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SOURDOUGHS FOR SWEET BAKED PRODUCTS: MICROBIOLOGY, CHARACTERIZATION, SCREENING AND STUDY OF EXOPOLYSACCHARIDES PRODUCED BY MICROBIAL STRAINS

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1. INTRODUCTION

Cereals represent the most important food crop. Their cultivation dates back to 7000 B. C. for wheat and barley, 4500 B.C. for rice and maize, 4000 B.C. for millet and sorghum, 400 B.C. for rye and 100 B.C. for oats. At present, the total global production of food crops amounts is 3.6 billions tonnes and the 60% are cereals. In develops countries the 70% of the cereals were used for animal feed. The other part was used for human nutrition. This last one is almost all consumed in cereals fermented foods. The first use of cereal fermentation was represented by a porridge that was made of pounded or ground grains, which were later baked.

1.1 Cereals fermentation

Normally, when considering the variety of foods made with cereals, it will be easier thinking about just fermentation foods. Generally, fermentation is a process that proceeds under the influence of activities exerted by enzymes and /or microorganisms (Hammes et al., 2005). To know the microbial ecology of cereals fermentation it is necessary know about the fermentation substrates like the grains or seeds of the various cereal plants. During the cereals fermentation, the enzymes, bacteria, yeast and mould can play a role alone or with the combination of all of them togheter, and finally they contribute to the creation of a great variety of products.

The aim of fermentation process is to achieve this aims:

- Conditioning for wet milling by steeping of maize (Johnoson, 2000) and wild rice (Oelke and Boedicker, 2000).
- Influencing sensory properties.
- Saccharification by use of koji prior to alcoholic fermentation or producing a sweetened rice.
- Preservation.
- Increasing food safety by inhibition of pathogen bacteria.
- Enhancing the nutritive value by removing antinutritive compounds (as phytate, enzyme inhibitors, tannins) and improving the availability of components, for example by affecting the physio-chemical properties of starch.
- Withdrawing same undesired components as mycotoxins, endougenous toxins, cyanogenic compounds and flatulence producing carbohydrates.

The cereal fermentation is influenced by different variables (Hammes and Gänzle, 1998):

- Type of cereals determining the fermentable substrates.
- Water content.
- Degree and moment of comminution of the grains.

- Components adding to the fermenting substrates.
- Source of amylolytic activities (Hammes et al., 2005).

The kind of cereal used is the major variable, in fact, the amount and quality of carbohydrates, nitrogen source, growth factors, minerals, buffering capacity and the efficacy of growth inhibitors are affected.

1.2 Sourdough

The traditional sourdough fermentation is represented by the combined activity of hydrolytic activities of the grain and LAB and yeast. Essentially it consisted of a mixture of flour and water that is fermented by microorganisms present. At this aim the grain used must not been heat treated before to assure the presence of hydrolytic activities. When added water, microorganisms present will become metabolically active, multiply and with incubation, the most competitive microorganisms will be dominant. In sourdough, generally, LAB occurring at numbers > 10^8 CFU g^{-1}, while the yeast are lower, around 10^6-10^7 CFU g^{-1}. The LAB: yeast ratio is generally 1:100 (Ottogalli et al., 1996). Unlike the others fermentation process, where the LAB homofermentative play a fundamental role, for sourdough fermentation heterofermentative LAB are dominating, especially when sourdough are prepared by traditional techniques. The LAB present may originate from a selected natural contaminants in the flour or from starter culture. A big number of studies were demonstrated that more than 50 LAB species (mostly species of the genus *Lactobacillus*) and more then 20 species of yeast (especially species if the genera *Saccaromyces* and *Candida*) can been involved during the fermentation steps. *Saccaromyces cerevisiae* is frequently for the use of the baker's yeast; *S. exiguus* (*Torulaspora holmii* or *Candida holmii* or *S. minor*), *C. humilis* (*C. milleri*) and *Issatchenkia orientalis* (*C. krusei*) are usually associated with LAB in sourdough fermentation. Otherwise a large variety of yeast was isolated from sourdough, the variability depends to the degree of dough hydration, the type of the cereals used and the leavening temperature. Also, the number and species of yeast present in a sourdough depending on the degree of yeast tolerance to the organic acids produced by the LAB and by the availability sources of carbon (Pulvirenti et al., 2004). The sourdough microflora is composed of stable associations of lactobacilli and yeasts, in particular due to metabolic interactions. These microbial associations may endure for years, although the fermentation process runs under non-aseptic conditions (De Vuyst and Neysens, 2005). Ecological factors are determining to select the microflora during sourdough fermentation and also depend on both endogenous and exogenous factors (Hammes et al., 1996; Vogel et al., 1996). Endogenous factors are determined by the chemical and microbiological composition of the dough, exogenous depends especially, from the temperature and redox potential.

Many effects are due to the process parameters such as dough yield, addition of salt, amount and composition of starter, number of propagation steps and fermentation time. The impact of this parameters causes the selection of the characteristic microflora and at the same time prevent the growth of potential pathogen or alterative microorganisms.
The use of sourdough during the baked good production improved dough machinability, nutritional properties, organoletic features and prolonged the shelf-life. The disadvantages to use sourdough are the long time and the labour consuming.

1.2.1 Spontaneous sourdough fermentation

Sourdough is rich of a fermentable carbohydrates and it has a initially pH ranged from 5.0 to 6.2, that determined a spontaneous development of LAB, derived from cereals or flours. The traditional sourdough process does not involve the fortuitous microflora but used a mother doughs that they are continuously propagated for long period. The mother dough represents the natural microbial inoculum for the subsequent doughs (De Vuyst and Neysens, 2005). During this process, the LAB immediately dominate on the Gram negative enterobacteria. In this kind of sourdough fermentation are present both lactobacilli (homofermentative as *Lb. casei, Lb. delbrueckii, Lb. farciminis*, *Lb. plantarum,* and heterofermentative as *Lb. brevis*, *Lb. buchneri* and *Lb. fermentum*) and pediococci (*P. acidilactici*, *P. pentosaceus*). Instead, genera *Weisella* and *Leuconostoc* may play a role during the first part of the fermentation and pediococci species are more frequently in the end of the fermentation. Finally, in this kind of sourdough, the most common species of yeast that can find are *S. turbidans*, *S. marchalianus*, *S. albida*, *S. exiggus*, *S. cerevisiae* and *Saturnispora saitoi* (Stolz, 1999).

1.2.2 Sourdough fermentation through backslopping

When this technique is applied, can find a spontaneous microflora and in particular, mostly heterofermentative LAB. The so called sourdough lactobacilli *Lb. sanfranciscensis* (Kline and Sugihara, 1971), *Lb. pontis* (Vogel et al., 1994), *Lb. panis* (Wiese et al., 1996), *Lb. paraalimentarius* (Cai et al., 1999), *Lb. frumenti* (Müller et al., 2000a) and *Lb. mindensis* (Ehrmann et al., 2003) are typical of this sourdough, because their competitive metabolism has adapted to this environments. Instead, species like *Lb. brevis* and *Lb. plantarum* can be considered like a ubiquitous and *Lactococcus* species can be used deliberately. In this case, also, same factors can contribute to the LAB dominance. First, their carbohydrates metabolism is highly adapted to the main source of energy in the dough, maltose and fructose. Second the growth requirements with a respect of temperature and pH conditions. Third, the lactobacilli have stress response mechanisms

to overcome acids, temperature, osmolarity, oxidation and starvation (De Angelis et al., 2001). In the end, the production of antimicrobial compounds improves their competitiveness and contributes to their presence in the sourdough.

1.2.3 Classification of sourdough

Sourdoughs have been classified into three types, based on the kind of technology applied for their production, as used in artisan and industrial process (Böcker et al., 1995):

- Type I sourdough or traditional sourdough.
- Type II sourdough or accelerated sourdough.
- Type III sourdough or dried sourdough.
- Type 0 dough

The type I sourdough is produced with a traditional techniques and are characterized by a continuous and daily refreshment to keep the microorganisms in active state, for a high metabolic activity and to obtain a good leavening with a production of gas. The process is performed at temperature ranged from 20-30°C. The LAB frequently isolated from this kind of sourdough are *Lb. sanfranciscensis*, *Lb. pontis*, *Lb. fructivorans*, *Lb. fermentum* e *Lb. brevis* and the yeast specie is *C. humilis*. The type I sourdough include pure culture, sourdough starter isolated from different origin (type Ia), mixed culture sourdoughs made from wheat and rye and prepared with multiple stage fermentation process (type Ib) and finally the sourdough made in a tropical regions fermented at high temperature (Type Ic).

The type II sourdough is a semi-fluid silo preparation and was born to satisfy the industrial demands. In this, case the sourdough process is obtained by a continuous propagation and long-term one-step fermentations to guarantee more production reliability and flexibility. This process is carried out at a fermentation temperature of 30°C for 2-5 days and after 24 h of fermentation the sourdough has a pH value of <3.5. In this case the microorganisms are in the last stationary phase and their metabolic activity is restricted. The LAB species occurs are the obligate homofermentative as *Lb. acidophilus*, *L. delbrueckii*, *Lb. amylovorus*, *lb. farciminis* and *Lb. johnsonii* and heterofermentative specie as *Lb. fermentum*, *Lb. frumenti Lb. panis*, *Lb. pontis*, *Lb. reuteri*, and *Lb. brevis* and *Weisella* species, too (Müller et al., 2001; Vogel et al., 1999).

Type III sourdough is dried dough in powder form which are initiated by defined starter cultures (De Vuyst and Neysens 2001). This kind of sourdough is used especially as a acidifier supplements and to increase the aroma. It contains LAB that are resistant to the drying process as *Lb. brevis* or same facultative heterofermentative species as *P. pentosaceus* or *Lb. plantarum*. This form of sourdough is convenient, simple in use and result in standardized end products.

Sourdoughs type I and type II required the addition of baker's yeast as leavening agent.
In the end, type 0 dough consisted of dough which uses baker's yeast to obtain the leavening and it not made with a sourdough fermentation. Anyways, yeast preparation often contain LAB, belonging mainly to the genera *Pediococcus, Leuconostoc* and *Lactococcus* spp. (Jenson, 1998), which can contribute only to a small degree to the acidification and aroma development (Corsetti and Settanni 2007).

1.2.4 Microbial interaction

Knowledge to develop and increase the stability association between LAB and yeasts in sourdough it is necessary to prevent the loss of variety of regional specialities and at the same time, to meet consumer and industry demands. The stable association between LAB and yeast in sourdough fermentation exists because of their growth requirements with respect of temperature, pH, and organic acids as well as metabolic interaction. Although in some cases, LAB and yeasts can compete for the available substrates, resulting in heterogeneous populations that reflect the media resources and environmental conditions (De Vuyst and Neysens 2001). This can change the mother completely and quickly in the case of propagation and backslopping (Ottogalli et al., 1996). The importance of antagonism and synergism between these microorganisms is due to the metabolism of carbohydrates and amino acids and the production of carbon dioxide (Gobbetti e Corsetti 1997; Gobbetti et al., 1994a, b).
The typical example of a mutual interaction between LAB and yeasts in sourdough is *Lb. sanfrancisciensis* and *S. exiguus* or *S. humilis*, in San Francisco French bread and in Panettone. In fact, *Lb. sanfrancisciensis* used as a preferred source of energy maltose, while *S. exiguus* and *S. humilis* can not use maltose but sucrose, glucose or fructose as a source of energy. In the other hand, amino acids production by yeasts stimulates *Lb. sanfranciscensis* growth (Gobetti et al, 1994c). The lack competition for maltose is essential for this stable association. The sourdough yeasts do not affect the cell yield of *Lb. sanfrancisciensis*, because pH is the limiting factor for growth of the lactobacilli (*Lb. sanfrancisciensis* does not grow below pH 3.8).
The cell yield of the maltose negative yeasts is lower in the presence of lactobacilli because their growth is inhibited by the accumulation of metabolic end products. However, the glucose concentration in the flours remains high enough to support yeast growth throughout the fermentation (De Vuyst and Neysens 2001). This kind of association in sourdough fermentation can also influence the CO_2 and therefore the leavening (Gobbetti et al., 1995). Finally, the interactions influence also the synthesis of volatile compounds and therefore the aroma of final products.

Anyways, these kinds of interaction are helpful in self-protection sourdough because they can inhibit the effects of other pathogen and alterative microorganisms.

1.2.5 Impact of sourdough on the texture of baked products

The main advantage of the microbial population in dough is that dough formed by the addition of water to ground cereals will be fermented by the microorganisms naturally present to become a sourdough characterized by acid taste, aroma and increased volume due to gas formation (Hammes and Gänzle, 1998). Generally, the sourdough is used to improve flavour but its addition also as an effect on the dough and the final baked product structure. In fact, there is a wide consensus with regard to the positive effects of sourdough addition for bread production, including improvemnts in bread volume and crumb structure (Coresetti et al., 2000; Clarke et al., 2002; Crowley et al., 2002; Arendt et al., 2007), flavour (Thiele et al., 2002), nutritional values (Liljeberg and Björck, 1994; Liljeberg et al., 1995) and shelf-life (Corsetti et al., 1998b; Lavermiccola et al., 2000, 2003; Dal Bello et al., 2007). The influence of sourdough on the structure depends of the mechanisms at work in sourdough and of its application that both are complex. In fact, for example the variety of flour characteristics and process parameters contribute to conferring particular effects on the metabolic activity of the sourdough microflora. To obtain a good final product, it is necessary to characterize the microorganisms responsible of two of main activities: the acidification and the rate of substrate breakdown. The selection of a characteristic microbiota, during continuous propagation of sourdough, is due by different parameters as dough yield, addition of salt, amount and composition of the starter, number of propagation steps and fermentation time (De Vuys and Neysens, 2005). In fact, as show by Gül et al. (2005), individual strains and combination thereof strongly affect the final bread texture. Therefore, the ecological composition of each sourdough influences the final quality of baked products.

1.2.6 Acidification effects

The pH values of ripe sourdough is variable, but for wheat sourdoughs it ranges from 3.5 to 4.3 (Collar et al., 1994a; Wehrle and Arendt, 1998). The main factor regulating acidification is the amount of fermentable carbohydrates. One of the most important effect on the acidification is the nature of the flour, in particular its ash content (Collar et al., 1994b).

The acidification of the sourdough and the partial acidification of the bread dough will impact on structure-forming components like gluten, starch and arabinoxylans (Arendt et al., 2007). Acids affect the mixing behaviour of the doughs, in fact a dough with a lower pH values needs a shorter

mixing time and have a less stability than normal dough. Moreover, the presence of acids influences also the softness and elasticity of gluten, increasing those (Schober et al., 2003).
The acidification has a secondary effect on the dough, including changes in the activity of cereal or bacterial enzymes associated. For example, wheat flour proteases have a optimal activity around pH 4.0 (Kawamura and Yonezawa, 1982).

1.2.7 Protein fraction changes during sourdough fermentation

The protein fraction plays a crucial role for baked goods quality. The proteolysis process provides to the formation of precursor compounds for the formation of aroma volatiles during baking as well as substrates for microbial conversion of amino acids to flavour precursor compounds. The gluten proteins in wheat flour determine dough rheology, gas retention and bread volume (Weegels et al., 1996). The sourdough influence the structure and the rheology, in fact, Di Cagno et al. (2002) demonstrated a decrease in resistance to extension and an increase in both extensibility and degree of softening. Acidification due to growth of LAB also alters the gluten network. At pH below 4.0 there is a sizable positive net charge and the increased electrostatic repulsion enhances protein solubility and prevents the formation of new bonds (Schober et al., 2003). The reduction of intermolecular and intramolecular disulfide bonds solubilises gluten proteins and allows greater access by proteolytic enzymes allowing for more efficient proteolysis (Arendt et al., 2007). The proteolysis activity gives an improvement in final product flavour and also it can change the rheology and the texture. The gliadin macropolymer is a major determinant of the volume and texture of wheat breads in a straight dough process; however, when a sourdough fermentation was used it can obtain a larger loaf volumes (Corsetti et al., 1198a).

1.2.8 Effect of sourdough on staling

For texture properties of a food is understanding as “that group of physical characteristics that are sensed by the felling of touch, are related to the deformation, disintegration and flow of the food under the application of a force and are measured objectively by functions of force, time and distances” (Bourne, 1982). Bakery products have a very short shelf-life; in fact, during their storage the freshness decreases and in parallel, the crumb will become hardness. All of these aspects contribute to a loss of consumer acceptance. This deterioration process it knows like a staling that it has been defined as “a term which indicates decreasing consumer acceptance of bakery products caused by changes in crumb other than those resulting from the action of spoilage organisms” (Bechtel et al., 1953). During this process the changes of texture of crumb are: the crumb becomes

harder, crumbly an opaque. The crust staling is caused by moisture migration from the crumb to the crust (Lin and Lineback, 1990) with a consequent soft and leathery texture.

It was demonstrated that the use of LAB in sourdough fermentation have a positive effects on staling process. One effect is an improvement in a loaf specific volume, which is associated with a reduction in the rate of staling (Axford et al., 1968; Maleki et al., 1980) and a reduction in crumb softness during the storage also (Corsetti et al., 2000; Crowely et al., 2002). This effect of sourdough is dependent from the strain performing the fermentation. The enzymes produced by LAB can influence starch molecules, causing a variation in the retrogradation properties of the stearch. The proteolytic enzymes can affect the final quality of the baked product, in fact. It was studied the addition of a protease can increases the shelf-life (Van Eijk and Hille, 1996). The proteases also support the liberation of water associated protein network that increasing the alpha amylase activity.

1.2.9 Sourdough for sweet baked products

Sweet leavened baked products obtained from sourdoughs are developed especially in northern Italy and they are also typical and traditionally made for religious feasts by small and industrial sized bakeries. They are usually, Panettone cake in Milan and Pandoro in Verona manufactured for Christmas, while Colomba is a Milanese cake for Easter. There are also, local products as Bisciola in Valtellina, Lagaccio biscuits in Genoa, Focaccia Dolce in the Venetian region and finally, some snacks for breakfast like Brioches and Cornetti and other small industrial cakes for infants. Despite their geographical origin, these cakes have a national and international diffusion. The production processes for all this products are different but they are in common the use of a particular cycle of preparation starting from a sourdough (or “madre”-mother sponge), reproduced in a continuous way that consists of a natural mixed cultures obtained by spontaneous selection of the original microflora of the flour. The main ingredients used for this kind of products are flour, water, eggs sugar, butter and/or margarine and in the case of Panettone and Colomba candied fruit and raisins, too.

Nowadays, a lot of studies were focalized the attention to the study of microflora isolated from bread sourdoughs; otherwise, the microbiota study of sweet baked products are still limited in comparison to the others. Galli and Ottogalli (1973) were the first one to carry out the microbial characterization of sourdough for Panettone, identifying strains belonging to *Lb. brevis* and *T. holmii* strains; while for Pandoro characterization, Zorzanello and Sugihara (1982), were the first one. Afterwards, Galli et al. (1988) characterized the microflora of sourdoughs for Brioche and

Panattone were they can identify strains belonging to *Lb. sanfranciscensis*, *Lb. brevis* and *Ln. mesenteroides* species and yeasts ascribed to *C. stellata* and *S. exiguus* species.
In this kind of technology, sometimes, it is a traditional practice to add vegetable matters as grape most, figs, lemon or orange peels, bran etc., to the starting dough to prepare the mother culture and therefore this can influencing the final microflora (Foschino et al., 2004). Some sourdough used to make Panettone and Pandoro are more then six years old and are preserved according to one's private custom carried on from generation to generation. All of these characteristic make the microflora characterization and the reproducibility more difficult.

1.3 Molecular approach to investigate sourdough microflora: PCR-DGGE

The use of molecular approach to identify and characterize the sourdough microflora can decrease the variability and instability of certain phenotypic characters and the dependence of culturing conditions. Culture-dependent method does not necessarily provide reliable information about the microbial communities and indeed these communities can have species that would be not cultivable with usual culture method. Molecular methods are also characterised by rapidity and reliability. Genetic fingerprinting techniques can provide a profile representing the genetic diversity of microbial communities. In addition, the PCR-DGGE has a great potential for comprehension of the community dynamics in response to variations in technological parameters. One of the most important features of a molecular approach is the possibility to monitor the presence and persistence of microorganisms in the ecosystem without any cultivation. Denaturing Gradient Gel Electrophoresis (DGGE) of rDNA amplicons was established in evaluation or compositions and activity over time in complex ecosystem (Ehrmann and Vogel, 2005; Heilig et al., 2002; Muyzer and Smalla, 1998). DGGE is the most common methods used among the culture-independent fingerprinting techniques. It is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but different sequences. This is because these fragments can be separated in a denaturing gradient gel based on their differential denaturation (melting) profile (Ercolini, 2004). The final result is a fingerprinting which contains different bands relative to a microbial species present in a sample analyzed. The technique was introduced firstly, in microbial ecology by Muyzer et al., (1993). The application of PCR-DGGE in microbiology environmental is extremely wide; in fact, this method is versatile and has been used in many fields of microbial ecology. For this reason, was applied for soli studies (Norris et al., 2002; Avrahami et al., 2003; Nicol et al., 2003); sea (Bano and Hollibaugh, 2002), gastrointestinal tract (Zoetendal et al., 2002), insects (Reeson et al., 2003) and more other kind of environmental.

