expected, quickly declined. At any rate, the lowest amount of residual glucose was recorded in the control, in which fermentation was led uniquely by fresh bakery yeast. The same trend could be recorded for fructose as well: the sugar was largely consumed but no difference could be detected in the amount of fructose at the end of fermentation within the four trials and either by comparing sourdoughs produced with granulated and fresh cells. Unexpectedly, the concentration of maltose at the end of fermentation was significantly higher than that recorded in doughs at the onset of fermentation. Indeed, such phenomenon has already been described (Lefebvre *et al*., 2001). Maltose content can increase during the sourdough fermentation due to the hydrolytic activity of indigenous amylases on the starch fraction damaged during the milling process (Mathewson, 2000). Similar results were even obtained by using *Lb. plantarum* strain as starter by Gobetti *et al.* (1994).

Due to the utilization of fermentable carbohydrates and the LAB population increase, lactic acid - nearly absent before fermentation -increased in all combinations. Values were very high after 16 h of fermentation to be around 8-10 g/L. Of course, this may explain the recorded reduction in pH values. Ethanol content, as expected, was absent in all combination at the beginning of fermentation but its concentration proved to be extremely variable after fermentation within analysed samples. It was around 2 and 4 g/L in viable E75-B72, and C710-B72 combinations but reached around 5 g/L in sourdoughs obtained by using the same strains combinations as granulated.

