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Biotechnological characterization of lactic acid bacteria and yeast isolates from spontaneous sourdough for the development of bread with postbiotic-like potential

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Dedication

“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because the lead little by little to the truth.”

— Jules Verne

To my parents, sister, and girlfriend for being with me during this process, just as they have accompanied me in different situation throughout my life, always present and encouraging me to peruse my dreams and goals. Thank you for being an example of discipline, tenacity, perseverance, and commitment. I always learn from your example and experience, and I hope to one day be able to give you back all what you have given to me.

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ABSTRACT

Sourdough (SD) is defined as the fermentation of flour, water, and other ingredients by lactic acid bacteria (LAB) and yeasts, which can be grown spontaneously from the flour and the environment or be inoculated as starter microorganisms. This a well-known biotechnological process that has shown the capability to improve the sensory, rheological, and shelf-life properties of baked goods. SD is an ecosystem characterized by a higher cell count of LAB over yeasts in terms of microbiological composition. In the last years, the presence of postbiotic-like components has been associated with SD fermentation and SD bread. Postbiotics refer to a group of inanimate microorganisms and/or their components that confer health benefits to the host. Examples of postbiotic-like components are exopolysaccharides, short-chain fatty acids (SCFAs), bacteriocins, biosurfactants, amino acids, and cell surface proteins, among others. Based on the previous information, the objective of this work was to characterize the biotechnological properties of LAB and yeasts isolated from spontaneous wholewheat SD and raspberry to generate defined microbial starter cultures useful for the development of novel functional breads with enhanced technological, and postbiotic properties. By MALDI-TOF technique 25 LAB and yeast isolates were correctly identified. The main identified species were *Candida glabrata, Saccharomyces cerevisiae, Pediococcus pentosaceus, Companilactobacillus paralimentarius* and *Latilactobacillus curvatus*. Biotechnological and functional properties were assayed for the 25 isolated microorganisms and three commercial probiotic strains for

comparative purposes. LAB and yeast isolates showed the ability to release free amino acids by the action of proteolytic enzymes, and the ability to release phenolic compounds. Yeast isolates denoted a higher leavening capacity than LAB isolates, whereas LAB isolates presented a high phytase activity compared to yeast isolates. The results from a statistical analysis using Principal Component Analysis and Cluster Analysis and the microbiological performance allowed the selection of *P. pentosaceus* BIOTEC032*, C. glabrata* BIOTEC021*, S. cerevisiae* BIOTEC026 *and Hanseniaspora. opuntiae* BIOTEC045 to be used as starter cultures in the formulation of wheat breads. Eight types of wheat bread were elaborated using selected starter cultures (in combination or not with SD fermentation). Cell counts of LAB and yeasts, pH, TTA, biotechnological and postbiotics properties, physical characteristics and sensory evaluation were performed for the 8 bread treatments. The loaves of bread containing LAB in combination with SD, particularly Bac+Yeast SD and Bacteria SD, exhibited the best results for the postbiotic-like properties (total phenol content, phytase activity, antioxidant activity and presence of exopolysaccharides), and microbiological and acidification features. For the physical characteristics, the bread with a yeast combination (Yeast bread) had the softness property, whereas Bac+Yeast SD and Bacteria SD treatments were the hardest loaves. Finally, differences in color and sensory acceptance between the eighth treatments were not detected ($P < 0.05$). These results confirm that the use of defined consortiums of LAB and yeast as starter cultures for SD bread production enhances the nutritional, biotechnological and potential postbiotic properties of the baking breads.

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction

Cereals have represented a substantial source of macronutrients, micronutrients, and energy for humans since the being of agriculture. Fermentation with lactic acid bacteria (LAB) and yeasts represents the most popular way to improve the functionality, nutritional value, taste, appearance, and safety of cereal foods. Cereal fermentation contributes to the enrichment of the human diet by increasing the content of proteins, essential amino acids, and short-chain fatty acids (SCFAs); it additionally plays a vital role in aflatoxin removal and detoxification of cereals. Furthermore, the presence of LAB during fermentation leads to the production of larger quantities of vitamins (group B, folate, and vitamin K), free amino acids, and micronutrients in the final products. LAB and yeasts are often involved in the preparation of traditional cereal-based food worldwide, and one of the most important examples is the sourdough (SD) fermentation process to generate bread-making products (Petrova & Petrov, 2020).