In conclusion, both phenotypic and genotypic characterization can be used of a polyphasic approach. In fact, the identification of pure cultures and the characterization of mixed microbial communities are both useful to understand the complex microbial communities in sourdoughs are occurring. PCR-DGGE has been successfully applied to the study of the LAB composition of fermented cereal-based products (Ampe et al., 2001; ben Omar and Ampe, 2000; Garofalo et al., 2008; Gatto and Torriani, 2004; Meroth et al., 2004; Meroth et al., 2003a; Miambi et al., 2002; Randazzo et al., 2005; Scheirlinck et al., 2008; Van der Meulen et al., 2007b) and to compare sourdough LAB communities subjected to different fermentation processes (Meroth et al., 2004; Meroth et al., 2003a). This method also was applied to study the yeasts community during sourdough fermentation (Garofalo et al., 2008; Gatto and Torriani, 2004; Meroth 2003b). In particular, Garafalo et al., (2008) were studied the LAB and yeasts population in sourdoughs used for Panettone production and they carried out the dominance in all three samples of *Lb. sanfranciscensis*, *Lb. brevis* and *C. humilis.*

1.4 Microbial exopolysaccharides

Polymer from plant, animal, and microbial origin play an important role in food fermentations (Tombs and Harding, 1998). Most of the biopolymers used in food industry are polysaccharides from crop plants (e.g. starch) or seaweeds (e.g. carrageenen) and animal proteins like caseinate and gelatin. For industry use this kind of polymer are chemically modified. A good alternative of biothickners are the microbial exopolysaccharides. The first description of exopolysaccharides formation by wine-spoiling LAB dates back to Pasteur (Pasteur 1861, as cited by Leathers, 2002). After that, Orla-Jensen (1943) described EPS formation from sucrose by *Leuconostoc* spp., mesophilic lactobacilli and pediococci and indicated the role of EPS formation in the spoilage of apple cider and beer. These polysaccharides are extracellular polysaccharides that they are associated with the cell surface in the form of capsule or secreted into the environmental in the form of slime (De Vuyst et al., 2001). The first kinds of exopolysaccharides are called capsular (CPS) and the other slime (EPS). Exist also cell wall exopolysaccharides (WPS), that in contrast with EPS, are not released into the medium and are associated with the cell envelope and they are covalently bound to the peptidoglycan layer (Delcour et al., 1999). In general, some strains can produce both kind of polysaccharides, whereas others strains ar able to produce only one kind. It can exist different phenotypic forms of EPS, ropy and mucoid, determined by environmental conditions. Ropy EPS is defined by viscous ropes longer than 5mm, originating from the colony when the colony is touched. Instead, mucoid EPS imparts a slimy appearance to the colony but does not produce viscous ropes (Knoshaug et al., 2000). EPS from LAB can also divide in two groups:

homopolysaccharides (HoPS) and heteropolysaccharides (HePS). In particular, the HoPS are composed of one type of monosaccharides and the HePS are composed of a backbone of repeated subunits (Monsan et al., 2001; De Vuyst et al., 2001).In Gram-negative bacteria polysaccharides are presented in the form of the O-antigens of the lipopolysaccharides (LPS). EPS are diffused widely among bacteria, microalgae and less among yeasts and fungi (Sutherland, 1990-1998; Crescenzi, 1995). EPS have different function as a protection against toxic and/or limiting environments and other antagonisms (Sutherland, 1972; Whitfield, 1988; Weiner et al., 1995; Roberts, 1996; Forde and Fitzgerald, 1999; Looijesteijn et al., 2001); but also as a protection against desiccation, phagocytosis, phage attack and antibiotics; as a stimulating adhesion to solid surfaces and formation o biofilms; as a sequestering of essential cations, as colonization and in cellular recognition. Some EPS may contribute to patnogenicity (Forsen et al., 1985; Roberts, 1995-1996; Whitfield and Valvano 1993). The bacteria can not use EPS like a food reserve because they are not able to utilize them (Cerning, 1990). A good example of industrially microbial EPS is dextran from *Leuconostoc mesenteroides*, xanthan from *Xanthomonas campestris* and EPS of the gellan family from *Sphingomonas paucimobilis* (Sutherland, 1986, Roller and Dea, 1992; Crescenzi 1995; Banik et al., 2000). The EPS use in food industry is limited from economics factors, which requires a thorough knowledge of their biosynthesis and an adapted bioprocess technology, the high costs of their recovery and the non-food bacterial origin of most of them. For these reason, strains recognized as safe (GRAS) food grade microorganisms, in particular LAB, dairy propionibacteria and bifidobacteria, which are able to produce EPS in large amounts, are an interesting alternative for food uses of EPS. Therefore, this kind of bacteria can use for the in situ production of EPS, in particular, for fermented foods to improve their rheology, texture and body. It has been suggested that EPS from LAB can confer health benefits (De Vuyst et al., 2001). Their benefits for human health consist in the availability to remain for longer in the gastrointestinal tract, thus enhancing colonization by probiotic bacteria (German et al., 1999). In addition, the EPS have been claimed to have antitumor effects (Kitazawa et al., 1998), immunostimulatory activity (Hosono et al., 1997; Chabot et al., 2001) and to lower blood cholesterol (Nakajima et al., 1992). Finally, LAB have a potential for development and exploitation as food additives or functional food ingredients with both economical an health benefits.

1.4.1 Classification of exopolysaccharides from LAB

EPS from LAB can divide in two groups: homopolysaccharides and eteropolysaccharides. HoPS are constituted of one type of constituting monosaccharides (D-glucopyranose or D-fructofuranose) and can be clustered into four groups: α-D-glucans, β-D-glucans fructans and others, like polygalactan.

Strain-specific differences depend on the degree of branching and the different linking sides. In fact, most of the HoPS are characterised to be synthesized by extracellular glycansucrases using sucrose as the glycosyl donor. Some examples of HoPS are cellulose, dextran, mutan, alternan, pullulan, levan and curdlan. HoPS have high molecular weights.

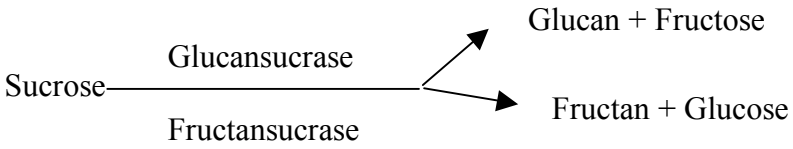
HePS are compose of a backbone o repeated subunits, that are branched (at positions C2, C3, C4 or C6) or unbranched, and that consist of three to eight monosaccharides, derivates of monosaccharides or substituted monosaccharides. The monosaccharides can be present in α- or β-anomer in the pyranose or furanose form. LAB can secrete different kinds of HePS in according to the sugar composition and the molecular mass that can be ranged from 1.0 X 10^4 to 6.0 X 10^6 (Cerning, 1995; De Vuyst and Degeest, 1999). The most frequently monosaccharides constituting the HePS are D-galactose, D-glucose and L-rhamnose that they are almost ever present but in different ratio. Some HePS can contain the acetylated amino sugars as N-acetylgalactosamine or N-acetylglucosamine and also residues such as glucoronic acid and *sn*-glycerol-3-phosphate. Small amounts of xylose, arabinose, mannose and uronic acids can be also present but are probably due to contamination from cell wall and/or medium components that persist during isolation and purification (De Vuyst et al., 2001). The media and culture conditions may be one of the most factors influencing the HePS monomer composition and variations in glycosidic bonds (De Vuyst and Degeest, 1999; Degeest et al., 2001b). Exopolysaccharides are synthesized in different growth phases and under variety of conditions. HoPS are synthesized outside the cell in the presence of a donor molecule, sucrose and an acceptor. Instead, HePS synthesis differs from HoPSsynthesis in that they are produced at the cytoplsmic membrane utilizing precursosrs formed intracellulary. In this case, sugar nucleotides play an essential role due to their role in sugar interconversions as well as sugar activation, which is necessary for monosaccharide polymerization (Cerning, 1990).

1.4.2 Biosynthesis of Homopolysaccarides from LAB

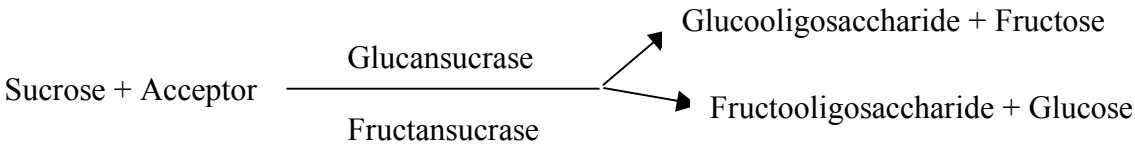
In the LAB three different system for sugar uptake are known (de Vos and Vaughan, 1994): 1) primary transport system or a direct coupling of sugar translocation with ATP hydrolysis via a transport-specific ATPase; 2) secondary, sugar transport systems or a coupling of sugar transport with transport of ions or other solutes, both as symport and antiport transport systems; 3) group translocation systems or a coupling of sugar transport with phosphorylation via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). In the most of LAB strains all sugar are transported via sugar-specific PEP-PTS system, with only one ATP consumed.

Most of the HoPS share the feature of being synthesized by extracellular glycansucrase using sucrose as the glycosyl donor. Most of these polymers are not produced by glycosyltransferases

wich use nucleotide-sugar precursors, but by transglycosylases (glycansucrases) which are able to use the energy of the osidic bond of sucrose to catalyse the transfer of a corresponding glycosyl moiety:



Over the synthesis of high-molecular-mass polymers, glycansucrase catalyse the production of low-molecular-mass oligosaccharides when efficient acceptor molecules, as maltose are present during the reaction (Koepsell et al., 1952):



In general, three reactions are catalyzed by glycosyltransferases, the hydrolysis of sucrose, the formation of oligosaccharides with a degree of polymerization ranged from 2 to 6 and the formation of polysaccharides with a relative molecular mass, more then 10^6. This enzyme catalyze a (1) hydrolysis reaction using water like an acceptor molecule. In an acceptor reaction (2) sucrose or kestose act as acceptors, yielding in the β-(2-1) linked oligosaccharides kestose and nystose, respectively. The polymerisation (3) of fructose to a levan chain yields a high-molecular-mass polymer with β-(2-6) linkages in the main chain that may be branched with β-(2-1) linkages (Tieking and Gänzle, 2005). Fructan and glucansucrase enzymes are extracellular or cell wall bound enzymes. Glucansucrases generally are composed of four domains: domain A, an N-terminal export signal; domain B, a variable region with no role in catalysis; domain C, the highly conserved catalytic core and domain D, a C-terminal core, which are responsable for glucan binding (Remaud-Simeon, 2000). Instead, fructosyltransferases consist of an export signal (domain A), a N-terminal variable domain (domain B) and the catalytic domain with conserved residues (domain C). These two enzymes are a optimal activity at pH 5.4.

1.4.2.1 Fructans

Two types of fructose HoPS are produced by fructosyltransferases from sucrose: levan and inulin, which have β-2.6 and β-2.1 osidic bonds, respectively (Monsan et al., 2001). About the synthesis of fructose a little is known. The proposal mechamism of catalysis for fructosyltransferases is a two step mechanism in which an acidic group and a nucleophilic group of the enzymes are involved in the transfructosylation reaction (Sinnot, 1990).

Levansucrase catalyses the transfer of D-fructosyl residues from fructose to yield the β-2.6 osidic bonds which characterized a levan (Figure 1A). Instead, fructooligosaccharides containing β-2.1

osidic bonds have a nutritional value, in fact, they are non digestible and also have a interesting prebiotic properties for both animals and humans (Tokunaga et al., 1193; Bouhnik et al., 1999; Diplock et al., 1999). These kinds of fructooligosaccharides are called "Inulin-type" (Figure 1B).

1.4.2.2 Glucans

The enzymes glucansucrases catalyse the synthesis of a variety of glucans containing mostly α-1.6, α-1.3, α-1.4 and α-1.2, linked D-glucosyl units. Extracellular glucansucrases are mostly produced by LAB belonging to the genera *Leuconostoc*, *Streptoccocus* and *Lactobacillus*. Different strains can produce more than one glucansucrase: for example, *S. mutans* 6715, are able to produce three distinctive enzymes (Shimamura et al., 1983), four are produced by *S. sobrinus* (Walker et al., 1990), three by *Ln. mesnteroides* NRRL B-1355 (Smith et al., 1998). The catalytic mechanism of glucansucrases has not been clarified. The main step of the transfer of the D-glucosyl unit is the formation of a covalent glucosyl-enzyme intermediate. Moreover, in the absence of sucrose in the reaction medium, glucansucrases can catalyse disproportion reactions involving oligosaccharides as substrates (Lopez-Munguia et al., 1993). Glucan polymer synthesis follows a processive mechanism. This is deduced from the observations that intermediate oligosaccharides cannot be detected in the reaction medium during the synthesis, and high molecular weight polysaccharides are obtained at early reaction times (Monsan et al., 2001). Several kinds of glucans are obtained from the action of glucansucrase. The mayor example is the dextran. In 1862 Pasteur discovered the microbial origin of the gelification of cane sugar syrups and after that, in 1874, the corresponding product was named "dextran" (Figure 2A), due to its positive rotatory power. The microorganism responsible of the gelification was *Ln. mesenteroides*. Hehre (1941) demonstrated that dextran could be synthesized from sucrose by a cell-free filtrate. The correspondent enzyme is the dextransucrase (Hestrin et al., 1943) that produces glucan which contain at least 50% of α-1.6 osisdic bonds within the main chain (Bucholz and Monsan et al., 2001). Dextransucrase catalyses the transfer of the glucosyl unit of sucrose to different "acceptor molecules" which are normally the growing dextran chain (Lacaze et al., 2007). The degree of branching involving α-1.2, α-1.3 and α-1.4 linkages in different kind of dextrans. All of the dextrans are more or less ramified and the branching very much depends on the subspecies. Dextrans molecular weights ranged from 1.5×10^4 to 2×10^7. Other kind of glucan is the mutan (Figure 2B). Mutansucrase produces a water in-soluble glucan containing more than 50% of α-1.3 glucosidic linkages, mainly associated with α-1.6 linkages. The last more famous glucan is the alternan (Figure 2C). Alternansucrase synthesizes the glucan, which contains alterning of α-1.6 and α-1.3 glucosidic linkages, with sime degree of α-1.3 branchings. This enzyme activity is bound to the bacterial cells and is more thermostable than the

dextransucrase activity. Finally, some LAB can produce called β-1.3 glucan. In fact, *Lactobacillus* subsp. G-77 has been reported to produce two glucose homopolysaccharides when grown on a glucose medium (Duenas-Chasco et al., 1998). One of the exopolysaccharides was shown to be a 2-substituted-(1-3)-β-D-glucan identical to that described for the exopolysaccharides from *P. damnosus* 2,6 (Duenas-Chasco et al., 1997). This is the first time that some LAB can produce β-glucan. The mechanism of synthesis was not been clarify bit it does not involve any glucansucrase, because sucrose was not present in the medium used. The second homopolysaccharides is a dextran-type polysaccharide with α-1.2 branching of a single D-glucose unit.

1.4.3 Biosynthesis of Heteropolysaccarides from LAB

The biosynthesis of HePS is an energy-demanding process. First, one ATP is necessary for the conversion of each hexose substrate molecule to a hexose phosphate, if the hexose is not transported via a PEP-PTS. A further high-energy phosphate bond is needed for the synthesis of each sugar nucleotide and one ATP is required for the phosphorylation of the isoprenoid lipid carrier. Finally, polymerization and transport need much energy. HePS are made by polymerization of repeating unit precursors formed in the cytoplasm (Cerning, 1990-1995; De Vuyst and Degeest, 1999). These are set up at the membrane by the sequential addition of activated sugars to the growing repeating unit that is most anchored on a lipid carrier. After completion of a HePS repeating unit becoming polymerized into a final HePS. Enzymes and proteins are involved during biosynthesis of EPS.

Mesophilic LAB are able to produce more EPS under sub-optimal growth conditions, while HePS production from thermophilic LAB strains are associated to the growth. The biosynthetic pathway can be broken down into four separate reaction sequences. These are the reactions involved with sugar transport into the cytoplasm, the synthesis of sugar-1-phosphates, activation of and coupling of sugars, and the process involved in the export of the EPS. These proces is schematized in Figure 3. Glucose-1-phosphate and fructose-6-phosphate are key intermediates linking HePS biosynthesis to the general energy metabolism (Boels et al., 2001a; Ramos et al., 2001). Glucose-1-P is converted to the sugar nucleotides dTDPrhamnose, UDP-galactose, or UDP-glucose, Fructose-6-P serves as precursor for UDP-GalNac and GDP-fucose (Boels et al., 2001b). The repeating unit is assembled from the sugar nucleotides by sequential activity of dedicated glycosyltransferases and in attached to the membrane carrier undecaprenylphosphate during assembly. This lipid II carrier is also involved in assembly and export of murein repeating units. Export of the repeating units is thought to occur through a “flippase”, followed by extracellular polymerisation. The enzymes involved in this process from LAB are homologous to proteins involved in biosynthesis of the O-antigens of Gram negative bacteria (Jolly and Stingele, 2001).

The biosynthesis of activated sugars like UDP-glucose, UDP-galactose and TDP-rhamnose is necessary for both sugar interconversion reactions (epimerization, decarboxylation and dehydrogenation) and glycosyltransferase activities. Two different ways exist for the biosynthesis of ribonucleotides: 1) de novo synthesis from internalized or newly synthesized precursors and 2) salvage pathways, from the catabolism of pre-existing nucleotides. Deoxyribonucleotides are generated from reduction of ribonucleotides. It has been shown that 5-phosphorylribose 1-pyrophosphate, an intermediate in biosynthesis of nucleotides. In bacteria, these genes involved in the metabolism of nucleotide may be either clustered or isolated along the chromosomal DNA. An example is the *deo* operon from *Lc. Lactis* involved in the degradation of nucleotides (Duwat et al., 1997).

1.4.3.1 Molecular organization of genes involved in HePS biosynthesis by LAB

A complex genetic organization is responsible for HePS biosyhthesis. Besides the specific *eps*/*cps* genes, HePS biosynthesis also requires a number of "housekeeping" genes for synthesis of sugar nucleotides from which HePS is built (De Vuyst et al., 2001). The gene organization was the first time described for *S. thermophilus* Sfi6 (Stingele et al., 1996-1999a) (Figure 4). The similarity between the HePS gene clusters from different LAB is most remarkable (Jolly and Stingele, 2001). These gene clusters, which are well conserved at the 5′ region, code for regulation, chain length determination, biosynthesis of the repeating unit, polymerization, and export (Broadbent et al., 2003; Van der Meulen et al., 2007). This strain had a *eps* gene cluster of 14,5-kb *eps*ABCDEGHIJKLM comprises 13 genes. The gene cluster *eps*ABCDEGHIJKL has also been identified, cloned and sequenced (Griffin et al., 1996; Almiron-Roig et al., 2000). The genes *eps*A, *eps*B, *eps*C and *eps*F that have a variable divergence with related sequences are mosaic genes. The two distal region *eps*AB and *pgm* and a small central region that contains *orf*14.9 are costant and present in most *S. thermophilus* strains studied. The other region are variable, however not all strains were found to be ropy in skim milk (Bourgoin et al., 1999). Generally, for thermophilic LAB the *eps* genes are chromosomal; instead, for mesophilic LAB almost all genes are associated with plasmids. The *eps* genes in *S. thermophilus* strains may have undergone numerous rearrangements by homologous recombination between distantly related or unrelated sequences, as a result of horizontal transfers of DNA with the transferred sequences replacing a part of the original ones. These exchanges may explain for the variability of the *eps* loci and also the appearance of novel structures. The general organization, transcriptional direction and deduced functions of the genes in different *eps* gene clusters seem to be highly conserved. The genes seem to be organized in four functional regions (Van Kranenburg et al., 1997): a central region with genes showing homology

with glycosiltransferases, two regions flanking the central region that show homology to enzymes involved in chain length determination, polymerization and export and a regulatory region located at the 5' end of the gene cluster.

The instability and variability in HePS production is maybe at the genetic level as well as for the ropy texture. Also, it is possible that some spontaneous mutation occurred with consequent weaker production or even an altered HePS composition. Finally, not all ropy strains are suitable for large-scale industrial fermentations and ropy strains in use have to be periodically re-isolated to maintain HePS production. The genetic instability could be due to mobile genetic elements like insertion sequences or to a generalized genomic instability, including DNA deletions and rearrangements.

1.4.3.2 Factors influencing the HePS production by LAB

The total yield of EPS produced by the LAB depends on the composition of the medium (carbon and nitrogen sources, growth factors) and the conditions in which the strains grow like temperature, pH, oxygen tension and incubation time. The first media used to study the EPS production was milk, after that test was assed on MRS medium (Cerning et al., 1990-1992; Garcia-Garibay and Marshall, 1991; De Vuyst et al., 1998). Also whey and whey-based media have been used (Knoshaug et al., 2000). In the last years semi-synthetic and synthetic media have been investigated. To study the influence of nutrients on growth, metabolic pathways and the biosynthesis of EPS is more appropriate a chemically media with carbohydrate source, amino acids, vitamins, nucleic acid bases and mineral. These kinds of media allow the quantitative and qualitative production of the HePS and the investigation of the exact composition of the HePS produced. Instead, media containing complex nutrients like beef extract, peptone and yeast extract are not suitable because of interference of these compounds with the monomer and structure analysis of the HePS (Degeest et al., 2001). Enhanced HePS production and growth were obtained on the basis of the ingredients and media used. In fact, when casein was added to skim milk cultures of cultures of *Lb. delbrueckii* subsp. *bulgaricus* (Cerning et al., 1990), instead, the addition of hydrolyzed casein to MRS does not increase the EPS production. An other example was a supplementation of milk and milk ultrafiltrate with glucose or sucrose stimulates the HePS production of *Lb. casei*. Finally, not only the nature of the carbon source and the combination of monosaccharides, but also their concentration can have a stimulating effect on the HePS biosynthesis (Gamar et al., 1997). To achieve a good yield of EPS, it was shown that an optimal balance between the carbon and nitrogen source is necessary (Degeest and De Vuyst, 1999-2000; De Vuyst et al., 1998). Also the vitamins can influence the EPS production relative to cell growth. Optimal conditions of temperature, pH oxygen tension, agitation speed and incubation time can improve HePS yields (Kojic et al., 1992; Looijestein and

Hugenholtz, 1999; Petry et al., 2000). Some studies were show that low temperatures induce slime production (Mozzi et al., 1995a-1996a; Breedveld et al., 1998). For example, Gamar-Nourani et al., (1998), found that a temperature shift (from 37°C to 25°C) at the beginning of the exponential growth phase enhances the HePS production by *Lb. rhamnosus* C83. Other studies demonstrated that higer HePS production by LAB strains at higer cultivation temperatures (De Vuyst et al., 1998) and under conditions optimal for growth, for istance with to respect to pH (De Vuyst et al., 1998; Grobben et al., 1998) and oxygen tension (De Vuyst et al., 1998; Petry et al., 2000). In general, the agitation does not influence growth HePS production. Optimal pH conditions for production of the HePS are often close to pH 6.0 (Mozzi et al., 1994; Looijestein and Hugenholtz, 1999, De Vuyst et al., 1998). It was also show that the HePS production under growth conditions with continuously controlled pH is significantly higher than in acidifying batch cultures; therefore, it seems that the effect of pH adjustment is greater that that of supplementation with nutrients (Gassem et al., 1997, Degeest et al., 2001). The pH effect could be a problem when considering industrial exploitation of the HePS-producing LAB strains during fermentation.

The yield of intracellularly synthesized HePS by different LAB strains varies from 0.045 to 0.350 g/L when the bacteria are grown under non-optimized culture conditions. When the bacteria was in optimal culture conditions result in HePS yields from 0.150 to 0.600 g/L, depending on the strain (Cerning 1990; Ricciardi e Clementi, 2000).

1.4.4 Preparation, isolation and characterisation of EPS

The starting point for EPS production is the preparation of a culture inoculum and this is the first point where we can have some contaminations. For EPS isolation are necessary subculturing steps to remove unwanted high-molecuar mass material. To characterize EPS, is necessary an isolation of polysaccharides, without alter the chemical and physical properties. Exist different methods to do it; the first is the use of pronase (Cerning et al., 1986-1988); an other one involved the addition of trichloroacetic acid for the precipitation (Garcia-Garibay and Marshall, 1991); at the end, sometimes to precipitate used different concentration of ethanol (Korakli et al., 2002). LAB can synthesise mixtures of EPS. They can produce EPS with different structures or with identical structures but different molecular masses.

Before a polysaccharide can be considered characterised, it is necessary to have information about molecular mass, to identify the composition and composition of the monomers and to determine the linkage pattern of the monomers (Laws et al., 2001). To determine the molecular mass exist different methods, one of them it is the chromatography using refractive index detection (Cerning et al., 1986). Also to detect the monomer composition exist a large variety of techniques. Some

examples are the methanolysis and per-trimethylsilylation that provides samples that can be analysed by GLC. In the end, to determine pattern of the monomers can be used a NMR spectroscopy or HPLC analysis.