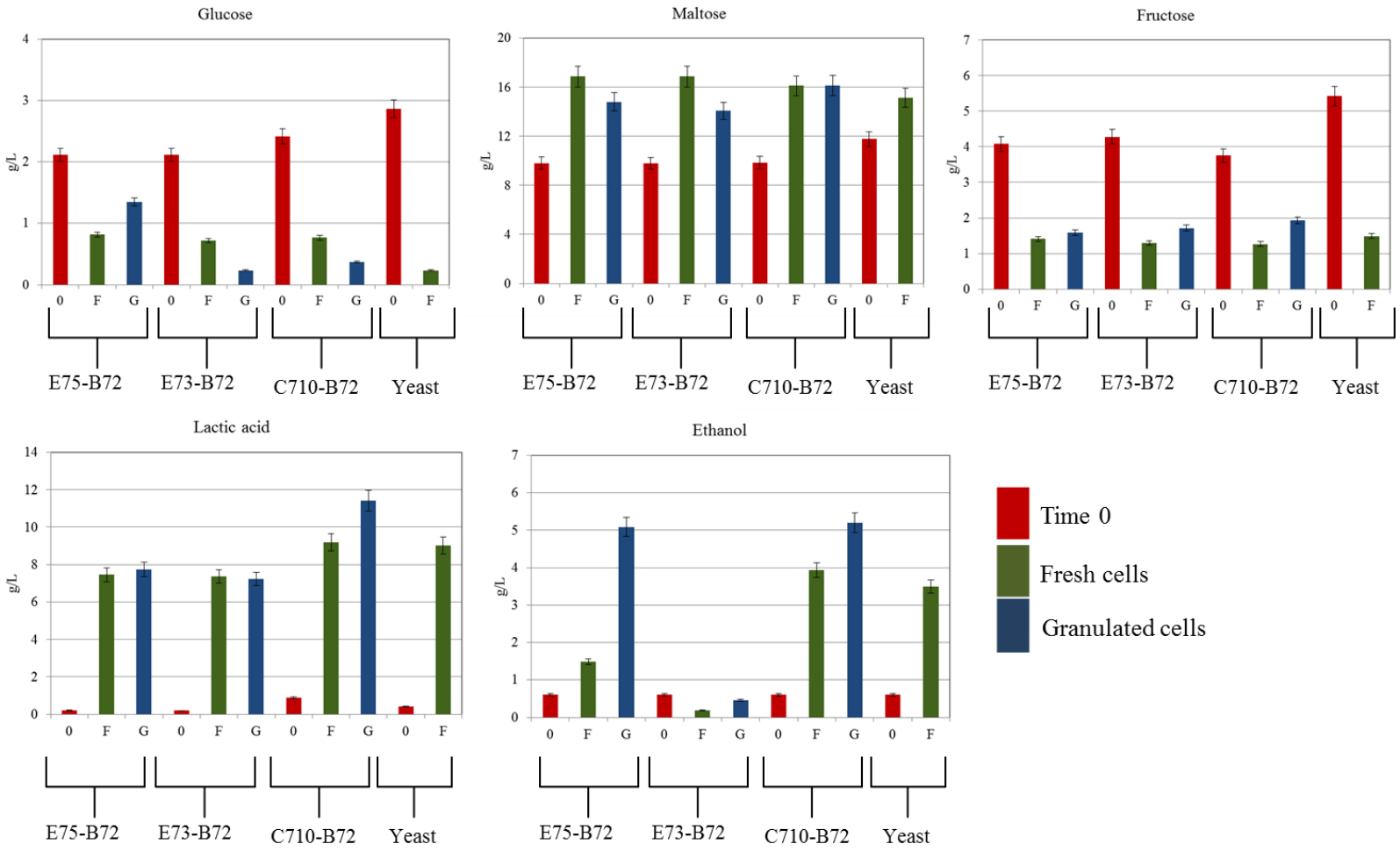


Figure 13. Carbohydrates consumption and lactic acid/ethanol production after 16 h of fermentation. HPLC analysis was performed on dough at time 0 and on sourdoughs produced by using the three starters as fresh (F) or granulated (G) cells.

No correlation could be found between ethanol accumulation, which is to be considered an index of a more pronounced alcoholic fermentation and, as consequence, of a greater CO^2 production and the height of the dough (Figure12).

3.8. Sensory evaluation

The results of the sensory tests on the breads are reported in Table 4. In image 3 is shown the way by which samples were presented to panellists. With reference to the appearance of the crust, outcomes obtained by comparing breads of the different trials exhibited noticeable differences (Figure 14). Breads obtained by using the traditional sourdoughs were almost identical to those obtained by using the starter, independently by the kind of formulation. The three types of bread proved to be characterized by a crust more crispy, thick and coloured, if compared to bread produced with bakery yeast only. This last type of samples also presented a crust more elastic. No difference was instead recorded with reference to the crumb. Only bread produced with the yeast alone exhibited a crumb more gummy than the other three types.

With regard to both odour and aroma, undeniably the sourdough descriptor was perceivable only in breads produced by using the natural sourdoughs. At any rate, for all the considered descriptors, the aroma of the breads produced by using the starter as fresh cells, values indicated by the eight panellists were slightly higher.

More significant differences could be retrieved by analysing the data of taste rating. All samples – without exceptions -were judged unsatisfactory for the salt, since no salt was added into the dough. Breads produced by adopting the starter as fresh cells were more bitter and more acid. This trait is appreciated by Italian consumers since associated to traditional breads. According to the evaluation of the overall acceptance, the breads produced by using the starter were the most appreciated by panellists, but only when cells were added to the dough without granulation. The samples less esteemed were those produced by using the bakery yeast only.

	Crust			
Trial	Color	Thickness	Elasticity	Crispness
A -yeast	2,38±1,30	2,63±1,19	3,00±1,07	1,88±0,83
B - E75-B72	3,25±0,71	2,88±1,36	2,13±1,81	3,75±1,39
C- Sourdough	3,38±0,74	3,00±1,20	1,50±1,20	3,88±0,99
D- Granulated	3,13±1,13	1,88±1,55	2,38±1,51	3,38±0,92

	Crumb			
	Gummy	Color	Alveolation	Alv. Unif.
A -yeast	3,25±1,04	3,00±0,76	2,50±0,93	2,63±0,92
B - E75-B72	2,25±1,28	2,75±0,71	2,75±1,28	2,63±1,06
C- Sourdough	2,63±1,30	3,00±1,31	2,88±1,13	2,00±1,41
D- Granulated	2,75±1,28	2,88±0,83	2,63±1,19	2,50±1,07