The term SD bread refers to bread leavened with a SD starter, where SD is a mixture of flour and water fermented with LAB and yeasts, which can be grown spontaneously from the flour and the environment or be inoculated as selected starters. Spontaneous SD is the oldest-known bread-leavening agent, and it consists of dough left at room temperature for several hours, which is fermented by endogenous microorganisms. The addition of new flour and water to the dough, known as the backslopping technique, regenerates the ecosystem of LAB and yeasts to develop the typical sour taste. In terms of technological functionality, yeasts are responsible for carbon dioxide production, whereas LAB are mainly responsible for the production of lactic acid, acetic acid, or both, and the generation of aromatic precursors compounds (Luis & Moncayo, 2019).

Since the 1990’s, research on SD biotechnology has been increasing, mainly focusing on the technological effects of SD on flavor, rheology, and shelf life.

However, in recent years scientific research has also moved towards the functional and nutritional properties of SD fermentation, such as lowering the glycemic index of bread, reducing starch digestibility, and formation of organic acids and other bioactive molecules (Gobbetti et al., 2019). Furthermore, the degradation of phytic acid by phytase enzyme during SD fermentation has been the object of study in the last years because phytic acid degradation allows an increase in minerals, free amino acids, and protein bioavailability. Additionally, the presence of endogenous cereal proteases or exogenous proteases from LAB in SD mitigates the allergy and intolerance responses like celiac disease (Gobbetti et al., 2014). Nowadays, SD fermentation is studied for the potential health effects due to the presence of postbiotic-like components, which refer to a group of inanimate microorganisms and/or their components that confer health benefits to the host. Some postbiotic-like components reported in SD are non-viable microorganisms along with SCFAs, bacteriocins, biosurfactants, secreted proteins/peptides, amino acids, flavonoids, and exopolysaccharides, among other molecules (Salminen et al., 2021).

According to the UN Food and Agriculture Organization statistics, the global consumption of cereals and cereal-based foods will increase from 2.6 bln tons in 2017 to 2.9 bln tons in 2027 (Petrova & Petrov, 2020). In the case of Mexico, it is estimated that the annual consumption of bread per capita reaches 33.5 kg, and the market generates a profit of $41 thousand million pesos (*MarketDataMéxico*, 2020). Analyzing the Mexican consumer preferences, data from the National Camara of the Bakery Industry form 2020 pointed out that 70 to 75% of the sales correspond to white bread and the rest 30 to 25% to cookies, cakes, and sweet bread. Moreover, the new updated version of the NOM-051 for the labeling of foods represents a challenge for the bakery industry in developing new bread products with better nutritional profiles, since the Mexican market rarely offers baked products with high nutritional quality.

Based on the previously mentioned, there is an opportunity in the Mexican bakery industry to develop SD breads using LAB and yeast consortiums as starter cultures and enhancing the nutritional and health properties of the final products.

1.2. Hypothesis

The biotechnological characterization of LAB and yeasts isolated from spontaneous wholewheat SD allows the design of microbial consortiums to be used as starter cultures in the elaboration of functional wheat breads with enhanced technological and potential health properties.

1.3. General Objective

Characterize the biotechnological properties of LAB and yeasts isolated from spontaneous wholewheat SD to generate defined microbial starter cultures useful for developing novel functional breads with enhanced technological, and postbiotic properties.

1.4. Specific Objectives

The specific objectives for the present thesis were the following:

1) Isolate and identify LAB and yeasts from a spontaneous wholewheat using MALDI-TOF mass spectrometry.
2) Evaluate the biotechnological and functional properties such as leavening capacity, phytase activity, proteolytic activities, and total phenol content, among others, in LAB and yeast isolates.
3) Define microbial consortiums of LAB and yeasts based on their biotechnological and functional properties using a Principal Component Analysis and Cluster analysis, and validate the performance of the best candidates in bread doughs.
4) Develop functional wheat breads through the combined use of a defined microbial consortium and the SD fermentation technique, and evaluate their microbiological and acidification features, postbiotic properties (total phenol content, phytase activity, antioxidant activity and presence of exopolysaccharides), physical characteristics (specific volume, colorimetry, and texture profile analysis) and sensory acceptance.