1.4.5 EPS from LAB in food

The commercial exploitation of EPS, as materials for enhancing the texture and mouthfeel of food, requires the synthesis of EPS having desirable physical properties and for the EPS to be available in sufficient quantities to match demand. At the present, EPS from LAB are not really exploited by industrial manufactures. In the last years, few exceptions were developed among the HoPS produced by LAB (Sutherland, 1990; Tombs and Harding, 1998). In fact, the dextran and its derivates find several commercial uses like in the manufacture of gel filtration products and as blood volume extenders. Other uses of dextran are in paper and metal-palting processes and as food syrup stabilizers, as conditioner, stabilizer and dough improvers. Also levan can be used in food application like a biothickener. Fructo-oligosaccharides (FOS) have interesting properties for food apllications as they have a low sweetness compared to sucrose, are essentially calorie-free and noncariogenic (Yun, 1996). The application of FOS and inulin in food are based essentially on their prebiotic properties (Tieking and Gänzle, 2005). One of the first application in food processing was in Scandinavian fermented milk drinks like viili display firm, thick, slimy, consistency (Toba et al., 1990). Also in some Europe countries dairy starter cultures that contain slime-forming LAB strains are commercially available. Ropy, thermophilic LAB starter cultures for yoghurt production are used. For the production of kefir, effervescent drink fermented from grains, some strains producing EPS were used. The intentional and controlled use of HePS from LAB as natural food additives or of functional starter cultures could result in a safe, natural end-product. This can represents an important strategies to develop novel food products, especially, food products with enhanced rheological properties, improved texture and stability and/or water retention capacity. An example of application is during yoghurt manufacture, to resolve the problems of low viscosity, gel fracture or high syneresis.

1.4.5.1 Application of EPS from LAB in dough processing

The addition of plant polysaccharides is a common practice in the production of baked products to improve textural properties and shelf life. The use of EPS-producing sourdough starters meets the strict requirements of modern baking biotechnology for clean labels and consumer demands for a reduced use of additives (Di Cagno et al., 2006). Several studies demonstrated that fructan and FOS improve rheological properties of wheat doughs and bread quality (Takehiro et al., 1994; Yasushi

and Akifumi, 1993). Some studies provided evidence that EPS effectively improve dough reological parameters and final quality (Brandt et al., 2003; Tieking et al., 2003). It was showed that the addition of dextran to a level of 5g/kg flour affected the viscoelastic properties of wheat doughs and the volumes of the corresponding breads to a greater extend than addition of the same levels of reuteran or levan (Tieking et al., 2003). Also, the US patent 2983613 (Bohn, 1961) reported that the incorporation of a sufficient amount of dextrans in bakery products to soften the gluten content and to increase the specific volume. In this document it is possible to read that the bread added with dextran was about 20% bigger in volume than products which do not contain dextrans. The formation in situ of EPS is more effective; in fact, the formation in situ of EPS from sucrose results in further metabolites as mannitol, glucose and acetate, that may contribute to the improved brad quality (Korakli et al., 2003). EPS from LAB can affect one or more of the following technological properties of dough and bread: water absorption of the dough, dough rheology and machinability, dough stability during frozen storage, loaf volume and bread staling. When more water is in dough, in presence of dextran that is a hydrocolloid, it can bind high amounts of water. Also they can contribute to produce additional metabolites to improve flavour, texture and shelf life of bread. The texturizing and antistaling properties of EPS depend on their molecular size, charge, monosaccharide composition, degree of branching and types of glycosidic linkages.

2. MATERIALS AND METHODS

2.1. CHARACTERIZATION OF SWEET SOURDOUGH BY CULTURE-DEPENDENT AND INDEPENDENT METHODS

2.1.1 Sourdoug's analysis

A total of 6 different bakeries from several Naples provinces were selected for sampling. Nine samples of sourdoughs were collected aseptically and kept at 4°C for the following microbiological, acidic and molecular analysis. The sourdoughs analyzed were used for the production of brioches, croissants and "Colomba" cake (Table 1).

2.1.2 pH and TTA

The pH value was measured from an aliquot of 5 g of dough blended with 25 ml of distilled water. After homogenisation in a Stomacher, the pH was measured with an electrode (AACC, 1975).
10 g of dough with 25 ml of distilled water, homogenised in a Stomacher, was used for the TTA determination. This suspension was poured in a graduated cylinder and added distelled water to achieve a volume of 50 ml. Twenty ml of this solution were titrated against 0,1N NaOH, used like indicator phenolphthalein.

2.1.3 Microbiological analysis

For microbiological analysis, 10g of dough samples were homogenized in 90 ml of quarter strength Ringer solution (Oxoid) by using a Stomacher (Stomacher 400 Circulator, PBI), after that serial dilutions in Ringer solution were prepared until 10^{-6}. LAB and yeasts were enumerated and isolated on differential modified Chalmers Agar (Vanos & Cox, 1986). 0,1 ml of each dilutions were plated in double on a series of modified Agar-Chalmers plates that were incubated at 30°C for at least 4 days days aerobically and anaerobically.
The modified Chalmers Agar is able to distinguish between colonies of LAB and yeasts, which form the typical microbial association of starter for bread making and also, it is suitable to differentiate colonies belonging to different genera and in some cases, to distinguish also between different species of lactic acid bacteria employed in dough preparation in association with yeasts (Pepe et al., 2001).

2.1.4 LAB and yeasts isolation

The LAB and yeast isolated colonies were obtained from the counting plates and examinted to observe their dimension, edge, colour, elevation, consistency and $CaCO_3$ dissolution halo (Pepe et al., 2001). Those colonies were randomly isolated and purified by streaking on the some medium

(modified Chalmers Agar). Isolates were cultured in modified Chalmers Agar and recognised as *Lactobacillus* spp. by assessing their morphological (phase contrast microscopy) and biochemical characteristics (Gram stains and catalase activity). Gram positive and catalase negative cocci and rods were purified by successive sub-cultered. The purity was checked microscopically.

2.2 Identification by culture-dependent method

2.2.1 DNA isolation from LAB isolates

For the preparation of genomic DNA for PCR assay, one colony from the purification plates was picked and washed with 1ml of STE buffer (NaCl 100mM, Tris 10mM, EDTA 1mM).

The cells were harvested by centrifugation at 12,000 rpm for 2 min and the pallet was resuspended in 200µl of InstaGene^{TM} matrix solution and incubated at 56°C for 20 min. After that, the samples were agitated by vortexing for 10 s and boiled for 8 minutes. The DNA samples obtained were agitated by vortexing and centrifugated at 12,000 rpm for 2-3 min. Finally, The DNA samples were stored at -20°C.

2.2.2 DNA isolation from yeasts isolates

One colony from each purification plates was picked and washed with 1ml of STE buffer (NaCl 100mM, Tris 10mM, EDTA 1mM). The cells were resuspended with 150 µl of SPG buffer containing 10 mg/ml of LERS and 150 µl of ET buffer (EDTA 10mM; TRIS 1 mM). After that, the DNA samples were incubated at 37°C over night. After that, 60 µl of Triton-X 100 (15%) were added and the DNA samples were agitated by inversion. One volume of $NH_4^+CH_3COO^-$ 5M was added and the samples were incubated at -20°C for 5 min; subsequently a centrifugation at 14 000 rpm at 4°C for 10 min was performed. Then, 500 µl of supernatant were precipitated with 1 vol. (500 µl) of isopropanol and centrifuged at 14,000 rpm for 10 min at 4°C. Finally, the pellet was dried and resuspended in 100 µl of DNA Rehydratation Solution (Promega) by incubation at 55°C for 1h. The DNA samples were stored at -20°C.

2.2.3 PCR conditions

Identification of LAB isolated was obtained by 16S rRNA sequencing, as below described. Five µl of the DNA isolated were used directly as a template for PCR amplifications. Synthetic oligonucleotide primers described by Weisburg et al., (1991) fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (*E. coli* positions 8-17 and 1540-1524, respectively) were used to amplify the 16S rRNA. PCR mixture (final volume 50 µl) contained each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM$MgCl_2$, 2.5 µl

of 10x PCR buffer and 2.5 U of *Taq polymerase* (Invitrogen, Milan, Italy). PCR conditions consisted of 30 cycles (1 min at 94 °C, 45 sec at 54 °C, 2 min at 72 °C) plus one additional cycle at 72°C for 7 min as a final chain elongation.

The internal transcribed spacers present between the 18S and 26S rDNA genes (ITS1-5,8S-ITS2) were amplified to characterize the yeast strains isolated. The Synthetic oligonucleotide primers described by White et al., (1990) ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the 26S rRNA. The PCR mixture used for the amplification was the same as described above. The amplification program was 95°C for 5 min, 40 cycles of 95°C for 1min, 58°C for 2 min, 72°C for 3 min and an elongation step at 72°C for 10 min.

Identification of yeasts isolated was obtained by D1-D2 domain of 26S rDNA sequencing. To analyze the yeast population, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett 1998) were used. The PCR mixture used was the same to that described above. The amplification program was 95°C for 5 min, 30 cycles of 95°C for 1min, 52°C for 45 sec, 72°C for 1 min and an elongation step at 72°C for 7 min.

The amplifications were performed in a programmable heating incubator (MJ Research Inc., Watertown, MA, USA).

The presence of PCR products was verified by agarose (1.5 % w/v) gel electrophoresis in 1X TBE buffer, at 100V for 2 h, purified by using a QIAquick gel extraction kit (Qiagen S. p. A., Milan) and sequenced by using the primer fD1 (Weisburg et al., 1991). The DNA sequences were determined by the dideoxy chain termination method by using the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequences were analysed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, F) and research for DNA similarity was performed with the GenBank and EMBL database (http://www.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, et al 1997).

2.3 OPTIMIZATION OF THE METHOD USED FOR THE IDENTIFICATION BY CULTURE-INDEPENDENT TECHNIQUE

2.3.1 Production of standardized sourdough

To study the functionality and the efficiency of different protocols for DNA isolation, standardized dough was made.

For this aim, lactobacilli (*Lb. brevis* H6, *Lb.plantarum* E5 and *Lb. sanfranciscensis* B9) were grown in MRS broth (Oxoid) and, incubated overnight at 30°C. *Saccharomyces cerevisiae* T22 was

cultured in Malt extract (Oxoid), for 2 day at 30°C. After that, the cells were collected by centrifugation (5000g), washed with sterile distilled water and resuspended to obtain 5 ± 0.5 x 10^9 microorganisms ml^{-1} (direct microscopic counts). The standardized dough was prepared by Kneading mixer (model KPM50 Professional by KitchenAid, St Joseph, MI, USA) for 5 min at room temperature and at medium speed, 500 g wheat flour, 280 g top water and starter suspension to achieve viable counts of about 5.0 x 10^7CFU g^{-1} of both yeasts and LAB in the final dough (Coppola et al, 1998). The dough was leavened for 24 h at 30°C. The sourdough obtained was used for PCR-DGGE analysis.

2.3.2 DNA isolation from sourdough

Three different methods of DNA extraction were used.

For all isolation methods 10 g of sourdough (before the fermentation and after 24 h of fermentation), prepared as described above, was cut in sterile conditions and diluited (1/10 and 1/100) in quarter strength Ringer's solution (Oxoid). Two ml of the 10^{-1} and 10^{-2} dilutions were centrifuged at 14,000 x g for 5 min.

Wizard method. The pellet was suspended in 100 µl of TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) and used for DNA extraction. The 100 µl suspension was mixed with 160 µl of 0.5 M EDTA/Nuclei Lysis Solution in 1/4.16 ratio, 5 µl of RNAse (10 mg/ml) and 15 µl of pronase E /20 mg/ml, Sigma-Alderich) and incubated for 60 min at37°C. After incubation, 1 vol. of ammonium acetate 5M was added to the sample and incubated at -20°C for 15 min. Then, the samples were centrifuged at 14.000 x g for 5 min at 4°C. The supernatant was precipitated with 0.7 vol. of isopropanol and centrifuged at 14.000 x g for 5 min. Finally, the pellet was washed with 500 µl of glacial ethanol (70%), dried and resuspended in 50 µl of DNA Rehydration Solution by incubation at 55°C for 45 min. The DNA samples were stored at -20°C.

Nucleo Spin Food Protocol (Macherey-Nagel, Germany). The DNA isolation was carried out in according to supplier's recommendations and applied as follows. Two ml of the 10^{-1} and 10^{-2} dilutions were used and 550 µl of lysis buffer CF (preheated to 65°C) was added and mixed carefully, after that 10 µl of proteinase K was added and mixed again for 2-3 min. Incubate at 65°C for 30 min; afterwards, the mixture was centrifuged for 10 min at 14.000 rpm. Then, 300 µl of clear supernatant was pipetted into a new 1.5 ml tube and 300 µl of buffer C4 and 200 µl of ethanol were added, the mixture obtained was vortexed for 30 sec. To bind the DNA 750 µl of the mixture were placed into a NucleoSpin Food column and centrifuged for 1 mi at 13.000 rpm. Instead, to wash and dry silica membrane, 400 µl of buffer CQW and centrifuged for 1 min at 13.000 rpm; after that, the supernatant was discarded and 700 µl of buffer C5 onto the NucleoSpin Food column ancentrifuged

for 1 min at 13,000 rpm. For the last washing, 200 µl was pipetted onto the NucleoSpin Food column and centrifuged for 2 min at 13.000 rpm to remove the buffer completely.Finally, to eluete the DNA, the NucleoSpin Food column was placed in a new 1.5 ml centrifuge tube and 100 µl of elution buffer CE (preheated to 70°C) onto the membrane. Incubate for 5 min at room temperature and the mixture was centrifuged for 1 min at 13.000 rpm to collect the DNA.

DNeasy Plant Mini Kit Protocol (Qiagen S. p. A., Milan). The DNA isolation was carried out in according to supplier's recommendations and applied as follows: The pellet from the first two dilutions was resuspended with 400 µl of buffer AP1and 4 µl of RNase A (stock solution 100mg/ml) and agitated by votexing vigouously. The mixture was incubated at 65°C for 10 min. After that, 130 µl of buffer AP2 was added and incubated on for 5 min. Then, to apply the lysate to theQIAshredder Mini Spin Column placed in 2ml collection tube and centrifuged for 2 min at 14.000 rpm. The flow-through fraction from the previous step was transferred to a new tube and 1.5 volumes of buffer AP3/E were added to the cleared lysate and mixed by pipetting. 650 µl of mixture from the previous step were applied to the DNeasy Mini Spin Column sitting in 2 ml collection tube and centrifuged for 1 min at 8000 rpm and the flow-through was discarded. Therefore, 500 µl of buffer AW were added and centrifuged for 1 min at 8000 rpm. After that, 500 µl of buffer AW were added and centrifuged for 2 min at 14.000 rpm to dry the membrane. The DNeasy Mini Spin Column was transferred in a 1.5 ml microcentrifuge tube and 100 µl of buffer AE were pipetted directly on the DNeasy membrane. The mixture was incubated for 5 min at room temperature and centrifuged for 1 min at 8000 rpm to eluete the DNA.

Their efficiency were evaluated by: a) DNA analysis by electrophoresis on 1% agarose gel run at 100V in 1x TBE buffer and ethidium bromide staining; b) evaluation of DNA amplificability, by PCR of 16S rDNA and D1-D2 domain of 26S rDNA (Kurtzman and Robnett 1998), for detection of LAB and yeasts, respectively; c) PCR-DGGE analysis of V3 region of the 16S rDNA and D1-D2 domain of 26S for LAB and yeasts, respectively, to detect the biodiversity in the dough samples.

2.3.3 PCR conditions

The primers V3f (5'-CCTACGGGAGGCAGCAG-3') and V3r (5'-ATTACCGCGGCTGCTGG-3') spanning the 200 bp V3 region of the 16S rDNA of *E. coli* (Muyzer et al., 1993) were used for LAB DGGE analysis. A GC-clamp was added to the forward primer, according to Muyzer et al., 1993. Amplification was performed in a programmable heating incubator (MJ Research Inc., Watertown, MA, USA). One or two µl of DNA were used. Each mixture (final volume 50 µl) contained each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM$MgCl_2$, 2.5 µl of 10x PCR buffer and 2.5 U of *Taq polymerase* (Invitrogen). Template

DNA was denatured for 5 min at 94°C. A "touchdown" PCR was performed (Muyzer et al., 1993) to increase the specificity to amplification and to reduce the formation of spurious by-products.
To analyze the yeast population, the primers 403f (5'-GTGAAATTGTTGAAAGGGAA-3') and 662r (5'-(GC)-GACTCCTTGGTCCGTGTT-3') (Sandhu et al., 1995) were used. A GC-clamp was added to the reverse primer, according to Muyzer et al., 1993. PCR mixture used for the amplification of yeast DNA was the same as for bacterial DNA. The reaction was run for 30 cycles: denaturation was at 95°C for 60 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min. An initial 5 min denaturation at 95°C for 60s and a final 7 min elongation step at 72°C were used. A "touchdown" PCR was performed (Muyzer et al., 1993) to increase the specificity of amplification and to reduce the formation of spurious by products. The initial annealing temperature was 60°C for 60 s, which was reduced by 1°C every cycle for 10 cycles. Finally, 20 cycles were performed at 50°C for 60 s. The denaturation and extension for each cycle were carried out at 94°C for 30s and 72°C for 3 min, respectively, while the final extension was at 72°C for 10 min.
The presence of PCR products was verified by agarose (1.7 % w/v) gel electrophoresis in 1X TBE buffer, at 100V for 2 h.

2.3.4 DGGE analysis

PCR products were analyzed by DGGE using a Bio-Rad Dcode apparatus and the procedure first described by Muyzer et al., 1993. Samples were applied to 8 % (wt/vol) polyacrylamide gels in 1X TAE buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing a 15 to 55% (for LAB analysis) and 20 to 50% (for yeasts analysis) urea-formamide denaturing gradient (100% corresponded to 7M urea and 40% (wt/vol) formamide) increasing in the direction of electrophoresis. The gels were analyzed by electrophoresis for 10 min at 50 V and for 4h at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water and photographed under UV transillumination.

2.3.5 Sequencing of DGGE fragments

DGGE fragments to be sequence were excised from the gel with a scalpel and purified by QIAquick gel extraction kit (Qiagen S. p. A., Milan) according to supplier's recommendations. Twenty microliters of the eluted DNA of each DGGE band were reamplified by using the PCR conditions describe above. Purity was checked by analyzing 10 µl portions of the PCR products in DGGE gels as described above; PCR products which gave a single band comigrating with the original band were then purified and sequenced. The DNA sequences were determined and analysed as above described.

2.4 SCREENING OF BACTERIA ISOLATED FROM BAKED PRODUCTS FOR EXOPOLYSACCHARIDES PRODUCTION

2.4.1 Strains, media and growth conditions

One hundred ninety two strains of LAB and Bacilli were screened for EPS formation. In particular, 53 LAB strains isolated from sourdoughs for sweet baked goods, 107 LAB strains previously isolated from sourdough and pizza, 17 LAB previously islated from commercial bakery's yeast (Coppola et al., 1996-1998; Pepe et al., 2004), and 15 Bacilli (*Bacillus subtilis*) strains, previously isolated from bread (Table 2). All strains used for this screening were previously identified by biochemical and molecular methods.

LAB strains were grown in MRS broth (Oxoid) and Bacilli strains were grown in PCA (Plate count agar, Oxoid). All strains were incubated at 30°C for 24h.

2.4.2 Media optimization for EPS screening

To verify the EPS production in vitro preliminary experiments were done to studie which specific grown solid media give the best performance. For this aim, microbial strains were tested by streaking on following media were:

- Modified Agar Chalmers;
- Modified Agar Chalmers with sucrose (5%);
- Modified Agar Chalmers, without $CaCO_3$ and with different carbohydrates source like sucrose, maltose, glucose, galactose, fructose and lactose.

Solutions of the different sugars (50mg/ml), after sterilization by filtration with 0,45 µm pore size filters (Minisart®plus Sartourius AG, Goettingen, Germany), were added to the media.

For this aim, 12 *Leuconostoc*, 8 *Lactobacillus* and 1 *Streptococcus* strains (Table 3) were tested on all media described above. The EPS production was observed during the 3 days of incubations at 30°C.3 Colonies were assayed for the ropy EPS phenotype by touching them with a sterile toothpick. Colonies were scored as ropy if visible strings were observed (Vescovo et al., 1989).

2.4.3 Screening of bacteria strains for EPS production on modified Agar Chalmers with sucrose

The media that we optimized for the 12 *Leuconostoc* strains, modified Agar Chalmers with sucrose (5%) without $CaCO_3$, we used to obtain a primary screening for all strains that we want tested for EPS production. In this case too, all plates were incubated at 30°C and the EPS production was observed during the time (3 days).

2.4.4 Screening of bacteria strains for EPS production modified Agar Chalmers with sugars mixture

The positive strains for the primary assay were tested on modified Agar Chalmers, without calcium carbonate and with a mixture of (50 g/100ml of water) sucrose (3%), maltose (3%) and fructose (3%). This mixture was chose because the sugars concentration was similar to the sugar concentration in the sourdough used for sweet baked products. The strains were incubated at 30°C for 3 days, and the ropiness property was evaluated as described above.

In the end, an other media was used to asses the EPS production. This media would like to be similar to sourdough composition. For this aim, 100 g of sourdoughs (previously used for other experiments), were mixed with 350 ml of water. This mixture was homogenised for 5 min in a Stomacher and after that, was centrifuged at 6500 rpm for 10 min. Then, the supernatant was gently separated from the pellet and filtered with distillate water to achieve a final volume of 500 ml. to the media wad added yeast extract 3% (wt/vol) to increase the LAB growth and the final pH was 6.0.

2.4.5 DNA isolation from bacteria strains

The DNA from the 18 strains that showed a better EPS production was isolated.

The strains were incubated in MRS broth at 30°C over night. Therefore, the pellet from 2 ml of were harvested and washed with 1 ml of STE buffer (100mM NaCl, 10mM Tris pH 8.0, 1mM EDTA pH 8.0). The cells were risuspended in 250 µl of ET (50mM Tris-HCl, 5mM EDTA, pH 8.0) with lysozyme (2 mg/ml) and mutanolysin (40U/ml) and they were incubated at 37°C over night. After that, 75 µl of SDS (10%) were added and mixed gently by inversion. Two µl of Pronase (20mg/ml) were added and incubated at 37°C for 1h. After incubation, 1 vol. of ammonium acetate 5M was added to the sample and incubated at 4°C for 15 min and centrifuged at 14000 rpm for 15 min at room temperature. The pellet was dried and resuspended in 50 µl of DNA Reydration Solution by incubation at 55°C for 1h. Finally, 2 µl of RNase (10mg/ml) were added and the samples were incubated for 10 min at 37°C.

2.4.6 PCR conditions and DNA sequencing to screen *eps* genes involved in omopolysaccharides (HoPS) and eteropolysaccharides (HePS) biosynthesis

A screening for *eps* genes was performed using different primers targeting omopolysaccharides (glucansucrase and levansucrase) (Kralj et al., 2003; Tieking et al., 2003) and eteropolysaccharides (*eps*A, *eps*B, *eps*D/E, *eps*GTF) genes (Table 4) (Mozzi et al., 2006). The PCR mixtures (25 µl)

contained 1 µl of DNA, 2.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at 100 µM each, each primer at 1 µM in *Taq* buffer and 2.5 U of *Taq* polymerase. The programs detailed in Table 4. Amplicons were analyzed by electrophoresis in 1% (wt/vol) agarose gels in 1X TBE buffer. The 200-bp PCR products obtained with the eps D/E primers were run in 2% (wt/vol) agarose gels. A 1kb ladder (Invitrogen, Milan, Italy) was used to estimate the sizes of the bands. Amplifications were performed in a programmable heating incubator (MJ Research Inc., Watertown, MA, USA). PCR products of from *gtf* genes and *eps*GTF were purified by using a QIAquick gel extraction kit (Qiagen S. p. A., Milan) and sequenced. The DNA sequences were determined and analysed as above described.