	Odour				
	Intensity	Bread	Yeast	Sourdough	Unpleasant
A -yeast	1,63±0,74	2,13±0,99	1,75±1,04	1,63±1,06	1,13±1,25
B - E75-B72	1,88±1,13	2,38±1,06	1,38±0,74	1,63±0,74	1,13±0,83
C- Sourdough	2,63±0,92	2,38±1,30	2,00±1,51	3,00±1,31	1,38±1,30
D- Granulated	1,63±0,74	1,88±0,83	1,38±1,19	1,25±1,39	1,00±0,93

	Aroma				
	Intensity	Bread	Yeast	Sourdough	Unpleasant
A -yeast	1,63±0,92	2,13±1,13	1,63±1,30	1,38±1,06	1,38±1,51
B - E75-B72	2,38±1,30	2,50±1,51	1,50±1,20	1,38±1,19	1,25±1,28
C- Sourdough	2,75±1,58	1,88±1,36	1,88±1,36	3,38±0,92	1,38±1,51
D- Granulated	1,75±0,89	1,88±0,83	1,75±1,39	1,75±1,58	1,13±1,73

	Taste				
	Salty	Acid	Bitter	Persistency	Overall
A -yeast	0,13±0,35	0,50±0,76	0,25±0,71	1,25±1,16	2,00±1,00
B - E75-B72	0,38±2,07	1,25±1,49	1,13±1,64	2,38±1,77	2,71±1,89
C- Sourdough	0,63±0,74	2,88±1,46	0,63±0,92	2,50±1,31	2,43±1,40
D- Granulated	0,50±0,76	0,88±1,13	0,63±1,06	1,88±1,81	2,29±1,60