1.5. Thesis Structure

The present thesis is composed of 5 chapters. Chapter 1 consists of a general introduction to the topic addressed in this thesis. Chapter 2 reviews the role of LAB and yeast in SD fermentation and their evaluation of the postbiotic-like components generated during SD and the potential health benefits. Chapter 3 addresses the isolation and identification of LAB and yeasts from a wholewheat SD and the evaluation of the biotechnological properties of the isolated microorganisms. Furthermore, a Principal Component Analysis and Cluster Analysis are performed to create microbial consortiums. Chapter 4 presents the design of microbial consortiums based on the results of PCA and Cluster Analysis to formulate eight types of wheat bread, and evaluate their biotechnological and postbiotic properties, physical characteristics, and sensory analysis. Finally, based on the results from this thesis, Chapter 5 summarizes a series of general conclusions and recommendations for future studies.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Sourdough (SD) fermentation is a well-known biotechnological process that has been in use for 5,000 years and has shown the ability to improve the sensory, rheological, and shelf-life properties of baked goods. This biotechnology process encompasses a great variety of lactic acid bacteria (LAB) and yeast interactions (Poutanen et al., 2009). SD is the result of fermentation of a mixture of flour, water, and other ingredients by LAB and yeasts naturally occurring in the flour that propagate during backslopping (the traditional process in which a new mixture of flour and water is fermented by using as a starter the SD from a previous fermentation batch) (Rizzello et al., 2019). From a microbiological point of view, SD is an ecosystem characterized by an environment with a low pH, high carbohydrate concentration, oxygen limitation, and a LAB cell count exceeding that of yeasts (De Vuyst et al., 2014). LAB dominate the mature SD, while the yeast content is one/two logarithmic cycles lower (Rizzello et al., 2019). The major metabolic activities of the SD microbiota are acidification (LAB), flavor formation (LAB and yeasts), and leavening (yeasts and heterofermentative LAB species) (De Vuyst et al., 2014). Even though LAB and yeasts originate principally from the flour and environmental microbiota, the process of microbiota maturation during SD fermentation depends on various factors such as temperature, the chemical and enzymatic composition of the flour, redox potential, water content, and time (Rizzello et al., 2019).

Nowadays, the culture-based techniques used to characterize SD microbial diversity across studies investigation the distribution of SD bacterial and fungal taxa are variable and biased (De Vuyst et al., 2014; Landis et al., 2021; Michel et al., 2019). SD starters are maintained in many households, but these differ from those in bakeries due to heterogeneity among environments, production practices, and ingredients. Geographic location and maintenance practices are the main factors in SD biodiversity. Different SDs from the same region may be similar in composition due to the response to regional microclimates or the restricted dispersion of microbes (Landis et al., 2021). Bread producers often attribute distinct regional

properties to their breads, giving credit to the environment for their unique characteristics (Landis et al., 2021). Some of the microorganisms identified from SD microbiota characterization studies are shown in Table 1, differentiated by LAB (homofermentative, obligate heterofermentative, and facultative heterofermentative) and yeasts (Chavan & Chavan, 2011; De Vuyst et al., 2017; De Vuyst & Neysens, 2005; Gänzle & Gobbetti, 2013; Luis & Moncayo, 2019; Papadimitriou et al., 2019).

Fermented foods and functional ingredients (such as probiotics, prebiotics, and synbiotics) can be used as dietary interventions seeking health benefits. Probiotics are live microorganisms that confer a health benefit when administered in adequate amounts. Probiotic products may also deliver significant amounts of non-viable cells due to cell death during storage (Salminen et al., 2021). The interest in the potential effect of non-viable microorganisms and their components on health is rising. Fermented foods can contain numerous non-viable cells, especially after prolonged storage, thermal treatments, or processes (such as pasteurization or baking). Fermentation mediated by LAB produces different cellular structures and metabolites, such as cell surface components, lactic acid, short-chain fatty acids (SCFAs), and bioactive peptides, among other effector molecules associated with benefits to human health (Salminen et al., 2021).