2.5 CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM *LACTOBACILLUS PARABUCHNERI* FUA3154

2.5.1 Strain, media and growth conditions

The strain used for this part of my study is *Lb. parabuchneri* FUA3154. It is a gram-positive, non-motile and catalase-negative and usually the cells are rods-shaped and occur single or in pairs. This strain was isolated previous from "Brottrunk" a bread fermented beverage. *Lb.parabuchneri* FUA3154 was incubated in MRS medium modified as described by Stolz et al., and containing 100 g/L of sucrose as the sole source if carbon (mMRS-sucrose) or 5g/L of glucose, 5 g/L of fructose and 10 g/L of maltose as sole carbon sources (mMRS-maltose). The medium pH was adjusted to 6.2 before it was autoclaved, and sugars were autoclaved separately from the other medium components. The strain was incubated at 30°C for 24-48h in anaerobic condition.

2.5.2 Isolation and purification of EPS

The cells were removed from 24-48h old cultures by centrifugation (5000 rpm for 10 min) and the cell-free culture supernatant was used to screen for EPS production. To harvest the EPS add ethanol to achieve a 70% (wt/vol) concentration. After that, the samples were incubated on ice for 1h. Harvest EPS obtained by precipitation and centrifuged them at 5000 rpm for 10 min. Then, add 2ml of ethanol to the sample and centrifuged. The pellet was resuspended in 2ml of distilled water. EPS were purified by dialysis over night. For dialysis, Spectra/Por membranes (VWR International) with a molecular mass cut off of 3.500 Da were used. After dialysis, to obtain the first fraction of EPS add ethanol to achieve a 70% (wt/vol) concentration and incubate on ice for 1h. After that harvest the EPS and resuspended them with 1 ml of mQ water. To precipitate a second fraction of EPS, was added ethanol, to achieve a 40% (wt/vol) concentration, to the supernatant and was incubated on ice 1h. Finally, the EPS were harvested and resuspended with 1ml of mQ water. After purification the

EPS purify were freeze-dried using a Labconco FreeZoneone 4.5 Liter Freeze Dry System (Labconco; Kansas City, USA) at a collector temperature of -50°C. When the cells were freeze-dried, were weighted to quantify the EPS.

2.5.2.1 Enzyme assay

The purified EPS were submitted to enzymatic digestion. For the digestion Dextranase from *Chaetomium erraticum* (Sigma-Alderich) and β-glucanase (Sigma-Alderich) were used and the reaction was carried out at 55°C and 37°C respectively, over night. After that, the samples were run to the HPLC to study the oligosaccharides composition.

2.5.3 HPLC analysis

The monosaccharides composition was analyzed by high-performance liquid chromatography (HPLC) using an Agilent HPLC system, Aminex 87H column with mobile phase was: 2 mM H2SO4, flow rate 0.4 ml min-1, column temperature was maintained at 70°C; the injection volume was 200 µl. To determine the size was used a SEC column Zorbax PSM1000. The mobile phase was deionized water at a flow rate 0.5 ml min-1 and column temperature was 30°C. Finally the oligosaccharides were analysed directly from the supernatant by HPAEC-PAD using a Carbopac PA20 column combined with an ED40 chemical detector (Dionex, Oakville, Canada). Water (A), 1 M NaOH (B) and 1 M Na-acetate was used as solvents with the gradient shown in Table 5. For peak identification, an external standard of glucose, maltose and galactose was used. To study an oligosaccarides composition, for the peak identification, an external standard of cellobiose, cellooligossacarides and laminarabiose were used.

2.5.4 Screening of *eps* genes

The primers and the conditions used to performer the PCR are the same that we used before and they were reported above (Table 4).

In this case, one more pair of primer was used, the primer which encodes a β-D-glucan synthase gene: GTF-F 5′-CGGTAATGAAGCGTTTCCTG-3′ and GTF-R 5′-GCTAGTACGGTAGACTTG-3′ (Werning et al., 2006). The PCR mixtures (50 µl) contained 1 µl of DNA, 3.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at 250 µM each, each primer at 0.2 µM in *Taq* buffer and 2.5 U of *Taq* polymerase. The programs used was 1 x (95°C, 5 min); 30 x (95°C, 5 min; 55°C, 1 min; 72°C, 30,s), and 1 x (72°C, 10 min).

PCR products from *eps* D/E and *eps* GTF genes were purified by using a QIAquick gel extraction kit (Qiagen S. p. A., Milan) and sequenced. The DNA sequences were determined and analysed as

described above. Sequence alignments were conducted with the ClustalW algorithm (Thompson, et al., 1994).

2.5.4.1 DNA cloning

Purified PCR products from *eps*EFG gene were cloned in the vector pCR 2.1-TOPO by using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif) with chemically competent cells in accordance with the manufacturer's recommendations. Plasmids from transformants purified with the QIAprep Spin Miniprep Kit (Quiagen) were used for automated sequencing. The sequences on both strands from two clones were then determined with M13 forward and reverse primers by the DNA sequencing service of Department of Bioscience of University of Alberta (Canada). Similarity searches were performed with the GenBank and EMBL database (http://www.ncbi.nlm.nih.gov/Blast.cgi) (Altschul, et al 1997).

2.7.4.2 RNA isolation and reverse transcription PCR

Total RNA was isolated from cultures of *Lb. parabuchneri* FUA3154 grown to the exponential growth phase (optical density at 660 nm, 0.4-0.5) in mMRS-maltose.

The TRIzol (Invitrogen) RNA Isolation Kit protocol was used. The protocol was as following:

Five hundred µl were added to the cells to resuspend them. The solution was applied to 2ml tube with DEPC.treated glass beads. Binding for 2 min at max speed and repeated it for 2 times. After that, the samples were incubated and homogenized for 5 min at room temperature. One hundred µl of chloroform were added and incubated at room temperature for 2.3 min. The samples were centrifuged at 12000 g for 15 min at 4°C. Therefore, the upper phase was withdrew and 1 vol of isopropanol was added to precipitate the RNA and the mixture was incubated at room temperature for 20 min. In the following, the samples were centrifuged at 12000g at 4°C for 10 min. The supernatant was removed and the RNA was washed once with 500 µl of 75% ethanol; the samples were agitated by vortexing and centrifuged at 7500 g for 5 min at 4°C and all leftover ethanol was removed. To dissolve the RNA pellet 25 µl of RNA ase-free water were added. Five µl of RNA was used to measure the concentration e for cheking by electrophoresis. The rest RNA was stored at -80°C. To check RNA isolation, RNA samples were run on 0.8 % agarose gel. Before the running, RNA loading dye was prepared as follows: 50% glyverol, 10mM EDTA (pH 8.0), 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF. Three µl of RNA samples were mixed with 17 µl of formamide and incubated for 10 min at 65°C to denature the secondary structure and the samples were chilled on ice for 2 min. Two µl of RNA loading dye were added to the samples and run at 80 Volts for 40 min. The gel was stained in a DEPC-treated water containing ethidium

bromide. In the RNA preparations, DNA was digested by incubation with DNase-treated RNA (Promega). Reverse transcription (RT)-PCR was performed using 100 U Moloney murine leukemia virus reverse transcriptase and was primed with 20 µg of hexameric random primers ml^{-1} (rRNase H minus and random primers from Promega). From the cDNA library, an internal fragment of the *eps*EFG gene was amplified with a new pairs of primer, designed on the basis of the sequence obtained for the our strain: *eps*Ef (5'-ACGAGCTCCCACAATTTTGGA-3') and *eps*Er (5'-GCTGAGGCGACGTTT TTGTTC-3'); also the cDNA was amplificated for two houskeeping genes: RecAf (5'-AATGCTAAAAAGTGGCTTAAGGAACA-3'), RecAr (5'-CCCATTTGCAACACGAACTTTAT-3') and DnaKf (5'-CCTACTTCAAAGAGCCAAATCTTCTC-3') and DnaKr (5'-TGCCATTGGACGTTCACCTT-3'). The PCR mixture and conditions were the same used fro the amplifications descibed above.

3. RESULTS

3.1. CHARACTERIZATION OF SWEET SOURDOUGH BY CULTURE-DEPENDENT AND INDEPENDENT METHODS

3.1.1 Microbial counts and chemical determinations of sourdough samples

The plate analysis results of each sourdough are shown in Table 6.

In the sourdoughs I4b, I4c, I5, I6 and I7 were isolated both LAB and yeasts with different concentrations. In particular, the count for the LAB was in the range of 1.1 x 10^5 CFU g^{-1} (sourdough I6) and 7.0 x 10^7 CFU g^{-1} (sourdough I4b). On the other hand, viable yeast counts were showed more differences between the sourdoughs; in fact, was ranged from 5.0 × 10^4 (sourdough I5) to 3.0 × 10^8 (sourdough I4b) CFU g^{-1}.

Otherwise, in the sourdoughs I1, I2, I3 and I4, no yeasts were counted and in general the viable LAB counts were less then for the other samples (8.0 x 10^2 CFU g^{-1} - 4.0 x 10^3 CFU g^{-1}).

The viable LAB counts were greater then viable yeast counts, in fact, the ratio LAB/yeasts was in the range from 4000 to 0.1. Therefore, the sourdoughs I4b, I4c, I5, I6 and I7, showed very low ratios more favourable for to the yeasts. Although in the sourdoughs I4 and I5 the majority of LAB was limited. This result is in accord with Ottogalli et al., (1996), that reported the ratio LAB/ yeasts of 100/1. In all sourdough samples a high degree of acidification was observed, with pH values (Table 6) varying between 3.76 and 5.74 with a mean value of 4.61. The TTA values were ranged from 1.6 (sample I7) to 3.4 (sample I6).

3.2 Microbial identification by "culture-dependent" method

3.2.1 Phenotypic characterization of bacterial isolates

Ninety-seven strains, collected from the different sourdoughs were Gram positive and catalase negative reactions, thus, considered as presumptive LAB (Table 7). The isolates were physiologically divided into 6 different groups for their macro morphology, micro morphology, Gram reaction and catalase test. Some representative strains of every group (4 strains) was chosen for the genotypic characterization and were identified by 16S rDNA sequencing.

3.2.2 Phenotypic characterisation of yeast isolates

After isolation on modified Agar Chalmers, each strain was microscopically observed and its morphology recorded. In total, forty-nine strains were isolated and differentiated with their macro morphology, micro morphology and grouped in 5 different groups (Table 8). The identification and typing of the yeast isolates was achieved by using the PCR for the ITS of 5.8S for 2 representative strains for each group. All strains showed the same fingerprinting for the ITS amplification with

one band at 850 bp, with exception of a strain (100B) isolated from the sourdough I6, that showed one band at 400 bp that was sequenced.

3.2.3. Molecular identification of LAB strains

In all samples we isolated bacteria belonging to the genera *Lactobacillus* (Table 9), in particular with the species *Lb. casei*, *Lb. plantarum*, *Lb. sakei*, *Lb. curvatus*, *Lb. graminis*, *Lb. rennanqilfy* and *Lb. coryniformis*. Moreover we isolated same strains belonging to the genera *Leuconostoc* with the species *Ln. mesenteroides*, *Ln. pseudomesenteroides*, *Ln. lactis* and *Ln. garlicum* and in the end, same strains belonging to the genera *Lactococcus* with species *Lc. lactis* subp. *lactis* and *Lc. lactis* subp. *cremoris*. All *Lactobacillus* strains isolated were facultatively heterofermentative. In particular, in the sourdoughs I1 and I3 we isolated the species *Lb. casei* and *Lb. rennanqilfy*; in the sourdoughs I2 and I4, *Lb. casei* was the only species carried out; in the sourdough I4b we identified the species *Lc. lactis* sp. *lactis*, *Lc. lactis* sp. *cremoris*, *Ln. pseudomesenteroides* and *Lb. plantarum*; in the sourdough I4c the species *Lb. curvatus*, *Lb. sakei*, *Ln. mesenteoides*, *Ln. lactis* and *Ln. garlicum*; in the sourdough I5 we can identified just one strain at species level as *Lb. plantarum* and the others just at genera level as *Lactobacillus sp.*, as well as for the sourdough I6 . Finally in the sourdough I7 we isolated the species *Lb. coryniformis* subsp. *torquens*, *Lb. curvatus* subsp. *curvatus*, *Lb. graminis* and *Leuconostoc* sp (Table 9).

3.2.4. Molecular identification of yeast strains

A single ITS1- 5.8S- ITS2 DNA fragment of about at 850 bp (Figure 5), was observed in all isolates of yeasts. The only exception was one isolate from the dough I6 that was showed one fragment with a length about at 400 bp that was, also, submitted to the sequencing of the domain D1-D2 of 26S. All isolated with a ITS fragment at 850 bp were compared to available on-line sequences using BLAST software. They showed 100% homology with *S. cerevisiae* (Table 9), whereas that from the dough I6 aligned with *Metschnikowia pulcherrima* at 100%.

3.3 Microbial identification by "culture-independent" method

3.3.1 Comparison of different DNA isolation methods

The three different protocols of DNA isolation were applied to the standardized sourdough that was made with three *Lactobacillus* strains (*Lb. brevis* H6, *Lb. plantarum* E5 and *Lb. sanfranciscensis* B9) and one yeast strain *S. cerevisiae* T22.

This preliminary study was important because the substances present in the food matrix may affect PCR, and such elements can be co-extracted with the target DNA and thus interfere with PCR (Picozzi et al., 2006).

The DNA was isolated from the first two dilutions employing the three above described protocols and subjected to agarose gel electrophoresis. The Figure 6 shows the extraction results. It is possible to note the presence of nucleic acids from Nucleo Spin, Wizard and DNeasy Plant Mini Kit methods. The last one protocol didn't show DNA products except for the first dilutions for each sample that show a weak band with, at the same time, some impurity smear. Otherwise, the Nucleo Spin and Wizard protocols yielded high-molecular weight DNA, for the first dilutions of sourdough samples before and after the sourdough leavening, but same impurity smear, too. Finally, the DNA obtained by agarose electrophoresis showed the presence of nucleic acids in all three methods, even if, the Wizard protocol yielded bands better than to the other protocols. Moreover, all methods allowed PCR amplification (Figure 7.) of all samples, but also in this case the Wizard protocol gave better results. Finally, in the analysis PCR-DGGE (Figure 8.), the DNA extraction method did not influence the final result obtained with all three protocols, even if, better fingerprinting was detected in the samples extracted with Wizard method. In conclusion, the Nucleo Spin and Wizard protocols were chosen to apply to characterize by molecular method the sourdoughs.

3.3.2 PCR-DGGE analysis for LAB identification

Figures 9 and 10 show the DGGE fingerprints of the bacterial DNA extracted directly from the sourdough samples with Nucleo Spin Food and Wizard protocols, respectively. The number and the intensity of bands varied among samples. To identify the bacterial species, the bands were excised from the gel, re-amplified and run again on a denaturing gel to confirm their position relative to the original sourdough sample. Purification and sequencing of some faint bands did not provide successful (Figure 9 and 10; bands X). The closest relatives of the sequences analysed are reported in Table 10.

In general, all samples were not showed a big variety in their DGGE fingerprinting. The fingerprintings obtained was very similar among the different samples. Anyways, the sourdoughs I1, I2 showed the same DGGE profile as well as I3 and I4, I4c and I5. All thesed samples showed more variability since their fingerprintings presented at least 6 bands together with the sourdough I4b. The samples I6 and I7 indeed showed a pour fingerprinting with only one band but four of them were very weak. Most of the sequences obtained with Nucleo Spin Food protocol corresponded to portions of 16S rDNA of LAB; one exception are the bands C and I, present in all sourdough samples (Figure 9) which showed a similarity with a portion of the mitochondrial 18S

rDNA of *Triticum aestivum*. The sequence of the other bands showed a similarity with *Lb. sanfrancisciensis* (band E), *Lb. sakei* (band G), *Weisella cibaria* (band N) and *S. thermophilus* (band D). Moreover, with Wizard protocol, the DGGE profile obtained showed less number of bands (Figure 10). The sourdough samples I1, I2, I3 and I4 (data not shown) and I4c and I5 (Figure 10), had the same had the same fingerprinting that showed the presence also of some weak bands. Also thye samples I6 and I7 were similar but with a pour fingerprintings constituted by a single band. Moreover, the sequences of the bands (A, C, C2) (Figure 10) showed a similarity with a portion of the mitochondrial 18S rDNA of *Hordeum vulgare* subs. *vulgare*. Other sequenced bands were identified as *Lb. sanfrancisciensis* (bands C1, D, D1) and *Weisella cibaria* (band E). When we used Nucleo Spin Food protocol, for *Triticum* aestivum two bands with different migration were obtained in the DGGE gel; with Wizard protocol, for *Lb. sanfranciscensis* and *Hordeum vulgare* two o three bands were obtained in the DGGE gel.

3.3.3 PCR-DGGE analysis for yeast identification

DGGE fingerprints obtained by analysing the amplified 26S rDNA fragments wit primers 403f and 662r are shown in Figure 11. No diversity was found in yeast population, with both DNA isolation protocols. In fact, in all samples there was one main band with a good intensity (band A). In all DGGE profiles there were other bands (for the samples I1, I2, I3 and I4) not clearly visible in fingerprint replicates to be sequencing.

3.4 SCREENING OF BACTERIA FOR EXOPOLYSACCHARIDES PRODUCTION

3.4.1 Media optimization for EPS screening

Different kinds of media were tested to choose the one that give a better result. For this aim, firstly, 12 *Leuconostoc* strains were tested on modified Agar Chalmers (Table 3), but after 48h of incubation, the EPS production was not detected. Then, the same strains were assayed on modified Agar Chalmers without $CaCO_3$ with sucrose (5%) as only carbohydrate. This media allowed the EPS formation by 10 of *Leuconostoc* strains tested (Figure 12A) but, when the sucrose was substituted by fructose, glucose, maltose, lactose and galactose (5% wt/vol) no more developing of EPS was observed. Modified agar Chalmers with sucrose and without $CaCO_3$ allowed to put in evidence the EPS production in three of the eight *Lactobacillus* strains tested (*Lb. coryniformis* subsp. *torquens*, *Lb. curvatus*; Figure 12B) and in *Uncultured Streptococcus* 107 (data not shown). All tested strains were isolated from swedet sourdoughs.

3.4.2 Screening of bacteria strains for EPS production on modified Agar Chalmers with sucrose

On the basis of previous results, the investigation of screening for EPS synthesis on modified Agar Chalmers with sucrose and without $CaCO_3$, showed that 42 strains were EPS producers. Particularly, the 61% of *Leuconstoc* and *Weissella* strains were sensitized EPS. Otherwise, the *Lactobacillus Lactococcus* and *Enterococcus* strains were all negative. Finally, 6 *Bacillus subtilis* strains were able to produce EPS with ropy property too (Table 11).

3.4.3 Screening of bacteria strains for EPS production modified Agar Chalmers with sugars mixture

This screening was aimed to the selection of strains able to be "iper-EPS producers" and it was a preliminary step to transfer the process from the lab condition to the industry production. The results were showed in the table 12. 42 positive strains obtained by modified Agar Chalmers with sucrose were tested on modified Agar chalmers with a mixture of sugars and without $CaCO_3$. The results obtained by this test showed that the 55% of strains was able to produce EPS; in particular, the strains isolated from sourdough samples for sweet products, represented the 19% of the total of all strains tested and the 44% of the strains isolated from the same origin. Instead, the strains isolated from sourdough for pizza were the 21% of the total strains investigated and the 53% of the strains isolated from the same origin. In the end, the 6 *Bacillus subtilis* strains cheeked were all positive for EPS production on modified Agar Chalmers with a mixture of sugars and without $CaCO_3$.

The same strains assessed on the media with a sourdough extract showed negative results.

3.4.4 Molecular screening of *eps* genes for HoPS and HePS from bacteria strains

All strains (17) belonging to different species (*Leuconostoc, Lactobacillus* and *Bacillus*) were capable of producing HePS genes with exception of the strain *Ln. pseudomesenteroides* 79A. Most of the EPS positive strains possessed the *eps* genes, as verified with the primers used (Table 13). DNA of 10 *Leuconostoc* species strains gave positive results for the *eps*EFG gene. Instead, just four *Leuconostoc* strains showed 800-pb PCR products with *eps*A gene. With the *eps*D/E primers, all strains tested displayed positive results, except for the strain *Ln. pseudomesenteroides* 79A and the *Bacillus subtilis* strains. Regarding the *eps*B primers, the most of the strains yielded a PCR product. No PCR products were obtained with the DNA from *Ln. pseudomesenteroides* 79A, *Ln. mesenteroides* A 52, *Ln. gelidum* A43, *Ln. gelidum* A16, *Ln. amelibiosum* A21, *Ln. dextranicum* A28 and *Ln. lactis* A28. Otherwise, to determine the presence of glucansucrase and levansucrase genes (involved in HoPS biosynthesis) PCR amplification was performed with the 17 strains used before. In this case all *Leuconostoc* strains were able to produce HoPS, except for the strain from *Ln. pseudomesenteroides* 79A, *Ln. lactis/ Ln. garlicum* 68A12, *Ln. lactis*69B and *Ln. lactis*68B. The strains tested showed for the glucansucrase gene positive PCR products with the exception the strain *Lb. curvatus*69B2; for the levansucrase gene all strains showed a positive results except for the strains *Ln. lactis*95.

The *eps*GTF sequencing did not showed a similarity with nucleotides analysis present in database, but it was found a 99% similarity with a genes that codified for a wall cell protein of *Leuconostoc citreum* KM20. Finally, from the sequencing of *gtf* genes of the strains *Ln. mesenteroides* A52, *Ln. gelidum* A43 and *Ln. amelibiosum* A21, for the first two strains were obtained a 99% similarity with the gene codifying for the dextransucrases in *Ln. mesenteroides* L0309; and for the last one, was found a 96% of similarity with the gene codifying for a dextransucrase in *Ln. citreum*.

3.5 CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM *LACTOBACILLUS PARABUCHNERI* FUA3154

3.5.1 Characterization of exopolysaccharides by HPLC analysis

The EPS isolated from *Lb. parabuchneri* FUA3154 from MRS modified with maltose was quantified as 713 ± 371 mg l^{-1}.

From the enzyme assay with dextranase, we can not found significative results; in fact the peak obtained was glucose and the other peaks derived from the buffer used during the assay (Figure 13). Otherwise, the results obtained from β-glucanase assay, the peak showed the presence of glucose, too, but the other peaks that we can not characterize, because they did not corresponded with the standard, but they represented same oligosaccharides (β-glucan) linked with linkages β-1.3.

3.5.2 Screening of *eps* genes and sequencing

Firstly, the presence of *eps* (HePS) was verified by using four primer pairs (*eps* A, *eps* B, *eps* D/E and *eps* EFG). *Lb. parabuchneri* FUA3154 showed positive PCR products for the gene *eps*EFG, a 300-pb and 1,600-pb PCR products were obtained. With the *eps*D/E primers, the strain displayed a positive result. For the other primer pairs, the PCR products were all negative. Negative results were obtained from the PCR amplification of levansucrase and glucansucrase genes, that they are involved in omopolysaccharides biosynthesis. Finally, for the gene involved in the β-glucan synthesis, the strain was showed a positive PCR product.

To confirm the positive results for the *eps*EFG and *eps*D/E genes, the PCR products were purified and sequenced. For the gene *eps*EFG, the sequence obtained was failed and this is the way we decided to clone the gene and sequenced again, but in this case too, the sequencing was failed.

Otherwise, for the gene *eps*D/E was found a 98% similarity (180bp) with *eps* gene cluster in *S. thermophilus*.