Table 4. Total scores for the sensory attributes. Data are reported as mean ±sd

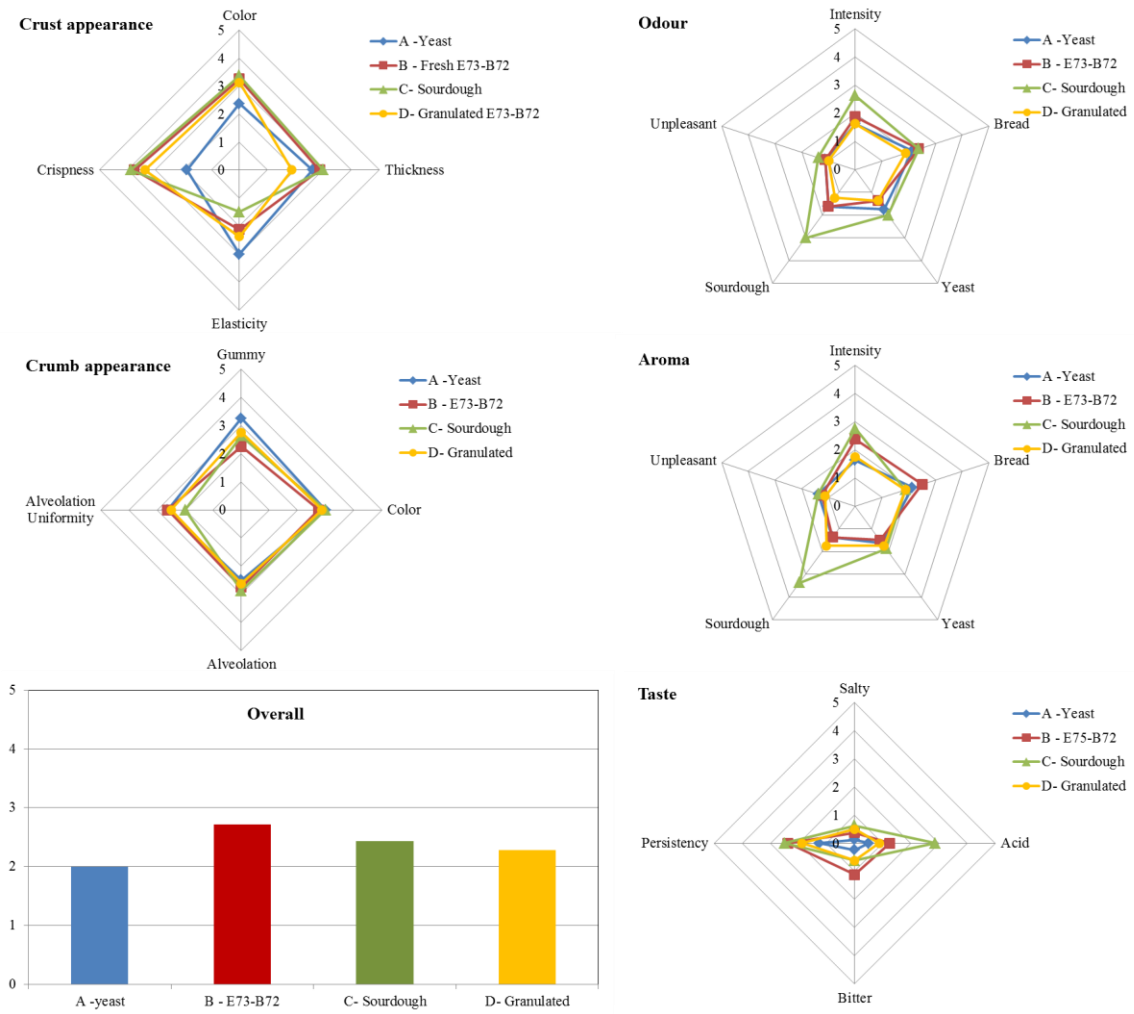


Figure 14. Sensory profiles of breads produced by using the bakery yeast (A), the starter E75-B72 as fresh cells, a traditional sourdough (C) or the starter after wet granulation (D).

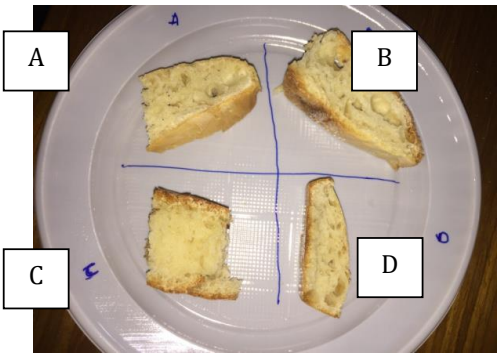


Image 3. Breads samples presentation during sensory test.

3.9. Freeze drying effect on the viability of sourdough bacteria

The combination E75-B72 was subject to freeze drying. Powders were used at 10% as inoculum to lead a fermentation. In figure 15 are reported LAB and yeast loads in doughs before and after freeze drying.

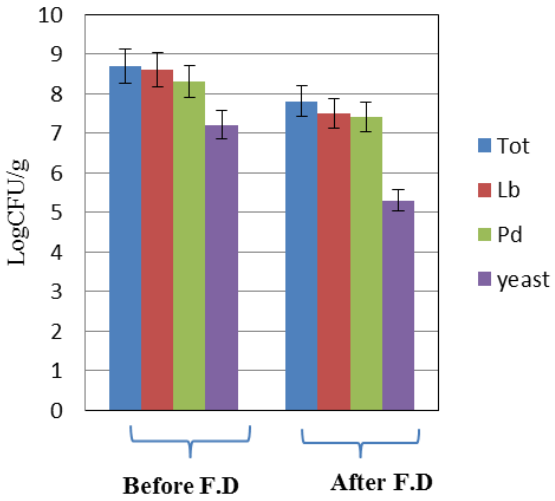


Figure 15. Total counts on dMRS (Tot,), selective loads of pediococci (Pd) and lactobacilli (Lb) as well as of yeasts before and after freeze drying.