Table 1. Lactic acid bacteria (LAB) and yeasts found in different types of sourdough.

Homofermentative LAB	Obligate heterofermentative LAB	Facultative heterofermentative LAB	Yeasts
Enterococcus casseliflavus	*Levilactobacillus acidifarinae*	*Companilactobacillus alimentarius*	*Saccharomyces cerevisiae*
Enterococcus durans	*Levilactobacillus brevis*	*Companilactobacillus paralimentarius*	*Kazachstania exigua*
Enterococcus faecalis	*Limosilactobacillus fermentum*	*Lactiplantibacillus plantarum*	*Kazachstania humilis*
Enterococcus faecium	*Limosilactobacillus reuteri*	*Lacticaseibacillus casei*	*Pichia kudriavzevii*
Lactobacillus amylovorus	*Limosilactobacillus pontis*	*Lacticaseibacillus paracasei*	*Torulaspora delbrueckii*
Lactobacillus amylolyticus	*Furfurilactobacillus rossiae*	*Lacticaseibacillus rhamnosus*	*Wickerhamomyces anomalus*
Lactobacillus delbrueckii	*Limosilactobacillus panis*	*Levilactobacillus spicheri*	*Pichia kudriavzevii*
Lactobacillus acidophilus	*Companilactobacillus crustorum*	*Lactiplantibacillus xiangfangensis*	
Companilactobacillus farciminis	*Latilactobacillus curvatus*	*Limosilactobacillus coleohominis*	
Lactobacillus johnsonii	*Limosilactobacillus frumenti*	*Companilactobacillus kimchii*	
Companilactobacillus crustorum	*Fructilactobacillus fructivorans*	*Lactiplantibacillus pentosus*	
Companilactobacillus heilongjiangensis	*Levilactobacillus hammesii*	*Schleiferilactobacillus perolens*	
Companilactobacillus mindensis	*Levilactobacillus koreensis*	*Latilactobacillus sakei*	
Companilactobacillus nantensis	*Levilactobacillus namurensis*	*Pediococcus acidilactici*	
Companilactobacillus nodensis	*Companilactobacillus nodensis*	*Lapidilactobacillus dextrinicus*	
Lactobacillus crispatus	*Limosilactobacillus oris*	*Pediococcus pentosaceus*	
Lactobacillus gallinarum	*Lentilactobacillus parabuchneri*		
Lactobacillus gasseri	*Fructilactobacillus sanfranciscensis*		
Lactobacillus helveticus	*Limosilactobacillus secaliphilus*		
Liquorilactobacillus nagelii	*Furfurilactobacillus siliginis*		
Ligilactobacillus salivarius	*Lentilactobacillus buchneri*		
Streptococcus constellatus	*Fructilactobacillus fructivorans*		
Streptococcus equinus	*Lentilactobacillus hilgardii*		
Streptococcus suis	*Fructilactobacillus fructivorans*		
	Lentilactobacillus kefiri		
	Apilactobacillus kunkeei		
	Fructilactobacillus lindneri		
	Limosilactobacillus mucosae		
	Limosilactobacillus fermentum		
	Secundilactobacillus collinoides		
	Limosilactobacillus vaginalis		
	Levilactobacillus zymae		
	Leuconostoc citreum		
	Leuconostoc gelidum		
	Leuconostoc mesenteroides		
	Weissella cibaria		
	Weissella confusa		
	Weissella hellenica		
	Weissella kandleri		

New terms have been used to name these non-viable microbial cells and metabolites in recent years, including paraprobiotics, parapsychobiotics, ghost probiotics, metabiotics, tyndallized probiotics, and bacterial lysates. However, the concept of postbiotics to promote health is emerging as an important microorganism-derived tool (Salminen et al., 2021). The term postbiotic can be considered as a composite of biotic, defined as “relating to or resulting from living organisms,” and post, which refers to “after life,” implying that postbiotics are non-living microorganisms. Thus, postbiotics are defined as a preparation of inanimate microorganisms and/or their components that provides benefits to the host’s health (Salminen et al., 2021). The definition of postbiotics requires identification to the strain level of the microbe/s used for their preparation, a deliberate termination of cell viability step, and the demonstration of a health benefit in a well-designed and conducted efficacy trial in the target host (Salminen et al., 2021). Since SD fermentation is performed by microbes that occur naturally in the flours used to prepare the dough, and even though the baking step represents a deliberate termination of cell viability, the presence of a consortia of unidentified microbes prevents SD from being regarded as a postbiotic. Therefore, the term “postbiotic-like” will be used in this review.