3.5.3 Expression of the *eps*D/E gene in *Lactobacillus parabuchneri* FUA3154

To determine whether the putative *eps*D/E gene was expressed, RNA isolation was carried out to obtain a cDNA. To study the effective expression of the gene, the cDNA was used for a Reverse Transcription PCR. For each pairs of primer (β-glucan, *dnaK*, *recA*, *eps*D/E and *epsE*) the cDNA was showed positive results (Figure 14). In particular, we obtained a positive results for *eps*D/E primer used before for the sequencing and for the primers that we designed, specifically, on the interesting gene. In conclusion, the strain *Lb. parabuchneri* FUA3154 was showed a really expression of *eps*D/E gene.

4. DISCUSSION

4.1 CHARACTERIZATION OF SOURDOUGH SAMPLES BY CULTURE-DEPENDENT AND CULTURE-INDEPENDENT METHODS

In the first part of this study, the microflora of nine sourdoughs, used for bakery sweet goods, collected in different bakery of Campania region, have been investigated by using culture-dependent and culture-independent techniques. This part of the work was mainly aimed to characterize LAB and yeasts population in sweet leavened baked products, because more studies were focalised on microbiological aspects of sourdoughs used in bread production; instead not to much papers have treated the microbial composition of mother sponges for the sweet baked goods.

The LAB count results were lower than the usually in sourdough fermentation, probably, because the samples collected were not belonging to the sourdough type I and II, but maybe they can be defined like sourdough type III or type 0, which the for the first type, production technology predict the use of bakery's yeast and some LAB species more resistant and obtained standardized end products. Type 0 dough consisted of dough which uses baker's yeast to obtain the leavening and it not made with sourdough fermentation and also the LAB species can be the species present in the flour and they can contribute to a small degree to the acidification and aroma development.

In the samples I1, I2, I3 and I4 did not counted yeasts species and also the viable count of LAB is low; this sourdoughs can be considered constituting just of LAB species, as described by Gobbetti et al., (1994d) for wheat sourdough of central and southern Italy. Anyways, in sourdough population exists a huge variability that can be due by a lot of different factors as degree of dough hydration, kind of cereal used and leavening temperature and time. Also in this case, the condition of storage samples can determine the yeasts death. An other way that we can not count yeasts in this sample could be the media that we used to count and isolate the microflora. In fact, this media was suitable for counting a mixed population consisting of different species of LAB used as starter in breadmaking. It permit a selection of strains of *Lb.s plantarum*, *Ln. mesenteroides* subsp. *mesenteroides*, *Lb. sanfranciscensis*, *Enterococcus faecalis* together with *S. cerevisiae* could be easily differentiated and counted with an acceptable recovery in comparison with reference media (Pepe et al., 2001) and in this case did not allowed a good isolation and selction of the other yeast and LAB species occured. In all sourdoughs, the LAB: yeast ratio was always pro LAB, with an exception for the sourdough I4b, I6 and I7. In this case, the yeast can dominated the fermentation overhanging the indigenous LAB flora as indicated by the high pH. The viable count yeast (ranging from 5.0×10^4 to 3.0×10^8 CFU g^{-1} were in agreement with those reported for other sourdoughs from wheat flour produced in other regions (Succi et al., 2003).

The pH values of ripe sourdough is variable, but for wheat sourdoughs it ranges from 3.5 to 4.3 (Collar et al., 1994a; Wehrle and Arendt, 1998). Almost, all sample were included in this range, just for the sourdoughs I4b, I6 and I7, the pH was higher, because the temperature of fermentation were higher than the others and also for the predominance of yeast as explain above (Table 6). The acidification properties of different doughs also varied with the starter cultures used or of the proportion of lactic and acetic acid produced by LAB (Coppola et al., 1998), as well as the microbial influence on the pH values, is affected, also, by the amount and the number of homofermentative and heterofermentative species and by characteristic strains. It is also true that it is impossible compare the pH values and microbiological characteristics of sourdoughs obtained with different ingredients and different production technologies (Coppola et al., 1996).

In the present study, the LAB isolated colonies were identified by culture-dependent techniques by 16S rDNA sequencing. The major genus isolated was *Lactobacillus* with the species *Lb*. *casei*, *Lb*. *plantarum*, *Lb*. *sakei*, *Lb*. *curvatus*, *Lb*. *graminis, Lb. rennanqilfy* and *Lb*. *coryniformis*. Also some species belonging to the genus *Leuconostoc*: *Ln*. *mesenteroides*, *Ln*. *pseudomesenteroides*, *Ln*. *lactis* e *Ln*. *garlicum* and in the end two species belonging to the genus *Lactococcus* as *Lc*. *lactis* subp. *lactis* e *Lc*. *lactis* subp. *cremoris* (Table 9). This kind of LAB is typically isolated as a dominant in sourdough fermentation (Foschino et al., 1999; De Vuyst, 2005; Corsetti et al., 2007). *Lb. sanfranciscensis*, *Lb. brevis* and *Lb. plantarum* are the most frequently lactobacilli isolated from sourdoughs. However, some strains, initially classified as *Lb. plantarum* may actually belong to *Lb. paraplantarum* or *Lb. pentosus* species, since both phenotypic determination tools, such as carbohydrate fermentation patterns, and genetic methods, such as 16S rRNA gene sequence analysis, are not able to distinguish among *Lb. plantarum* group species.

Gobbetti (1998) reported on the *Lb. sanfranciscensis*/*Lb. plantarum* association in Italian wheat sourdough.

The species belonging to the genus *Lactobacillus* were all facultatively heterofermentative, while the strains identified as *Leuconostoc* were all obligately heterofermentative and finally the only homofermentative species isolated were belonged to *Lactococcus* genus. This situation is characteristic for sourdoughs microflora; in fact, in contrast to the use of mostly homofermentative species of LAB in the majority of (fermented) food applications, heterofermentative species play a major role in sourdough fermentation (Salovaara, 1998). Generally, *Weisella* and *Leuconostoc* species may play a role during the first part of the fermentation. The presence of the species of *Lactococcus* genus can due to the probably used of bakery's yeast which can contain LAB, especially lactobacilli rather than *Pediococcus, Lactococcus* and *Leuconostoc* spp. (Jenson, 1998), which in this case contributes only to a small degree to the acidification and aroma development of

dough. Finally, the dough sampled can be definite as “mother with transitory association” (madre ad associazione labile) (Foschino et al., 1995), which the sourdough was fermented by LAB that they are variable in the species occurs and the yeast involved in the leaving process are also different in species. Also between the different microbial groups present do not establish a mutual association but their occurrence is due of the common origin, flour and to particular environmental conditions. This determines a continuous competition for different nutrient sources that causes a constant selection between the different microorganisms, improving the development of some strains with particular physiologic properties (growth temperature, capability of fermentation, acidic resistance). Forty-nine yeast strains were isolated from nine samples, in the first four samples, the yeast population was not carried out. The identification was obtained by D1-D2 domain of 26S rDNA sequencing, showed the some result: all strains were identified as *S. cerevisiae*. One exception was the strain 100B identified as *Metschnikowia pulcherrima*. No other papers reported the occurrence of this specie in sourdough habitat, but it was previously isolated from some fruits like grapes (Senses-Ergul et al., 2005) or from pests guts (Molnár O., Prillinger H., 2005). It can be considered as a contamination from the bakery environmental or because the producer added to the dough some fruits to increase the amount of sugar fermented. Among the species mostly isolated from sourdough, *S. cerevisiae* occurring very often also because it is often introduced through the addition of baker’s yeast. In common practice for sweet leavened baked products, the bakery yeast is added to the first refreshment to speed up the last leavening step, particularly when malt is used in place of sucrose (Garofalo et al., 2008). It is true that the amount of *S. cerevisiae* may be overestimated due to the lack of reliable systems for identifying and classifying yeasts from this habitat (Vogel, 1997). The persistence of *S. cerevisiae* is due to fermentation temperature, in fact, it was demonstrated that during a fermentation at 30°C for 24 h this the specie can dominate the process. In agreement with Hansen et al., (1989a), some strain of *S. cerevisiae*, are more adapted to growth with homofermentative LAB strains rather then homofermentative LAB, because the acidity produced by heterofermentative LAB can be toxic or can achieve levels that the yeast strains can not be support. This reasoning can be used to explain the results obtained for the samples I1, I2, I3 and I4, where *S. cerevisiae* was not isolated by culture-dependent method, instead in the other samples where always *S. cerevisiae* strains were associated with at least one homofermentative or facultatively heterofermentative strains. In the dough I4b, the association *S. cerevisiae*/*Lb. plantarum*/*Ln.c mesenteroides,* was present one of the best association to a good production of aroma and to reach high level of phytate biodegradation (Chaoui et al., 2003). These results emphasize microbial potential in improving the nutritional quality of cereal-based products. The predominance of *S.cerevisiae* was predictable and desirable, considering their role in bread-making

with particular reference to leavening and to the development of aroma compounds as described by different authors (Severini et al., 2002; Wick et al., 2001).

The second part of my work was the characterization of the nine sourdoughs by culture-independent techniques, in particular, PCR-DGGE. Before to apply this method, the DNA isolation protocol was optimized, because the substances present in the food matrix may affect PCR and such elements can be co-extracted with the target DNA and thus interfere with PCR. Three protocols (Wizard method, Nucleo Spin Food Protocol and DNeasy Plant Mini Kit Protocol) were used. To obtain the best solution a standardized dough was done and the DNA isolated was tested with PCR and PCR-DGGE analysis. The three DNA isolation methods gave comparable results for the DNA quality but when the DNA was used for PCR and PCR-DGGE analysis, the Wizard and Nucleo Spin Food protocols proved to give the best results. However, the Wizard and Nucleo Spin Food protocols were used for the following identifications, which the higher quality of the template DNA collected from these sweet doughs, with these two protocols is ascribable to the greater removal of lipids, proteins and carbohydrates, which can interfere with the DNA or even inhibit the PCR amplification.

The population dynamics of lactobacilli and yeasts in dough samples have been investigated by PCR-DGGE analysis. As far as the PCR-DGGE outcomes are concerned, low species diversity was detected in all of the samples investigated. This result is justified because, usually, in sourdough the low biodiversity is explained by the selection of well-adapted species which co-exist in a stable dynamic equilibrium (Meroth et al., 2003a). By comparing the fingerprints obtained with the two DNA isolation protocols, slight differences emerged in the compositions of the microbial communities. One exception was the detection of the species of *Lb. sakei* occurred in the samples I4c and I5 and *S. thermophilus,* occurred in the doughs I1, I2, I3, I4 and I4b, with Nucleo Spin Food protocol. The analysis of the DNA recovered directly from sourdough samples showed that *Lb. sanfranciscensis* was the dominant specie that it does not isolated by dependent-culture method. Also with this approach, *Weisella cibaria*, was detected. Although this specie is know to have important roles in growth association with lactobacilli during early sourdough fermentation. The differences highlighted with culture dependent and independent techniques can be explained with the following hypothesis. Firstly, the technological parameters, such as the addition of ingredients like butter, eggs, chocolate and raisins, could have exerted selection pressures towards the species as *Lb.s sakei*, *S.thermophilus*, *Lb. sanfranciscensis* and *Weisella cibaria*. *S.thermophilus* is not very frequently isolated from this kind of samples, but we can hypothesize a probable contamination or the use of yogurt during the manufacturing. The visualization of DNA bands ascribed to *Lb. sanfranciscensis* can be explained by the amplification of DNA released from lysed cells, which can

be stressed for metabolic or osmolar factors during sourdough fermentation and not able to multiply on the solid media. Also, *Lb. sanfranciscensis* growth is very fastidious: in fact, it requires fresh yeast extract, unsaturated fatty acids and it preferentially ferments maltose instead than glucose (Gobbetti and Corsetti, 1997).When *Lb. sanfranciscensis* was detected with wizard protocols, we found a not unique profile but differences in the migration distances. Foschino et al., (2001) were demonstrated that the analysis of 16S-23S rDNA spacer regions is unable to distinguish among the *Lb. sanfranciscensis* isolates.

The yeast populations carried out with PCR-DGGE was also very low in variability. In fact, like the results obtained by culture-dependent methods, only the specie *S. cerevisiae* was detected. Also the number of bands were law, in fact, for all samples just one band was present in DGGE profile, with the exception, the profiles for the samples I1, I2, I3, I4 and I4b where were present a second band but it did not possible to purify. This specie was always detected by PCR-DGGE when it was applied in sourdough samples (Garofalo et al., 2008; Gatto and Torriani, 2004; Meroth et al., 2003b). Indeed, it is well known that microbial community analyses based on PCR-DGGE allow the most representative species to be detected, owing to competition among heterogeneous templates (Meroth et al. 2003a; Ercolini 2004).

4.2. SCREENING OF BACTERIA ISOLATED FROM BAKED PRODUCTS FOR EXOPOLYSACCHARIDES PRODUCTION

The first aim of this part o the study, was to optimize a media to screen the LAB strains able to produce exopolysaccharides. At this aim, the first medium that was testes was modified Agar Chalmers, because it was the media used to isolate the LAB strains. All *Leuconostoc* strains tested did not showed positive results. Therefore, the second medium that we tried was modified Agar Chalmer plus sucrose (5%), because the extracellular glucansucrases, the enzymes involved in EPS biosynthesis, are specifically induced by sucrose (Mizutani et al., 1994). In this case the strains assayed did not showed EPS production, but just when we used modified Agar Chalmers with sucrose (5%) as the only source of carbon, and without $CaCO_3$ the strains were produced EPS. To select the media, the some strains were tested on modified Agar Chalmers with different sugars as carbon sources but no one produced EPS. This results confirmed data from literature, in fact, it is known that *Leuconostoc* species can produce EPS, when in the media is present sucrose at high concentration.

The EPS production was better after 48 hours of incubation.

After this media optimization, all LAB strains (192) and 15 *Bacillus* strains were tested on modified Agar Chalmers with only sucrose as a carbon source and without $CaCO_3$. In particular, the 61% of

the LAB strains, isolated from sourdough, were EPS+. In particular, among the positive strains, it was possible mote that there were all strains belonging to the genera *Leuconostoc* sp. and *Weisella* sp., while all strains belonging to the genera *Enterococcus* sp., *Lactococcus* and *Lactobacillus* sp., were negative, with the exception of two strains *Lb.curvatus* 69B2 e *Lb. coryniformis* subsp. *torquens* 133, which were positives. The synthesis of EPS from sucrose for the species belonging to the genera *Weisella* sp. and *Leuconostoc* sp. has been found previously. These strains were also tested for the ropy test by Vescovo et al., (1989) methods, but they were all negative. Instead, 6 Bacillus strains were EPS + and showed, also, the ropy properties. In the end, to confirm the previous results, a media with a mixture of sugars (maltose, sucrose and fructose) was used. This media could be similar to the sourdough composition used for the production of sweet baked goods. Only 23 strains were able to produce EPS on this media, instead all six positive strains of *Bacillus subtilis* showed a positive results. The choice of the media used to study the EPS production is very important because it will be critical factors for EPS isolation. A good substrate must be a good source of carbohydrate, amino acids, vitamins, nucleic acid bases and mineral, because this kind of media allow the quantitative and qualitative production of the HePS and the investigation of the exact composition of the HePS produced. Instead, media containing complex nutrients like beef extract, peptone and yeast extract are not suitable because of interference of these compounds with the monomer and structure analysis of the HePS (Degeest et al., 2001). Usually, the screening for EPS production is performed in broth media. The methodology that we optimized is advantageous because the strains were inoculated directly in a solid media and for the consequent EPS isolation it was not necessary the elimination of substances different from EPS like a high-molecular mass material and also the protocol is easier and quickly because it is not necessary used the dialysis.

The results obtained by screening on this kind of solid media could hypothesize that the strain EPS+ are able to produce especially polysaccharides like dextrans or fructan.

The visual inspection of bacterial colonies on agar paltes is the easiest method but sometimes it can be insensitive and only indicative (Welman et al., 2003), also with this method it is not possible screen the LAB strains that produce a low amounts of EPS. Different alternatives exist but in this study, we decided to perform a further screening by molecular investigations for both HoPS and HePS. The frequencies of fructan- or glucan-positive strains were highest in *Leuconostoc* strains. Glucans and fructans are formed from sucrose by the activity of a single enzyme, i.e., glucan- or fructansucrase. Glucansucrases and fructansucrases display a similar protein domain organization (van Hijum et al., 2006). Primers targeting the conserved catalytic domain have already been developed to screen both for genes encoding glucansucrases or fructansucrases in LAB (Kralj et al., 2003; Tieking et al., 2003).

In this study the strains were screened to for the presence of the genes involved in the production of these two enzymes. All strains tested were positive for *gtf* or *lev* genes to predict the production of omopolysaccharides, which could be dextran or fructan. This results can confirm also the results obtained by the screening in solid media, because in that case, the strains were able to produce EPS just when they grew on media containing sucrose and also because almost all positive strains were belonging to the genera *Weisella* and *Leuconostoc*, the mostly species producing glucansucrases and fructansucrases (Monsan et al., 2001). With the sequencing of the three strains we confirmed the PCR amplification.

Recently, the molecular genetics of HePS biosynthesis have been studied for different LAB species (De Vuyst et al., 2004; Provenchee et al., 2003). Several glycosyltransferases involved in the assemblage of the HePS repeating units have been discovered (Jolly et al., 2002; van Kranenburg et al., 1999; Mozzi et al., 2006). For this aim 17 LAB and bacilli strains were subjected to PCR analysis with 4 different pairs of primer codifying for the different genes involved in HePS biosynthesis. All strains tested showed a positive results for at least one gene except for the strain *Ln. pseudomesenteroides* 79A. During this molecular analysis, a lot of aspecificity were obtained that is because it will be necessary focalised more studies on the specific strains and design a new specific pairs of primer. A good correlation between the presence of *eps* genes with the specific primers described for HePS and the HePS phenotype was observed (Mozzi et al., 2006). An inspected result was the negative *eps*A amplification for *Bacillus subtilis* strains, because from literature data, it was knew that EpsA from *S. thermophilus* Sfi6 has been shown to have significant homology with LytR of *Bacillus subtilis* (Stingele et al., 1996). In this study, it will be necessary a characterization of EPS to can compare the results obtained. Anyways, screening with different sets of *eps* primers could be a rapid alternative way to seek HePS-producing strains, although the HePS phenotype has to be confirmed afterwards.

The result obtained by *eps*GTF sequencing can be because detection of this gene is difficult considering that little similarity exists between genes encoding similar functions from distantly related species (Provencher et al., 2003). However, single consensus primers targeting highly conserved regions do not account well for codon usage and are thus most useful for more closely related sequences (Rose et al., 1998). Also, the protein could be considered involved in the synthesis of glucosiltransferases that they are cell wall bound enzymes.

It is very interesting note that the strain *Lb. curvatus*69B2 was able to produce EPS, as possible to see from results obtained by both phonotypically and molecularly analysis. In fact, just in another one paper it was reported the production of EPS from *Lb. curvatus* (Van der Meuken et al., 2007).

4.3. CHARACTERIZATION OF EXOPOLYSACCHARIDES FROM *LACTOBACILLUS PARABUCHNERI* FUA3154

The study of EPS produced from the strain *Lb. parabuchneri* FUA3154 was complete. In fact, in this case, we started with a production of EPS in broth and consequently purification and quantification. Therefore, the EPS composition was evaluated with HPLC analysis and with molecular approach, too. The important result was the identification by HPLC permit to note the EPS monosaccharides and oligosaccharides composition. In fact, this strai was avìble to produce as a monosaccharide dextran and as oligosaccharides that it was not possible identified because the pick obtained did not correspond with the standard pick. Anyways, it was possible to highlight the presence of different oligosaccharides like β-glucans constituted by linkages β-1.3 and other polysaccharides with linkages α-1,6. *Lactobacillus* sp. G-77 was able to produce a exopolysaccharides was shown to be a 2-substituted-(1-3)-β-D-glucan identical to that described for the exopolysaccharides from *P. damnosus* 2,6 (Duenas-Chasco et al., 1997). This is the first time that some LAB strains produce β-glucan. The ability to produce this kind of homopolysaccharides is very important, because this strain could be use during sourdough fermentation with a consequent improvement of the final quality of baked products. In fact, β-glucan can increased loaf volumes and the viscosity of the dough (Lazaridou and Biliaderis, 2007). Finally, they also have a prebiotic characteristic and can contribute to human health.

The molecular screening for *Lb. parabuchneri* FUA3154, to investigate its capability to posses the genes involved in both HoPS and HePS biosynthesis. Positive results were obtained just for he genes *eps*D/E and *eps*GTF, involved both priming glycosyltransferase. The genes for HoPS biosynthesis were not present, but this it could be a contradiction with the data obtained with HPLC analysis. It was demonstrated that the gene was less conserved in these strains and this can generate the presence of false negatives that it is inevitable and has been reported previously (Tieking et al., 2003-2005; Mozzi et al., 2006). Also a genetic screening can generate false positives that generate a positive PCR signal, but the sequence of the amplified fragment does not correspond with the targeted gene (Van der Meulen et al., 2007). That could be the explanation for negative results obtained when the gene *eps*GTF was cloned and sequenced. A 200.bp sequence could be amplified in this work from *Lb. parabuchneri* FUA3154 by PCR targeting for *eps*D/E gene and the database searches revealed high similarity to *eps* gene cluster in *S. thermophilus*. This pairs of primer plus an internal fragment of *eps*E/F genes were amplified from cDNA libraries of *Lb.parabuchneri* FUA3154, indicating this gene expression in this strain. In this way, we obtained more specific information about the genetic properties of this strain during EPS production.

5. CONCLUSIONS

The microbial characterization of sourdough for sweet leavened baked products by culture-dependent and independent methods was very efficient to know and understand the biodiversity and species complexity in sourdough samples.

In conclusion, this work highlights the need to combine both culture-dependent and culture independent methods for a better description of complex microbial populations involved in the production of fermented foods, such as sourdough.

The screening and selection of strains able to produce EPS highlighted the useful of the method to obtain a rapid screening; but also it will be necessary confirm the EPS characterization by other methods like for example, HPLC analysis to know the nature of the EPS produced. In this way the results obtained from molecular screening will be confront with the EPS composition. Anyways, the molecular approach can be easier method to allow a fast and initial selection of strains able to produce EPS.

Future studies will be focalising to the characterization of monosaccharides and oligosaccharides composition of EPS from positive strains. Also some of these strains will be test in vivo during sourdough fermentation to choose a possible starter for a production of sweet leavened baked goods.

Finally, *Lb.parabuchneri* FUA3154 can produce polysaccharides with α-1.6 linkages and β-1.3 linkages. The expression of *epsE* gene shows the possible relationship between *eps* gene cluster and EPS production.

Future plans will be try to characterize the oligosaccharides obtained and also try some experiments to study the behaviour of this strain in vivo, during sourdough fermentation. In the end we will investigate the sequencing *eps* gene operon and looking for essential genes for EPS production

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Table 1. Origin of sweet sourdough samples.

SAMPLES NAME	ORIGIN
I 1	Portici
I 2	Ponticelli
I 3	Casalnuovo
I 4	Torre del Greco
I 4b	Torre del Greco
I 4c	Torre del Greco
I 5	Torre del Greco
I 6	Sorrento
I 7	Sorrento

Table 2. Strains used for EPS screening.