Freeze dried doughs showed 8.7 logs of total LAB and 7.8 logs of yeast. After drying, microbial counts dropped by nearly one log for LAB and of about 2 logs for yeasts. Dried doughs were used as starters by adding the powders to wheat flour in ratio 10%. Figure 15 illustrates the microbial loads at time 0 and after 16 h. Bacterial and yeast loads were able to increase by around 2 logs despite the increase in height of the sourdough was only 0.9 cm. Height difference between fresh and freeze dried starters is significant (p value=0.02 < 0.05)

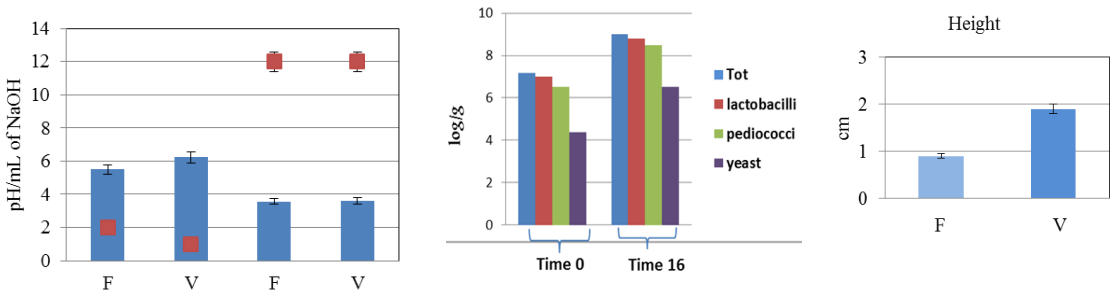


Figure 16. Bacterial and yeast loads in doughs realized by using the freeze dried sourdough E75-B72 as starter before and after fermentation. Height reached (F) was compared with that obtained by using fresh cells (V).

For the freeze dried dough the pH started with low value around 5 and it reached 3after 16 h of fermentation but for the viable cells it was normal around 6. TTA values were no significantly different: mL of NaOH were almost the same for sourdoughs obtained by using fresh or freeze dried cells

Similar result on pH values and microbial count were obtained by Robert *et al. (2006*) in which pH values dropped from 6.1 to reach about 3.9 and initial microbial count starts with 10^7cfu/g and reached about 10^9cfu/g after16 h of fermentation using freeze dried cultures of heterofermentative *Leuconostoc* strains and *Lb. plantarum*. .

CONCLUSION

In this study the potential of 43 bacteria strains to be use as suitable sourdough starters was assessed. First, the fermentation abilities of these strains were assessed, then the best fermentative strains were further classified on the bases of other features of technological interest The selection of three *Lb. plantarum* and *Pd. solli* strains was made on the bases of their biochemical and genotypical features in which *Pd. lolli* strains expressed urolytic activity while *Lb. plantarum* E75 have phytase activity, E73 was able to ferment xylose while C710 was unable to produce EPS. The combinations between *Lb. plantarum* strains and *Pd. lolli* were successful, because the leavening height with the bacterial combination was higher than each strain alone. On the other hand, E75-B72 has recorded the highest height both by using small and large flour samples comparing with the other two combinations.

The biochemical features, microbial count, gas measurements and volatile compounds production didn't differ significantly between combinations

In the second phase of the study, two methods were used for sourdough starters preservation and compared. The first method was the wet granulation and the second was the freeze drying method. Wet granulation was successful in obtaining better leavening height in comparing with fresh cells (p value < 0.05), while the leavening height obtained by using freeze dried sourdough starter was lower than the fresh cells. Wet granulation process has a very little effect on the viability of microbial cells and doughs obtained were characterized by a considerable amount of lactic acid like the fresh cell starters.

Sensory analyses univocally indicated that breads obtained by using the starter culture, above all as fresh cells, resemble those produced by means of the natural sourdough. Indeed, further efforts are required to point out a better strategy to stabilize the microbial combination during its shelf-life.

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