The advantage of postbiotic over probiotic microorganisms is that they present little or no interaction with the different compounds in the food matrix, which increases shelf-life and maintains the same sensory and physicochemical properties. Other advantages are that they remain stable over a wide range of pH and temperature, which allows ingredients with higher acidity to be added and treated by thermal processing in a way that their functionality is not compromised, minimizing the chances of microbial contamination after packaging and during storage (Nataraj et al., 2020). Currently, researchers are particularly focused on the discovery and characterization of new LAB strains able to biosynthesize active compounds such as exopolysaccharides (EPS), antimicrobial compounds, bioactive peptides, and SCFAs to exploit their functional properties in food (Păcularu-Burada et al., 2020a). Moreover, the selection of LAB and yeast strains (Table 1) to further design starter cultures must also consider important features, such as the

preservation technologies and overall nutritional-functional aspects of the final fermented products. This review focuses on the roles of LAB and yeasts in SD in the production of baked goods with enhanced properties and the presence of postbiotic-like components (SCFAs, EPS, biosurfactants, cell surface proteins, cell supernatants, organic acids, etc.). The role of LAB in the formation of postbiotic-like components during SD fermentation and baking, as well as the health benefits of SD bread, are also explored.

2.2. Role of sourdough fermentation in the production of baked goods

Bread is one of the most important staple foods consumed in the world (Koistinen et al., 2018). Its recipe comprises cereal flour and can include pseudocereals and/or legumes, water, salt, other minor ingredients, and a leavening agent (Rizzello et al., 2019). Cereals and legumes are valuable sources of proteins, fats, and dietary fiber. Through lactic acid fermentation, the properties of these ingredients can be improved, which also enhances the sensory characteristics of the final products. In recent years, the use of SD has become increasingly standardized, and the interaction of the microbial cultures has been studied with the aim of employing fermentation in baking for leavening, flavor formation, and improving stability (Gobbetti et al., 2019). Moreover, SD fermented with LAB is a source of proteolytic enzymes, activated by acid production, that are likely to eliminate gluten toxicity during bread making (Fekri et al., 2020). Furthermore, the phytic acid and other antinutritional factors from cereals and legumes are reduced by specific enzymes produced during fermentation, resulting in higher bioavailability of important minerals in baked goods (Fekri et al., 2020).

Nowadays, four types of SD can be made depending on the inocula and the final desired properties of baked goods, such as flavor, texture, smell, stability, and nutritional properties: types I, II, III, and IV (Fig. 1) (Galli et al., 2019).