Strain	Identification	Origin
51B	*Ln. pseudomesenteroides*	Sourdough for sweet products
53B	*Ln. pseudomesenteroides*	Sourdough for sweet products
60	*Ln. pseudomesenteroides*	Sourdough for sweet products
63	*Ln. pseudomesenteroides*	Sourdough for sweet products
59A1	*Ln. pseudomesenteroides*	Sourdough for sweet products
77A	*Ln. pseudomesenteroides*	Sourdough for sweet products
77B	*Ln. pseudomesenteroides*	Sourdough for sweet products
79A	*Ln. pseudomesenteroides*	Sourdough for sweet products
68B1	*Ln.mesenteroides*	Sourdough for sweet products
68B2	*Ln. lactis/ Ln. garlicum*	Sourdough for sweet products
68A	*Ln. lactis*	Sourdough for sweet products
68B	*Ln. lactis*	Sourdough for sweet products
68B12	*Ln. lactis*	Sourdough for sweet products
69B	*Ln. lactis*	Sourdough for sweet products
95	*Ln. lactis*	Sourdough for sweet products
95A	*Ln. lactis*	Sourdough for sweet products
68A12	*Ln. lactis*	Sourdough for sweet products
103	*Lnonostoc* sp.	Sourdough for sweet products
53C	*Ln.pseudomesenteroides*	Sourdough for sweet products
49	*Ln.pseudomesenteroides*	Sourdough for sweet products
49B1	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
49B2	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
79	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
47B21	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
47B	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
59B1	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
57B	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
77	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
59B	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
47B2	*Lc. lactis* subsp. *lactis*	Sourdough for sweet products
42	*Lb. casei*	Sourdough for sweet products
44A	*Lb. casei*	Sourdough for sweet products
44B	*Lb. casei*	Sourdough for sweet products
A	*Lb. casei*	Sourdough for sweet products
A1	*Lb. casei*	Sourdough for sweet products
A12	*Lb. casei*	Sourdough for sweet products
B2	*Lb. casei*	Sourdough for sweet products
E	*Lb. casei*	Sourdough for sweet products
33A	*Lb. casei*	Sourdough for sweet products
39	*Lb. casei*	Sourdough for sweet products
43	*Lb. casei*	Sourdough for sweet products
56A1	*Lactobacillus* sp.	Sourdough for sweet products
56B	*Lactobacillus* sp.	Sourdough for sweet products
127	*Lactobacillus* sp.	Sourdough for sweet products
108	*Lactobacillus* sp.	Sourdough for sweet products

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
69B2	*Lb.curvatus*	Sourdough for sweet products
68A1	*Lb.curvatus*	Sourdough for sweet products
68A2	*Lb.curvatus*	Sourdough for sweet products
64A	*Lb.curvatus/Lb. sakei*	Sourdough for sweet products
116A	*Lb.curvatus* subsp. *curvatus*	Sourdough for sweet products
133	*Lb. coryniformis* subsp. *torquens*	Sourdough for sweet products
109A	*Lb. coryniformis* subsp. *torquens*	Sourdough for sweet products
107	*Uncultured Streptococcus* sp.	Sourdough for sweet products
LM47	*Lb. sakei*	Pizza dough
LM227	*Lb. sakei*	Pizza dough
M77	*Lb. sakei*	Pizza dough
T56	*Lb. sakei*	Pizza dough
E5	*Lb. plantarum*	Commercial bakery's yeast
E7	*Lb. plantarum*	Commercial bakery's yeast
T9	*Lb. plantarum*	Pizza dough
T211	*Lb. plantarum*	Pizza dough
T231	*Lb. plantarum*	Pizza dough
E1	*Lb. paracasei*	Commercial bakery's yeast
E4	*Lb. pentosus.*	Commercial bakery's yeast
E10	*Lb. pentosus*	Commercial bakery's yeast

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
LA18	*Lb. viridiscens*	Pizza dough
LM210	*Ln. pseudomesenteroides*	Pizza dough
LM264a	*Ln. pseudomesenteroides*	Pizza dough
T229	*Ln. pseudomesenteroides*	Pizza dough
LM249	*Ln. gelidum*	Pizza dough
A16	*Ln. gelidum*	Pizza dough
A43	*Ln. gelidum*	Pizza dough
A25	*Ln. gelidum*	Pizza dough
A49	*Ln. gelidum*	Pizza dough
G14	*Weis. paramesenteroides*	Commercial bakery's yeast
G15	*Weis. paramesenteroides*	Commercial bakery's yeast
G18	*Weis. paramesenteroides*	Commercial bakery's yeast
A12	*Weis. paramesenteroides*	Pizza dough
A51	*Weis. paramesenteroides*	Pizza dough
A58	*Weis. paramesenteroides*	Pizza dough
A64	*Weis. paramesenteroides*	Pizza dough
A4	*Ln. oenos*	Pizza dough
A27	*Ln. mesenteroides*	Pizza dough
A52	*Ln. mesenteroides*	Pizza dough
A57	*Ln. mesenteroides*	Pizza dough
A65	*Ln. mesenteroides*	Pizza dough

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
A21	*Ln. amelibiosum*	Pizza dough
A28	*Ln. dextranicum*	Pizza dough
A90	*Ln. dextranicum*	Pizza dough
A7	*Ln. dextranicum*	Pizza dough
LA17	*Ln. carnosus*	Pizza dough
E30	*Lc. lactis* subsp. *raffinolactis*	Commercial bakery's yeast
LM66	*Lc. garviae*	Pizza dough
LA17	*Ln. carnosus*	Pizza dough
E30	*Lc. lactis* subsp. *raffinolactis*	Commercial bakery's yeast
LM66	*Lc. garviae*	Pizza dough
LM6	*Lc. garviae*	Pizza dough
LM10	*Lc. garviae*	Pizza dough
LA21	*Lc. lactis* subsp. *cremoris*	Pizza dough
LA23	*Lactoccocus* sp.	Pizza dough
G35	*Weisella* sp.	Commercial bakery's yeast
G17	*Lactococcus* sp.	Commercial bakery's yeast
A19	*Lactococcus* sp.	Pizza dough
M178	*Lactococcus* sp.	Pizza dough
M230	*Lactococcus* sp.	Pizza dough

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
T40	*Lactococcus* sp.	Pizza dough
T46	*Lactococcus* sp.	Pizza dough
T201	*Lactococcus* sp.	Pizza dough
A86	*E. faecium*	Pizza dough
M39	*Ln. gelidum*	Pizza dough
M47	*Leuconostoc* sp.	Pizza dough
T238	*Lb. pentosus*	Pizza dough
LM204b	*Leuconostoc* sp.	Pizza dough
E3	*Lactobacillus* sp.	Commercial bakery's yeast
LA25	*Leuconostoc* sp.	Pizza dough
T229	*Ln. pseudomesenteroides*	Pizza dough
K22	*Lb. plantarum*	Pizza dough
H19	*Lb. plantarum*	Pizza dough
H16	*Lb. plantarum*	Pizza dough
H5	*Lb. plantarum*	Pizza dough
H14	*Lb. plantarum*	Pizza dough
K13	*Lb. plantarum*	Pizza dough
H22	*Lb. plantarum*	Pizza dough
H4	*Lb. plantarum*	Pizza dough
H7	*Lb. plantarum*	Pizza dough

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
H20	*Lb. plantarum*	Pizza dough
93	n.i.	Pizza dough
H9	*Lb. plantarum*	Pizza dough
H21	*Lb. plantarum*	Pizza dough
LB26	n.i.	Pizza dough
A269	*Weis. paramesenteroides*	Pizza dough
H17	*Lb. plantarum*	Pizza dough
L5	*Lb.curvatus*	Pizza dough
Q2	*Lb. plantarum*	Pizza dough
M230	*Lactococcus* sp.	Pizza dough
H13	*Lb. plantarum*	Pizza dough
Q3	*Lb. plantarum*	Pizza dough
H10	*Lb. plantarum*	Pizza dough
L3	*Lb. plantarum*	Pizza dough
A78	*Ln. pseudomesenteroides*	Pizza dough
LM219	n.i.	Pizza dough
H6	*Lb. brevis*	Pizza dough
A23	*Leuconostoc* sp.	Pizza dough
T33	*Lc. lactis*	Pizza dough

n. i. = not identified

Table 2 (continued). Strains used for EPS screening.

Strain	Identification	Origin
L8	*Lb. plantarum*	Pizza dough
K21	*Lb. plantarum*	Pizza dough
ZS71	*Ent. faecalis*	Pizza dough
NS9	*Ent. faecalis*	Pizza dough
DL6	*Lb. plantarum*	Pizza dough
US69	*Ent. faecalis*	Pizza dough
BL6	*Lb. brevis*	Pizza dough
OS14	*Ent. faecalis*	Pizza dough
ES5	*Ent. faecalis*	Commercial bakery's yeast
PS23	*Ent. faecalis*	Pizza dough
AL5	*Lb. plantarum*	Pizza dough
CL9	*Lb. plantarum*	Pizza dough
EL7	*Lb. paracasei* subsp. *paracasei*	Pizza dough
CL5	*Lb. plantarum*	Pizza dough
GL2	*Lb. brevis*	Commercial bakery's yeast
GL3	*Lb. brevis*	Commercial bakery's yeast
FL2	*Lb. pentosus*	Pizza dough

Table 2 (continue). Strains used for EPS screening.

Strain	Identification	Origin
CL6	*Lb. plantarum*	Pizza dough
TS57	*Ent. faecalis*	Pizza dough
H11	*Lb. plantarum*	Pizza dough
H12	*Lb. plantarum*	Pizza dough
H8	*Lb. plantarum*	Pizza dough
NS11	*Ent. faecalis*	Pizza dough
NS10	*Ent. faecalis*	Pizza dough
OS17	*Ent. faecalis*	Pizza dough
H13	*Lb. plantarum*	Pizza dough
LM197	*Lactoccoccus* sp.	Pizza dough
AL2	*Lb. plantarum*	Pizza dough
ES3	*Ent. faecium*	Commercial bakery's yeast
H17	*Lb. plantarum*	Pizza dough
LA20	*Lc. lactis*	Pizza dough
LB26	n.i.	Pizza dough
A269	*Weis. paramesenteroides*	Pizza dough

n. i. = not identified

Table 2 (continue). Strains used for EPS screening.

Strain	Identification	Origin
H17	*Lb. plantarum*	Pizza dough
T1	*Bacillus subtilis*	Bread
F1	*Bacillus subtilis*	Bread
U1	*Bacillus subtilis*	Bread
A1	*Bacillus subtilis*	Bread
G1	*Bacillus subtilis*	Bread
S6	*Bacillus subtilis*	Bread
L2	*Bacillus subtilis*	Bread
K2	*Bacillus subtilis*	Bread
C3	*Bacillus subtilis*	Bread
D1	*Bacillus subtilis*	Bread
S9	*Bacillus subtilis*	Bread
N1	*Bacillus subtilis*	Bread
R1	*Bacillus subtilis*	Bread
I1	*Bacillus subtilis*	Bread
U2	*Bacillus subtilis*	Bread

n. i. = not identified

Table 3. Results media optimization for EPS screening.

Strain	Identification	Chalmers	Chalmers+Suc	Chalmers+Glu - $CaCO_3$	Chalmers+ Mal- $CaCO_3$	Chalmers+Gal - $CaCO_3$	Chalmers+Lac- $CaCO_3$	Chalmers+Suc- $CaCO_3$
51B	*Ln. pseudomesenteroides*	-	-	-	-	-	-	++
53B	*Ln. pseudomesenteroides*	-	-	-	-	-	-	+
60	*Ln. pseudomesenteroides*	-	-	-	-	-	-	-
63	*Ln. pseudomesenteroides*	-	-	-	-	-	-	++
59A1	*Ln. pseudomesenteroides*	-	-	-	-	-	-	-
77A	*Ln. pseudomesenteroides*	-	-	-	-	-	-	+
77B	*Ln. pseudomesenteroides*	-	-	-	-	-	-	++
68B1	*Ln.mesenteoides*	-	-	-	-	-	-	+
68B2	*Ln. lactis/ Ln. garlicum*	-	-	-	-	-	-	+
69B	*Ln. lactis*	-	-	-	-	-	-	++
53C	*Ln. pseudomesenteroides*	-	-	-	-	-	-	+

Table 3 (continued). Results media optimization for EPS.

Strain	Identification	Chalmers	Chalmers+Suc	Chalmers+Glu - $CaCO_3$	Chalmers+ Mal- $CaCO_3$	Chalmers+Gal - $CaCO_3$	Chalmers+Lac - $CaCO_3$	Chalmers+Suc- $CaCO_3$
49	*Ln. pseudomesenteroides*	-	-	-	-	-	-	+
133	*Lb. coryniformis subsp. torquens*	-	-	-	-	-	-	+/-
109A	*Lb. coryniformis subsp. torquens*	-	-	-	-	-	-	-
107	*Uncultured Streptococcus sp.*	-	-	-	-	-	-	+
57B	*Lact. lactis subsp. cremoris*	-	-	-	-	-	-	-
69B2	*Lb. curvatus*	-	-	-	-	-	-	++
68A1	*Lb. curvatus*	-	-	-	-	-	-	-
68A2	*Lb. curvatus*	-	-	-	-	-	-	-
64A	*Lb. curvatus/Lb. sakei*	-	-	-	-	-	-	-
116A	*Lb. curvatus subsp. curvatus*	-	-	-	-	-	-	-

Table 4. Pairs of primer used to screen the presence of *eps* genes.

Primer	Sequence (5'-3')	Gene target	PCR condition	References
eps EFG fw	GAYGARYTNCCNCARYTNWKNAAYGT	Priming glycosyltransferase (*Lb.delbrueckii* subs. *bulgaricus*)	30 cycles of 90°C (30s), 49°C (45s), 72°C(1min)	Mozzi et al., 2006
eps EFG rev	TGCAGCYTCWGCCACATG	Priming glycosyltransferase (*L. helveticus*)		Mozzi et al., 2006
*eps*D/E fw	TCATTTTATTCGTAAAACCTCAAATTGAYGARYT NCC	Priming glycosyltransferase (*L.casei* group and *S. thermophilus*)	5 cycles of n94°C (30s), 62°C (30s), 72°C (30s); 40 cycles of 94°C (30s), 52°C (30s), 72°C (30s)	Mozzi et al., 2006
*eps*D/E rev	AATATTATTACGACCTSWNAYYTGCCA	Priming glycosyltransferase (*L.casei* group and *S. thermophilus*		Mozzi et al., 2006
*eps*A fw	TAGTGACAACGGTTGTACTG	EPS regulation (*S. thermophilus*)	35 cycles of 94°C (15s), 40°C (30s), 72°C (1min)	Mozzi et al., 2006
*eps*A rev	GATCATTATGGACTGTCAC	EPS regulation (*S. thermophilus*)		Mozzi et al., 2006

Table 4 (continued). Pairs of primer used to screen the presence of *eps* genes.

Primer	Sequence (5'-3')	Gene target	PCR condition	References
*eps*B fw	CGTSCGSTTCGTACGACCAT	EPS chain length determination (*L.lactis*)	35 cycles of 94°C (45s), 46°C (1min), 72°C (1min)	Mozzi et al., 2006
*eps*B rev	TGACCAGTGACACTTGAAGC	EPS chain length determination (*L.lactis*)		Mozzi et al., 2006
gtf fw	GAYAAYWSNAAYCCNRYNGTNC	Glucansucrase	35 cycles of 95°C (5min), 95°C (30s), 42°C (45s), 72°C (1min)	Kralj et al., 2003; Tieking et al., 2003
gtf rev	ADRTCICCRTARTAIAVIYKIG	Glucansucrase		Kralj et al., 2003; Tieking et al., 2003
LevV fw	GAYGTNTGGGAYWSNTGGC	Levansucrase	35 cycles of 95°C (5min), 95°C (30s), 42°C (45s), 72°C (1min)	Kralj et al., 2003; Tieking et al., 2003
LevV rev	TCNTYYTCRTCNSWNRMCAT	Levansucrase		Kralj et al., 2003; Tieking et al., 2003

Table 5. Solvent gradient used for oligosaccharides analysis by HPLC.

Time [min]	Eluent A [%]	Eluent B [%]	Eluent C [%]
0	68.3	30.4	1.3
30	54.6	30.4	15
35	50	50	0
40	10	73	17
40.1	32.3	30.4	36.3
45	10	73	17
50	10	73	17
55	68.3	30.4	1.3
60	68.3	30.4	1.3

Table 6. Microbial enumeration and pH and TTA values.

Samples	LAB CFU g^{-1}	Yeast CFU g^{-1}	LAB/yeast	pH	ATT*
I 1	4.0×10^3	-	4000	4.51 ± 0,02	3.0
I 2	8.0×10^2	-	800	4.95 ± 0,01	3.1
I 3	4.0×10^3	-	4000	3.76 ± 0,01	2.7
I 4	1.0×10^3	-	1000	4.68 ± 0,06	1.9
I 4b	7.0×10^7	3.15×10^8	0.22	5.74 ± 0,03	3.2
I 4c	3.4×10^7	1.5×10^7	2.,26	3.76 ± 0,04	2.4
I 5	1.75×10^5	5.0×10^4	3.5	3.91 ± 0,01	4.7
I 6	1.1×10^5	3.0×10^8	< 0.1	5.03 ± 0,00	3.4
I 7	1.0×10^7	9.3×10^7	0.1	5.15 ± 0,02	1.6

*** ATT = acidità titolabile totale, ml di NaOH 0,1 N**

Table 7. Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I 1	24 (AN)	irregular	flat	clammy	pink	+	single cocci
I 1	45	irregular	flat	clammy	pink	+	single cocci
I 2	42	irregular	flat	clammy	pink	+	small rods in pairs or in chain
I 2	44A	irregular	flat	clammy	pink	+	small rods in pairs or in chain
I 2	44B	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I 3	A	irregular	flat	clammy	pink	++	single rods or in pairs
I 3	A 1	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I 3	A 12	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I 3	B 2	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I 3	E	irregular	flat	clammy	Pink	++	small rods in pairs or in chain
I 3	33A (AN)	irregular	flat	clammy	Pink	++	small rods in pairs or in chain
I 3	39	irregular	flat	clammy	Pink	++	small cocci in pairs or in chain
I 4	43	irregular	flat	clammy	Pink	+	small cocci in pairs or in chain
I4b	49B	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I 4	43	irregular	flat	clammy	pink	+	cocci in pairs or in chain
I4b	49B	irregular	flat	clammy	pink	++	cocci in pairs or in chain
I4b	49B1	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	49B2	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	59B2	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	79 (AN)	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	47	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	47B21	irregular	flat	clammy	pink with bordeaux centre	+	cocci in pairs or in chain
I4b	47B	irregular	flat	clammy	pink with bordeaux centre	+	cocci in pairs or in chain
I4b	59B1	irregular	flat	clammy	pink with bordeaux centre	++	cocci in pairs or in chain
I4b	51A	irregular	flat	clammy	pink with bordeaux centre	++	small rods in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I4b	56A1	irregular	flat	clammy	pink with bordeaux centre	++	small rods in pairs or in chain
I4b	56B	irregular	flat	clammy	pink with bordeaux centre	++	small rods in pairs or in chain
I4b	57B	irregular	flat	clammy	pink with bordeaux centre	++	small rods in pairs or in chain
I4b	59A2	irregular	flat	clammy	pink with bordeaux centre	++	small rods in pairs or in chain
I4b	51B	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain
I4b	53B	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain
I4b	53C	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	60	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	62B2	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	63	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	77A	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	49	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain
I4b	57	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain
I4b	59	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I4b	59A1	irregular	flat	clammy	bordeaux	++	cocci in pairs or in chain
I4b	58A	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4b	77A	irregular	flat	clammy	bordeaux	++	sigle cocci or in pairs
I4b	77B	irregular	flat	clammy	bordeaux	++	sigle cocci or in pairs
I4b	79A	irregular	flat	clammy	bordeaux	++	sigle cocci or in pairs
I4b	57A	irregular	flat	clammy	bordeaux	+	small rods in pairs or in chain
I4b	59B2	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4b	77	irregular	flat	clammy	bordeaux	+	small rods in pairs or in chain
I4b	59B	regular	flat	clammy	pink	++	cocci in pairs or in chain
I4b	78	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I4b	51A	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I4b	47B2	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I4c	69B2	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I4c	68A1	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I4c	68A2	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I4c	96B (AN)	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I4c	68B1	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4c	68B2	irregular	flat	clammy	bordeaux	+	cocci in pairs or in chain
I4c	68A	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	68A12	irregular	flat	clammy	bordeaux	+	small rods in pairs or in chain
I4c	68B	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	68B12	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	69B	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	95 (AN)	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	95A (AN)	irregular	flat	clammy	bordeaux	++	small rods in pairs or in chain
I4c	64A1	regular	flat	clammy	pink	+	single rods or in pairs
I4c	64A	irregular	flat	clammy	pale pink	+	small rods in pairs or in chain
I4c	96 (AN)	irregular	flat	clammy	pale pink	+	small rods in pairs or in chain
I5	83B (AN)	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I5	85 (AN)	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I5	81	irregular	convex	clammy	pink	++	small rods in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I5	83	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I5	85A	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I5	85B	irregular	flat	clammy	pink	++	small rods in pairs or in chain
I6	127	irregular	convex	clammy	pink with red centre	++	long tin rods
I7	132	irregular	flat	clammy	dark pink	+	small cocci in chain
I7	105	irregular	flat	clammy	dark pink	+	small cocci in chain
I7	119	irregular	flat	clammy	dark pink	+	small cocci in chain
I7	121	irregular	flat	clammy	dark pink	++	small cocci in chain
I7	107	irregular	flat	clammy	dark pink	++	small cocci in pairs or in chain
I7	131	irregular	flat	clammy	dark pink	++	small cocci in chain
I7	133	irregular	flat	clammy	pink with red centre	++	small rods in pairs or in chain
I7	116A	irregular	flat	clammy	pink with red centre	++	small rods in pairs
I7	111A	irregular	flat	clammy	pink with red centre	++	small rods in pairs
I7	103	irregular	flat	clammy	pink with red centre	++	cocci in pairs or in chain

Table 7 (continued). Phenotypic characterization of isolates presuntive LAB.

Sample	Strain	Differential morphological characteristics				Halo	Micro-morphology
		edge	elevation	consistency	colour		
I7	113	irregular	flat	clammy	pink	+	small cocci in chain
I7	108	irregular	flat	clammy	pink	+	long tin rods
I7	109A	regular	convex	clammy	pink	++	small rods in pairs
I7	114	regular	convex	clammy	pink	++	Small rods in pairs

Table 8. Phenotypic characterization of isolates presuntive yeasts.

Sample	Strain	Differential morphological characteristics				Halo
		edge	elevation	consistency	colour	
I 4b	47B22	regular	convex	creamy	pale pink	-
I 4b	46	irregular	convex	creamy	pink with yellow centre	-
I 4b	55	irregular	convex	creamy	pink with yellow centre	-
I 4b	56A2	irregular	convex	creamy	pink with yellow centre	-
I 4b	61	irregular	convex	creamy	pink with yellow centre	-
I 4b	71 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 4b	72 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 4b	73 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 4b	74 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 4b	75 (AN)	irregular	convex	creamy	Pink with yellow centre	-
I 4b	76 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	69A21	regular	convex	creamy	pale pink	-
I4c	69A2	irregular	convex	creamy	pink with yellow centre	-
I4c	87 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	90 (AN)	irregular	convex	creamy	pink with yellow centre	-

Table 8 (continued). Phenotypic characterization of isolates presuntive yeasts.

Sample	Strain	Differential morphological characteristics				Halo
		edge	elevation	consistency	colour	
I4c	91 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	92 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	93 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	94 (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	94A (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	94B (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	96A (AN)	irregular	convex	creamy	pink with yellow centre	-
I4c	98 (AN)	iIrregular	convex	creamy	pink with yellow centre	-
I 5	80 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 5	82 (AN)	irregular	convex	creamy	pink with yellow centre	-
I 5	82A (AN)	irregular	convex	creamy	pink with yellow centre	-
I 5	87	regular	convex	creamy	pink with yellow centre	-
I 5	89	regular	convex	creamy	pale pink	-
I 6	100A	irregular	convex	creamy	pink	-
I 6	100B	irregular	convex	leatery	purple	-

Table 8 (continued). Phenotypic characterization of isolates presuntive yeasts.

Sample	Strain	Differential morphological characteristics				Halo
		edge	elevation	consistency	colour	
I 6	122	irregular	convex	creamy	pink with yellow centre	-
I 6	123	irregular	convex	creamy	pink with yellow centre	-
I 6	124	irregular	convex	creamy	pink	-
I 6	126	irregular	convex	creamy	pink	-
I 6	127	irregular	convex	creamy	pink	-
I 6	135	irregular	convex	creamy	pink	-
I 6	136	irregular	convex	creamy	pink with yellow centre	-
I 6	138	irregular	convex	creamy	pink	-
I 6	139	irregular	convex	creamy	pink	-
I 6	140	irregular	convex	creamy	pink	-
I 7	104	irregular	convex	creamy	pink	-
I 7	109B	irregular	convex	creamy	pink	-
I 7	110	irregular	convex	creamy	pink	-
I 7	111B	irregular	convex	creamy	pink	-

Table 8 (continued). Phenotypic characterization of isolates presuntive yeasts.

Sample	Ceppo	Differential morphological characteristics				Halo
		edge	elevation	consistency	colour	
I 7	114B	irregular	convex	creamy	pink	-
I 7	115	irregular	convex	creamy	pink with yellow centre	-
I 7	116	irregular	convex	creamy	pink	-
I 7	118	irregular	convex	creamy	pink with yellow centre	-
I 7	130	irregular	convex	creamy	pink	-
I 7	134	irregular	convex	creamy	pink	-

Table 9. LAB and yeast identification by "culture-dependent" method.

Sample	LAB species (sequencing of 16S rDNA)	Yeast species (sequencing of D1-D2 26S rDNA)
I1	*Lactobacillus casei* *Lactobacillus rennanqilfy*	n. r.
I2	*Lactobacillus casei*	n. r.
I3	*Lactobacillus casei* *Lactobacillus rennanqilfy*	n. r.
I4	*Lactobacillus casei*	n. r.
I4b	*Lactococcus lactis* sp. *lactis* *Lactococcus lactis* sp. *cremoris* *Leuconostoc pseudomesenteroides* *Lactobacillus plantarum*	*Saccharomyces cerevisiae*
I4c	*Lactobacillus curvatus* *Lactobacillus sakei* *Leuconostoc mesenteoides* *Leuconostoc lactis* *Leuconostoc garlicum*	*Saccharomyces cerevisiae*
I5	*Lactobacillus plantarum* *Lactobacillus* sp.	*Saccharomyces cerevisiae*
I6	*Lactobacillus* sp.	*Saccharomyces cerevisiae* *Mechnikova pulcherrima*
I7	*Lactobacillus coryniformis* subsp. *torquens* *Lactobacillus curvatus* subsp. *curvatus* *Lactobacillus graminis* *Leuconostoc* sp.	*Saccharomyces cerevisiae*

n.r. = not relieved

Table 10. LAB and yeast identification by "culture-independent" method.

Sample	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) Wizard	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA)NS	Bands	Yeasts identify by PCR-DGGE (sequencing of region D1-D2 of 5,8S rDNA) NS
I1	---	n.d.	C	*Uncultured soil bacterium Triticum aestivum*	2	*Saccharomyces cerevisiae*
I2	---	n.d.	C	*Uncultured soil bacterium Triticum aestivum*	2	*Saccharomyces cerevisiae*
			C	*Uncultured soil bacterium Triticum aestivum*		
I3	---	n.d.	G	*Lactobacillus sakei*	2	*Saccharomyces cerevisiae*
			H	n.d.		
			I	*Uncultured soil bacterium*		

n. d. = not determined

Table 10 (continued). LAB and yeast identification by "culture-independent" method.

Sample	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) Wizard	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) NS	Bands	Yeasts identify by PCR-DGGE (sequencing of region D1-D2 of 5,8S rDNA) NS
I4		n.d.	C	*Uncultured soil bacterium Triticum aestivum*	2	*Saccharomyces cerevisiae*
			G	*Lactobacillus sakei*		
			H	n.d.		
			I	*Uncultured soil bacterium*		
I4b	A	*Uncultured bacterium clone h6 Hordeum vulgare subsp. vulgare*	C	*Uncultured soil bacterium Triticum aestivum*	2	*Saccharomyces cerevisiae*
	C	*Uncultured bacterium clone h6 Hordeum vulgare subsp. vulgare*	D	*Streptococcus thermophilus*		
	E	*Weissella cibaria*	N	*Weissella cibaria*		

n. d. = not determined

Table 10 (continued). LAB and yeast identification by "culture-independent" method.

Sample	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) Wizard	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) NS	Bands	Yeasts identify by PCR-DGGE (sequencing of region D1-D2 of 5,8S rDNA) NS
I4c	C1	*Lactobacillus sanfranciscensis*	C	*Uncultured soil bacterium* *Triticum aestivum*	2	*Saccharomyces cerevisiae*
	D	*Lactobacillus sanfranciscensis*	D	*Streptococcus thermophilus*		
	E	*Weissella cibaria*	E	*Lactobacillus sanfranciscensis*		
I5	C	*Uncultured bacterium clone h6* *Hordeum vulgare subsp. vulgare*	C	*Uncultured soil bacterium* *Triticum aestivum*	2	*Saccharomyces cerevisiae*
	C1	*Lactobacillus sanfranciscensis*				
	D1	*Lactobacillus sanfranciscensis*	D	*Streptococcus thermophilus*		
	C2	*Uncultured bacterium clone h6* *Hordeum vulgare subsp. vulgare*	E	*Lactobacillus sanfranciscensis*		

Table 10 (continued). LAB and yeast identification by "culture-independent" method.

Sample	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) Wizard	Bands	LAB identify by PCR-DGGE (sequencing of region V3 of 16S rDNA) NS	Bands	Yeasts identify by PCR-DGGE (sequencing of region D1-D2 of 5,8S rDNA) NS
I6	C	*Uncultured bacterium clone h6* *Hordeum vulgare subsp. vulgare*	C	*Uncultured soil bacterium* *Triticum aestivum*	2	*Saccharomyces cerevisiae*
	C2	*Uncultured bacterium clone h6* *Hordeum vulgare subsp. vulgare*	D	*Streptococcus thermophilus*		
			N	*Weissella cibaria*		
I7	C	*Uncultured bacterium clone h6* *Hordeum vulgare subsp. vulgare*	C	*Uncultured soil bacterium* *Triticum aestivum*	2	*Saccharomyces cerevisiae*
			D	*Streptococcus thermophilus*		
			E	*Lactobacillus sanfranciscensis*		
			G	*Lactobacillus sakei*		
	E	*Weissella cibaria*	H	n.d.		
			I	*Uncultured soil bacterium*		
			N	*Weissella cibaria*		

Table 11. Results of primary screening of bacteria strains for EPS production.

Strain	Identification	Origin	Chalmers+sucrose- $CaCO_3$	
			24 h	48 h
51B	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	+
53B	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	+
60	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	++
63	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	+
59A1	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	++
77A	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	++
77B	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	+
68B1	*Ln.mesenteoides*	Sourdough for sweet products	+	+
68B2	*Ln. lactis/ Ln. garlicum*	Sourdough for sweet products	+	++
69B	*Ln. lactis*	Sourdough for sweet products	+	++

Table 11 (continued). Results of primary screening of bacteria strains for EPS production.

Strain	Identification	Origin	Chalmers+sucrose- $CaCO_3$	
			24 h	48 h
53C	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	++
49	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	++
68A12	*Ln. lactis/ Ln. garlicum*	Sourdough for sweet products	+	+
69B2	*Lb. curvatus*	Sourdough for sweet products	+	+
133	*Lb. coryniformis subsp. torquens*	Sourdough for sweet products	+	+
107	*Uncultured Streptococcus sp.*	Sourdough for sweet products	+	+
A16	*Ln. gelidum*	Pizza dough	+	++
A43	*Ln. gelidum*	Pizza dough	+	++
A49	*Ln. gelidum*	Pizza dough	+	+
G14	*Weis. paramesenteroide*	Commercially Bakery'yeast	+	+
G15	*Weis. paramesenteroide*	Commercially Bakery'yeast	+	+
A51	*Weis. paramesenteroide*	Pizza dough	+	+
A58	*Weis. paramesenteroide*	Pizza dough	+	+
A64	*Weis. paramesenteroide*	Pizza dough	+	++

Table 11 (continued). Screening of bacteria strains for EPS production.

Strain	Identification	Origin	Chalmers+sucrose- $CaCO_3$	
			24 h	48 h
A49	*Ln. gelidum*	Pizza dough	+	+
G14	*Weis. paramesenteroide*	Commercially Bakery'yeast	+	+
G15	*Weis. paramesenteroide*	Commercially Bakery'yeast	+	+
A51	*Weis. paramesenteroide*	Pizza dough	+	+
A58	*Weis. paramesenteroide*	Pizza dough	+	+
A64	*Weis. paramesenteroide*	Pizza dough	+	++
G18	*Weis. paramesenteroide*	Commercially Bakery'yeast	+	+
A27	*Ln. mesenteroides*	Pizza dough	+	+
A57	*Ln. mesenteroides*	Pizza dough	+	++
A65	*Ln. mesenteroides*	Pizza dough	+	+
A21	*Ln. amelibiosum*	Pizza dough	+	++
A28	*Lactococcus* sp.	Pizza dough	+	++
A19	*Leuconostoc* sp.	Pizza dough	+	+
A23	*Bacillus subtilis*	Bread	+/-	+
T1	*Bacillus subtilis*	Bread	++	++
F1	*Bacillus subtilis*	Bread	+	+
U1	*Bacillus subtilis*	Bread	+	+
A1	*Bacillus subtilis*	Bread	++	++
G1	*Bacillus subtilis*	Bread	+	+
S6	*Bacillus subtilis*	Bread	+	+

Table 12. Results of screening of bacteria strains for EPS production on Modify Agar Chalmers with sugar mixture .

Strain	Identification	Origin	Chalmers+ SFM-$CaCO_3$	
			24 h	48 h
51B	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
53B	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
63	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	+/-
77A	*Ln. pseudomesenteroides*	Sourdough for sweet products	+	+
77B	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
79A	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
68B1	*Ln.mesenteroides*	Sourdough for sweet products	-	-
68B2	*Ln. lactis/ Ln. garlicum*	Sourdough for sweet products	-	-
68B	*Ln. lactis*	Sourdough for sweet products	+	++
69B	*Ln. lactis*	Sourdough for sweet products	++	++
95	*Ln. lactis*	Sourdough for sweet products	++	++
95A	*Ln. lactis*	Sourdough for sweet products	++	++
53C	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
49	*Ln. pseudomesenteroides*	Sourdough for sweet products	-	-
68A12	*Ln. lactis/ Ln. garlicum*	Sourdough for sweet products	++	++
69B2	*Lb. curvatus*	Sourdough for sweet products	++	++
133	*Lb. coryniformis subsp. torquens*	Sourdough for sweet products	-	-

Table 12 (continued). Results of screening of bacteria strains for EPS production on Modify Agar Chalmers with sugar mixture .

Strain	Identification	Origin	Chalmers+ SFM-$CaCO_3$	
			24 h	48 h
107	*Uncultured Streptococcus sp.*	Sourdough for sweet products	-	-
A16	*Ln. gelidum*	Pizza dough	+	+
A43	*Ln. gelidum*	Pizza dough	+	++
A49	*Ln. gelidum*	Pizza dough	-	-
G14	*Weis. paramesenteroides*	Commercially Bakery'yeast	-	-
G15	*Weis. paramesenteroidse*	Commercially Bakery'yeast	-	-
A51	*Weis. paramesenteroides*	Pizza dough	-	-
A58	*Weis. paramesenteroides*	Pizza dough	+	+
A64	*Weis. paramesenteroides*	Pizza dough	-	-
G18	*Weis. paramesenteroide*	Commercially Bakery'yeast	-	-
A27	*Ln. mesenteroides*	Pizza dough	-	-
A52	*Ln. mesenteroides*	Pizza dough	-	+

Table 12 (continued). Results of screening of bacteria strains for EPS production on Modify Agar Chalmers with sugar mixture .

Strain	Identification	Origin	Chalmers+ SFM-$CaCO_3$	
			24 h	48 h
A57	*Ln. mesenteroides*	Pizza dough	+	+
A65	*Ln. mesenteroides*	Pizza dough	+	+
A21	*Ln. amelibiosum*	Pizza dough	+	+
A28	*Ln. dextranicum*	Pizza dough	+	++
A19	*Lactococcus* sp.	Pizza dough	-	-
A23	*Leuconostoc* sp.	Pizza dough	+/-	+/-
T1	*Bacillus subtilis*	Bread	+	+
F1	*Bacillus subtilis*	Bread	++	++
U1	*Bacillus subtilis*	Bread	++	++
A1	*Bacillus subtilis*	Bread	++	++
G1	*Bacillus subtilis*	Bread	++	++
S6	*Bacillus subtilis*	Bread	++	++

Table 13. Screening of *eps* genes.

Strain	Identification	*eps*A	*eps*B	*eps*D/E	*eps*EFG	*gtf*	*lev*
79A	*Ln. pseudomesenteroides*	-	-	-	-	-	-
77B	*Ln. pseudomesenteroides*	-	+	+	+	+	+
A52	*Ln. mesenteroides*	+	-	+	-	+	+
95	*Ln. lactis*	-	+	+	+	+	-
A43	*Ln. gelidum*	+	-	+	-	+	+
A16	*Ln. gelidum*	-	-	+	+	+	+
A21	*Ln. amelibiosum*	-	-	+	+	+	+
A28	*Ln. dextranicum*	+	-	+	+	+	+
69B2	*Lb. curvatus*	-	+	+	+	-	+
68A12	*Ln. lactis/ Ln. garlicum*	-	+	+	+	-	-
69B	*Ln. lactis*	-	+	+	+	-	-
A57	*Ln. mesenteroides*	+	-	+	-	+	+
68B	*Ln. lactis*	-	+	+	+	-	-
63	*Ln. pseudomesenteroides*	-	+	+	+	+	+
T1	*Bacillus subtilis*	-	+	-	-	-	+
F1	*Bacillus subtilis*	-	+	-	+	-	-
A1	*Bacillus subtilis*	-	+	-	+	-	-

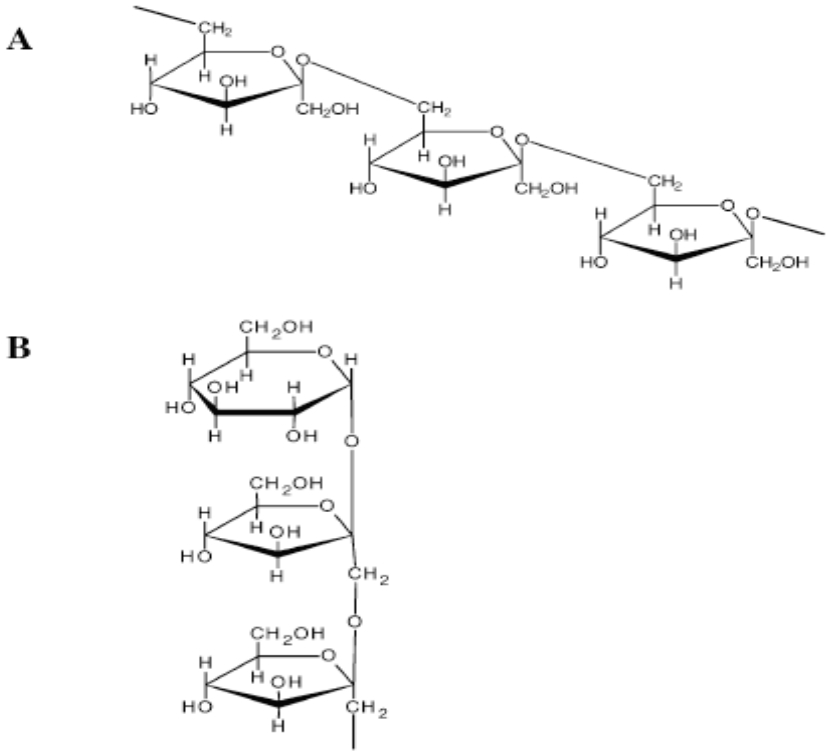

Figure 1. Structures of fructans: (**A**) levan; (**B**) inulin-type fructans.

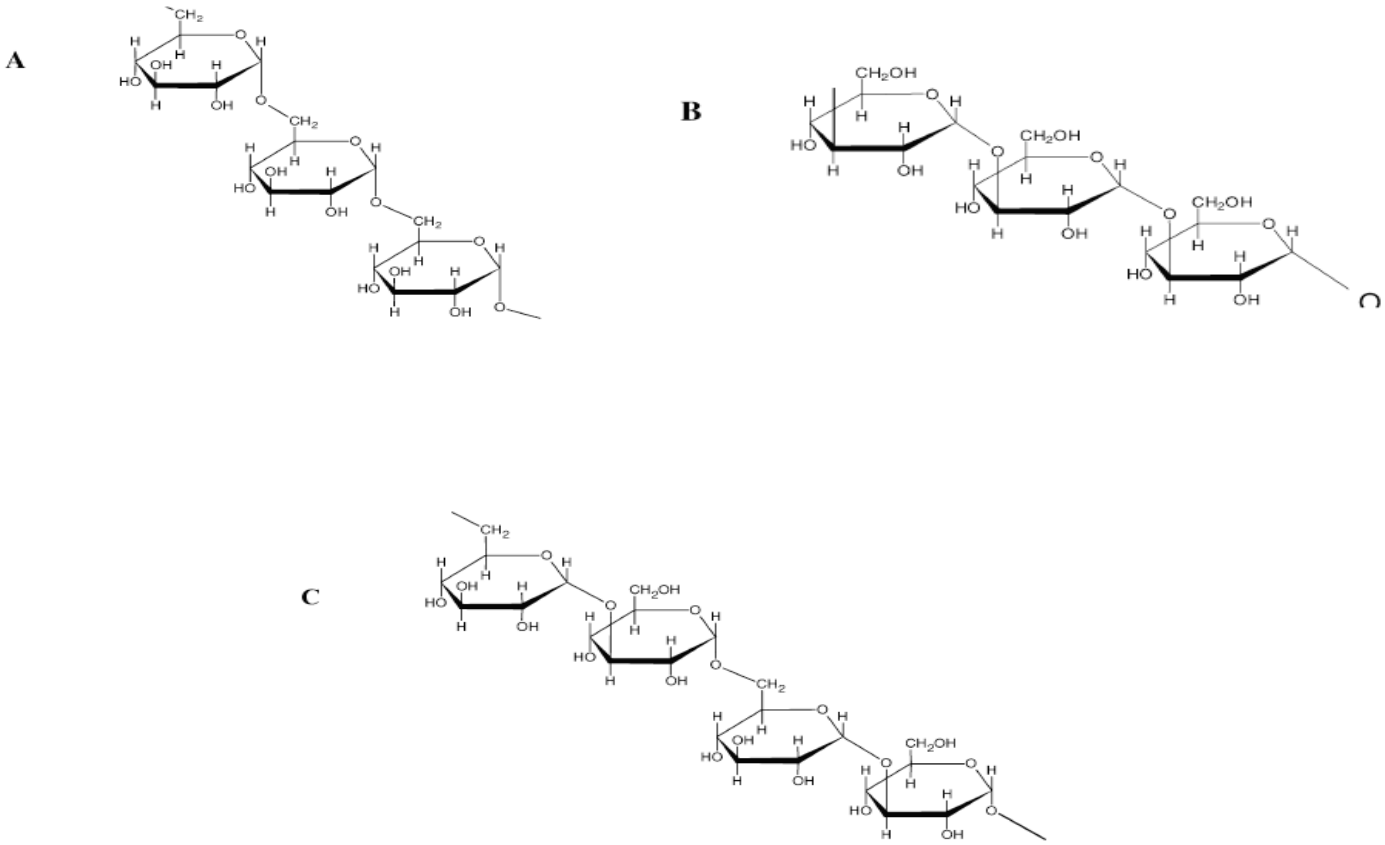

Figure 2. Structures of glucans: (A) dextran; (B) mutans and (C) alternan.

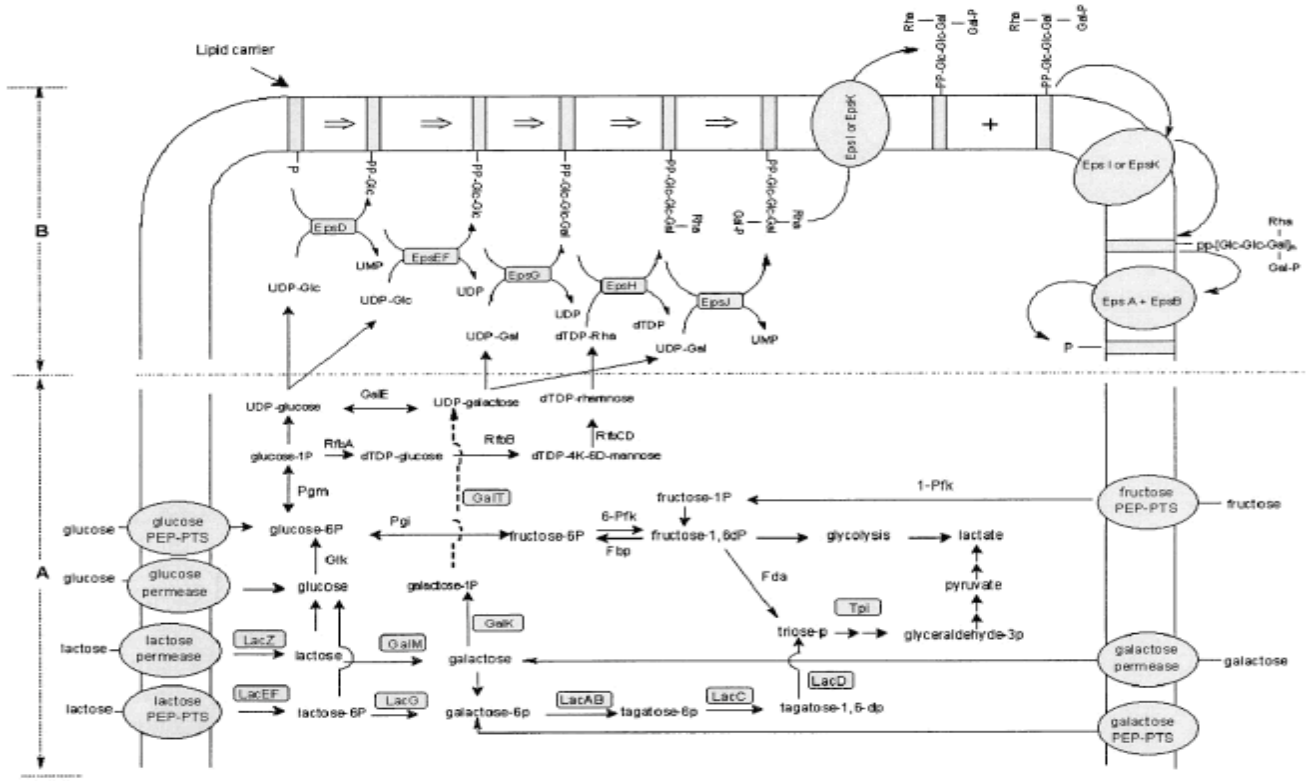

Figure 3. (A) Schematic representation of a number of possible pathways for sugar transport and metabolism in LAB. (B) Schematic representation of a possible pathway for EPS biosynthesis in L. lactis NIZO B40 starting from glucose-6-phosphate. From Laws et al., 2001.

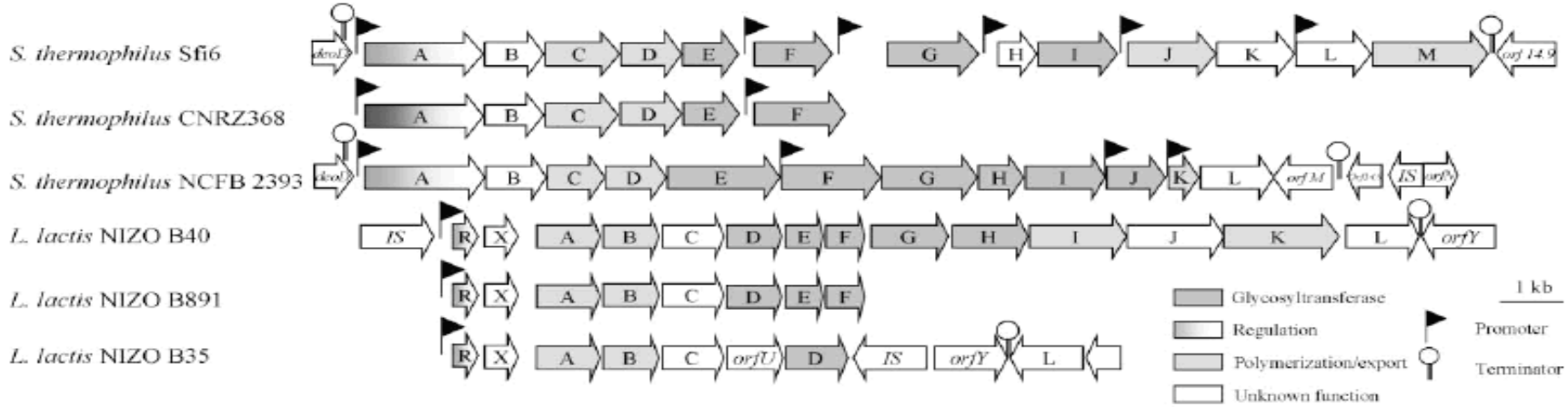

Figure 4. Organization ofthe eps gene clusters of heteropolys acharide-producing *Streptococcus thermophilus* and *Lactococcus lactis* strains. The (possible) functions of the different gene products are indicated (Figure from De vuyst et al., 2001) .

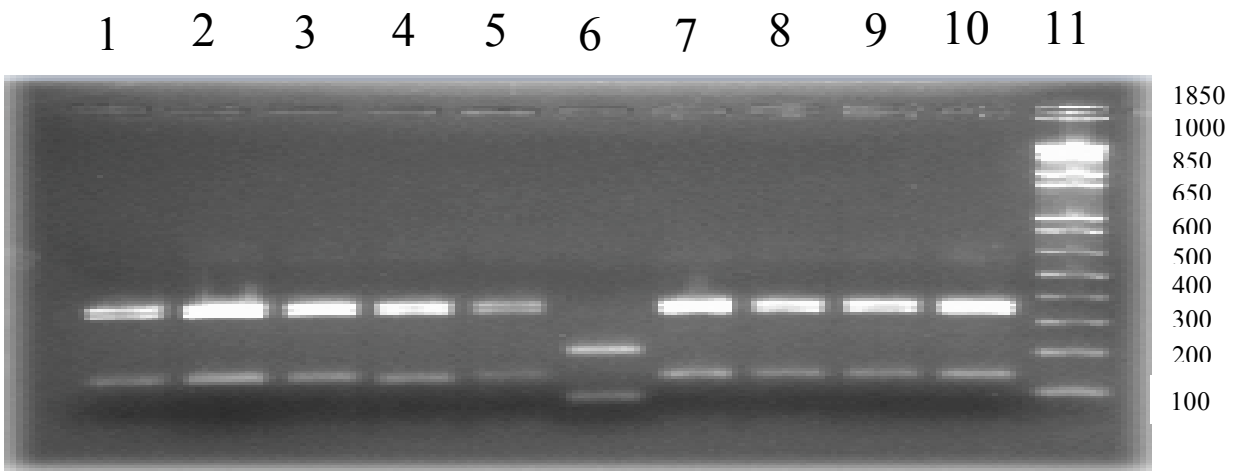

Figure 5. Example of digestion of ITS1- 5,8S- ITS2. Lanes 1-10 yeast strains; Lane 1 strain 61; lane 2 strain 69A21; lane 3 strain73; lane 4 strain 82; lane 5 strain 92; lane 6 strain 110B; lane 7 strain 111B; lane 8 strain 124; lane 9 strain 134; lane 10 strain 138; lane 11 1Kb ladder.

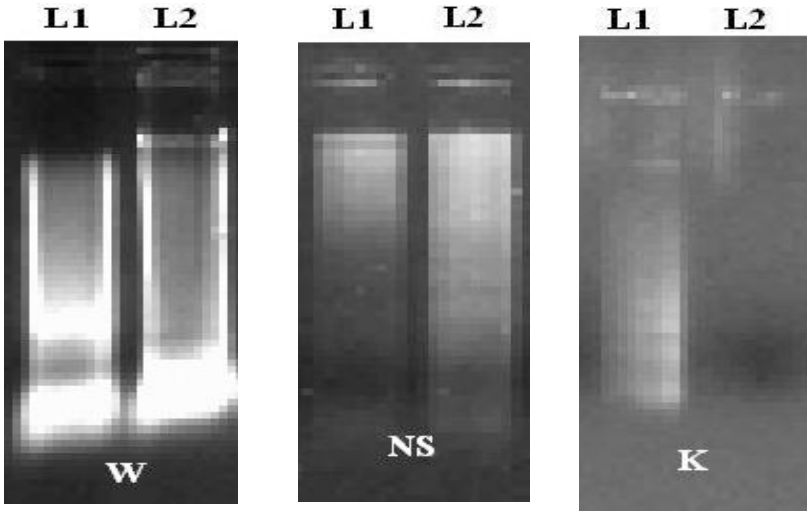

Figure 6. DNA isolation with three different protocols. The three mini gels show the DNA isolation from first two dilutions of the standardized sourdough (lanes L1-L2). W: Wizard protocol; NS: Nucleo Spin Food protocol and K DNeasy Plant Mini Kit.

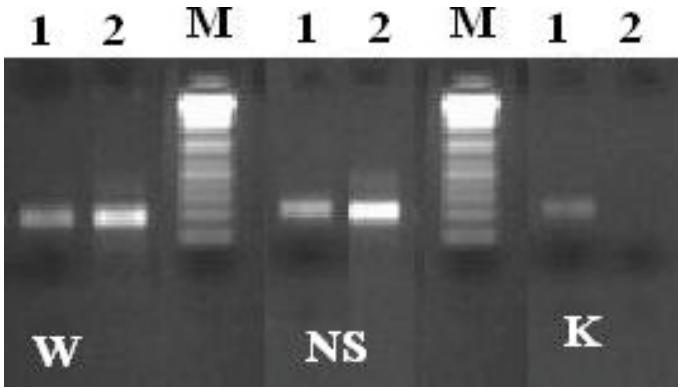

Figure 7. PCR amplification of V3 region of 16S rDNA of the first two dilutions (lanes 1-2) of standardized sourdough. W: Wizard protocol; NS: Nucleo Spin Food protocol and K DNeasy Plant Mini Kit. M: 1Kb ladder.

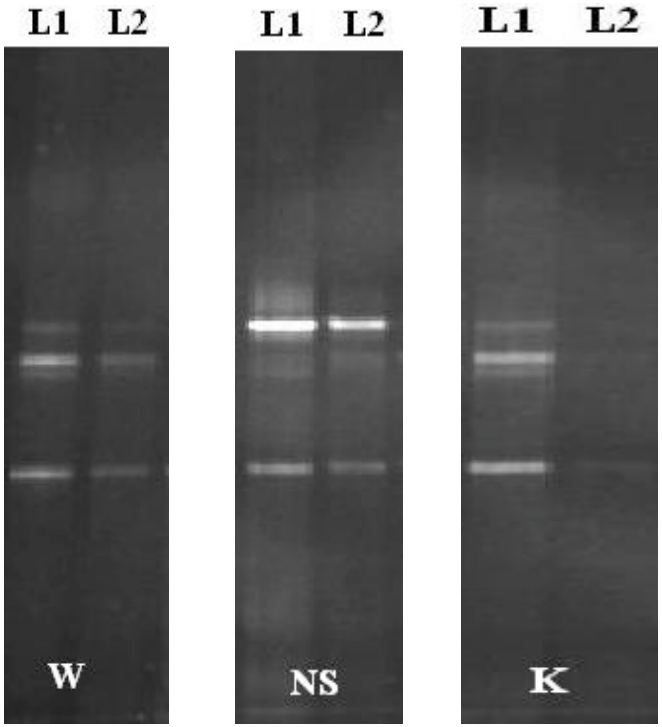

Figure 8. PCR-DGGE profiles from the first two dilutions of standardized sourdough. (lanes 1-2) of standardized dough. W: Wizard protocol; NS: Nucleo Spin Food protocol and K DNeasy Plant Mini Kit.

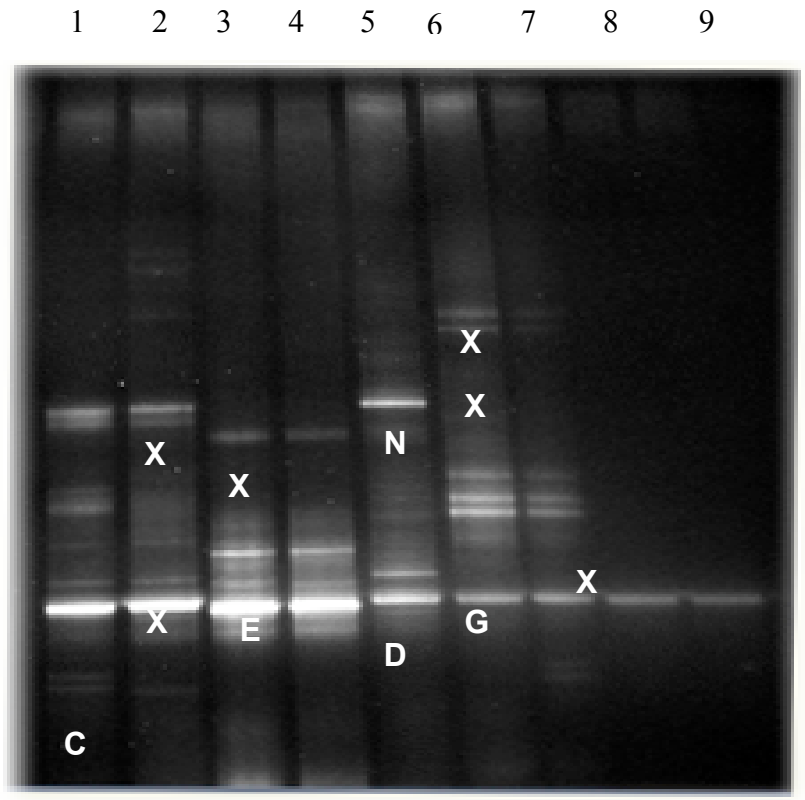

Figure 9. PCR-DGGE of 16S rDNA V3 region profiles from the 9 different sourdoughs obtained by Nucleo Spin Food protocol. Lanes 1-9: different sourdoughs. Lane 1:I1; lane 2: I2; lane 3: I3; lane 4: I4; lane 5: I4b; lane 6:I4c; lane 7: I5; lane 8: I6 and lane 9:I7. Each band was identified by a letter: C= *Triticum aestivum*; D= *Streptococcus thermophilus*; E= *Lactobacillus sanfranciscensis*; G= *Lactobacillus sakei*; I= *Triticum aestivum*; N= *Weissella cibaria*; X= not determinated.

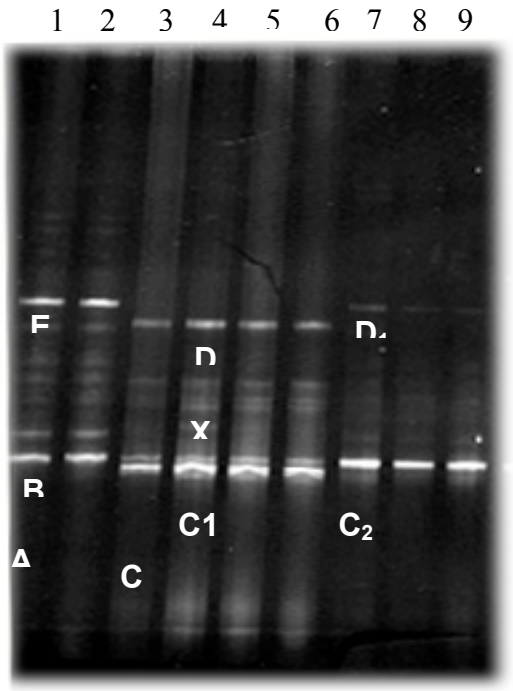

Figure 10. PCR-DGGE of 16S rDNA V3 region profiles from the 9 different sourdoughs obtained by Wizard protocol. Lanes 1-9: different sourdoughs. Lane 1:I4b (10^{-1}); lane 2: I4b (10^{-2}); lane 3: I4c (10^{-1}); lane 4: I4c (10^{-2}); lane 5: I5 (10^{-1}); lane 6:I5 (10^{-2}); lane 7: I6 (10^{-1}); lane 8: I6 (10^{-2}); and lane 9:I7. Each band was identified by a letter: A= *Hordeum vulgare subsp. vulgare* C= *Triticum aestivum*; C_1= *Lactobacillus sanfranciscensis*; C_2= *Hordeum vulgare subsp. vulgare*; D= *Lactobacillus sanfranciscensis*; D_1= *Lactobacillus sanfranciscensis*; E= *Weissella cibaria*;X= not determinated.

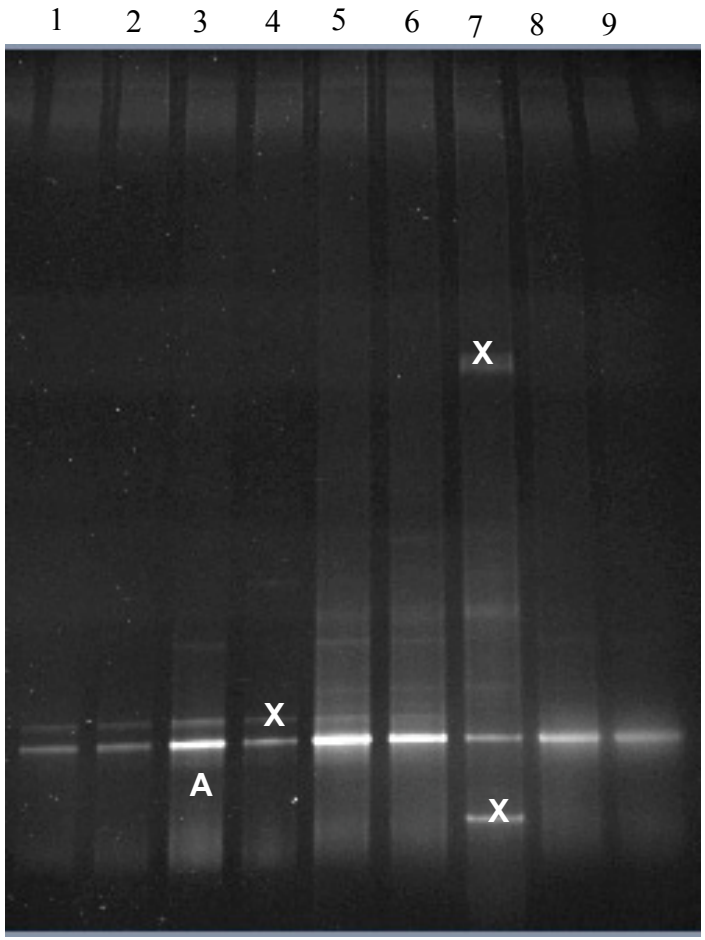

Figure 11. PCR-DGGE of yeast profiles from the 9 different sourdoughs obtained. Lines 1-9: different sourdoughs. Line 1:I1; lane 2: I2; lane 3: I3; lane 4: I4; lane 5: I4b; lane 6:I4c; lane 7: I5; lane 8: I6 and lane 9:I7. The band A was identified as *Saccharomyces cerevisiae*; X= not determinated.

A B

Figure 12. EPS production on modified Agar Chalmers with sucrose 5% (wt/vol) and without $CaCO_2$. A) *Leuconostoc lactis*/*Ln. garlicum* 68A12. B) *Lactobacillus curvatus* 69B2.

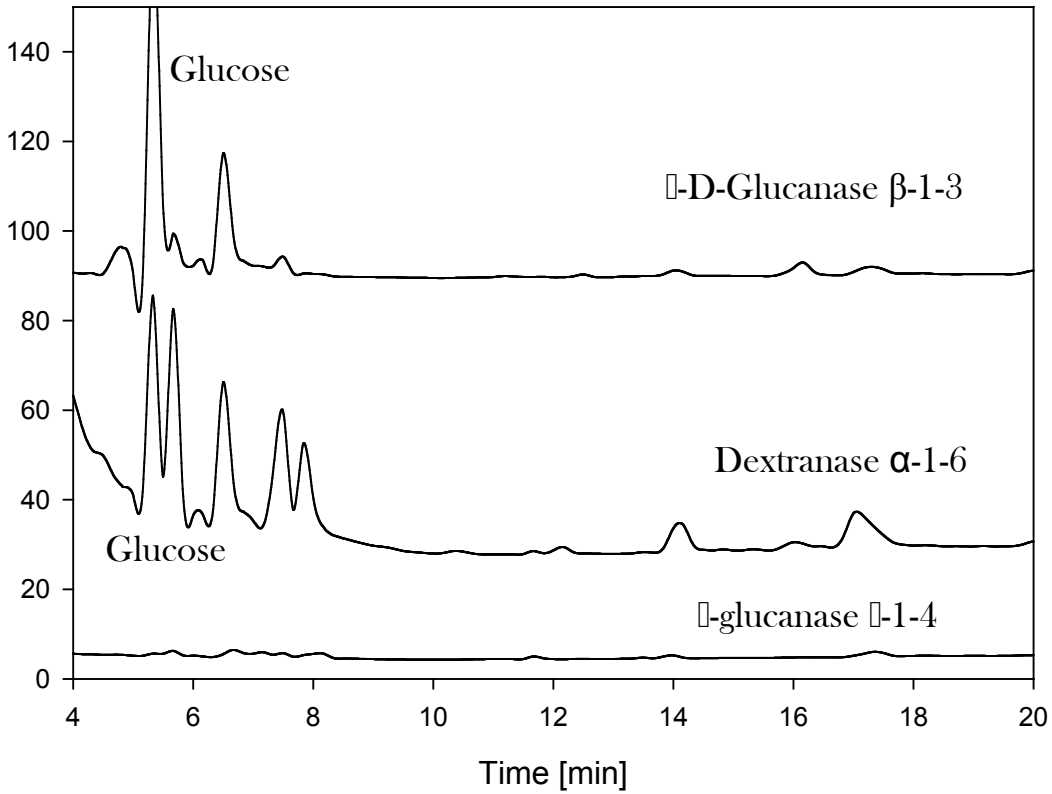

Figure 13. HPLC chromatograms from culture supernatants of *Lb. parabuchneri* FUA 3154.

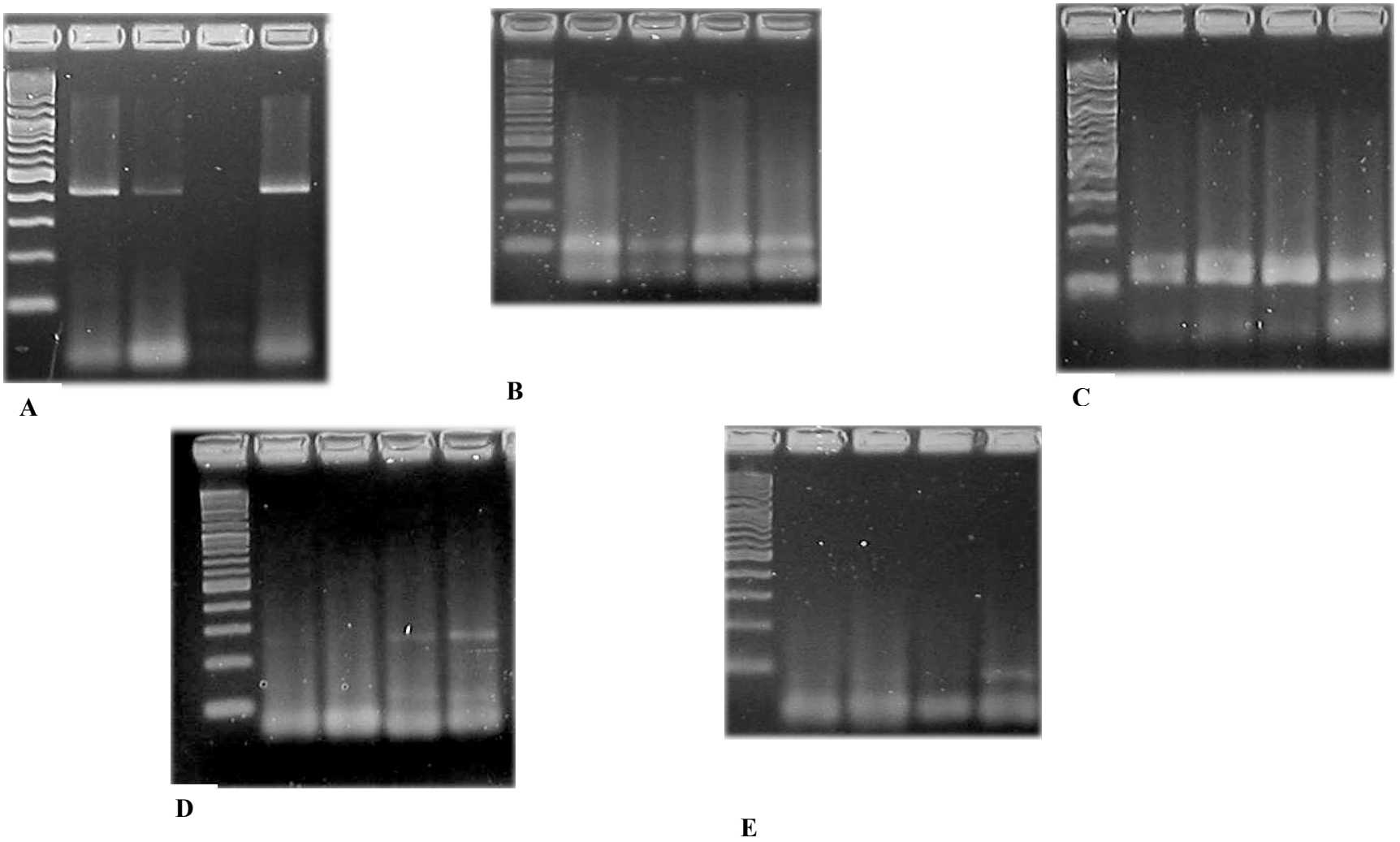

Figure 14. Results of RT-PCR of cDNA. A) Primers for β-glucan; B) Primers for housekeeping gene *dnaK*; C) Primers for housekeeping gene *recA*; D) Primers for *eps*D/E gene; E) Primers for *eps*E gene.