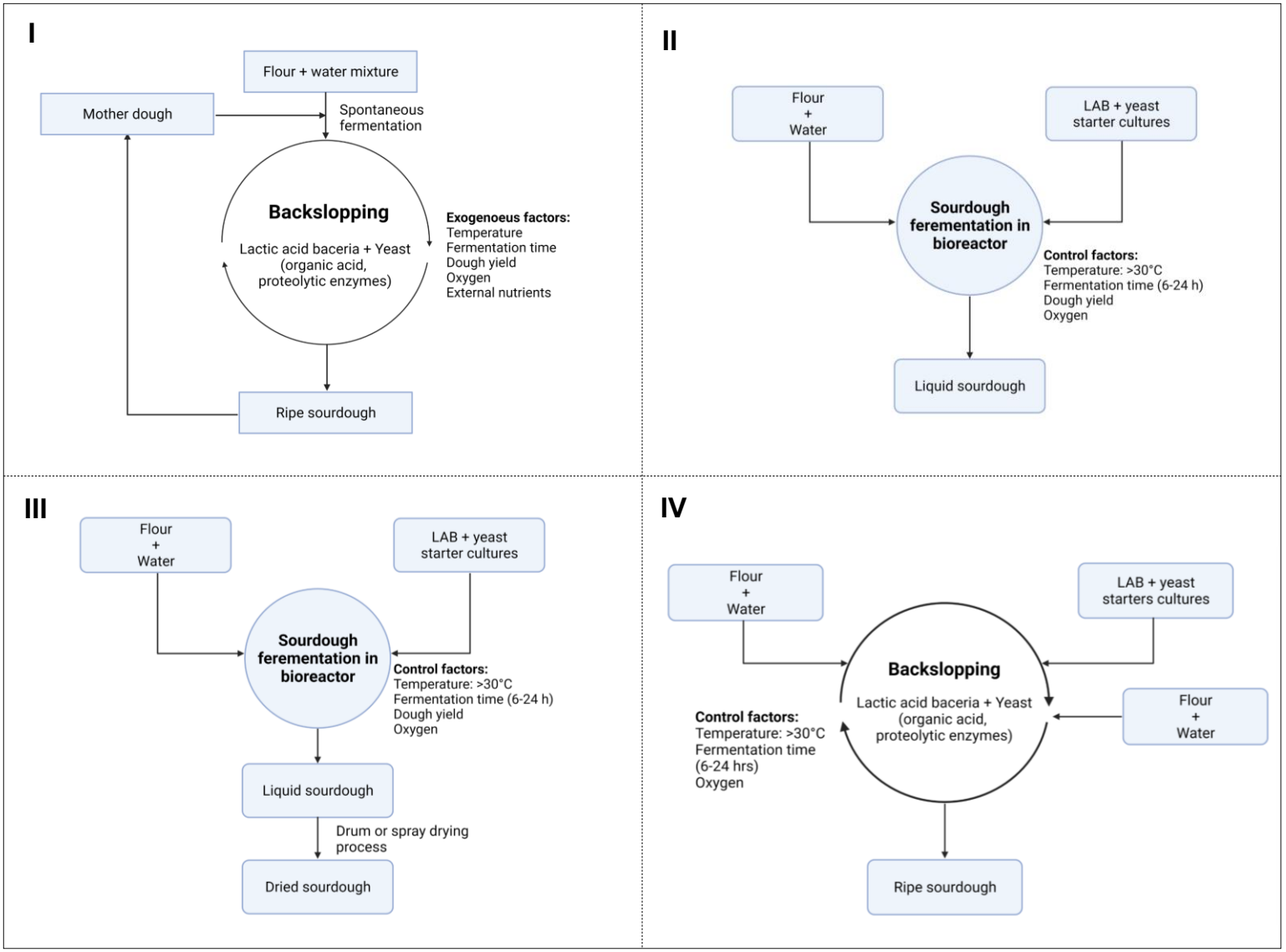

Figure 1. Types of sourdough processes depending on the inocula, and the final properties desired for breadmaking.

The type I or traditional SD process depends on the backslopping technique at a low incubation temperature (20–24 °C), which relies on a repeated cyclic of re-inoculation (6–24 h) with a new batch of flour and water from a previous one derived from a mother dough (Fig. 1, I). This type of SD is a pure craft, and the dough can be maintained for years. In terms of microbiology, type I SD harbors mixtures of distinctive yeast and LAB species or strains, representing a large diversity of natural SD starters. Backslopping results in the prevalence of the species/strains best adapted to the SD ecosystem. The type of flour used and its enzymatic, microbiological, nutritional, and textural qualities are of the utmost importance since these factors will determine the stability of the mature doughs (De Vuyst et al., 2014). The main drawback of the type I process is that it cannot be scaled up for industrial exploitation; moreover, it is time-consuming, requires trained and qualified staff, and is not fully controllable (Galli et al., 2019).

In the case of the type II process, doughs are characterized by fermentation with specific LAB strains (Fig. 1, II). Type II SD is generally not suitable for dough leavening but is used for dough acidification and as a flavor enhancer. This process is shorter than that of type I, with a unique fermentation step of 15–20 h, followed by a storage period of many days. The SD is generally liquid, and fermentation occurs in bioreactors or tanks. Thus, type II SD can be scaled to an industrial level (Corsetti, 2013). In this case, defined acid-tolerant LAB starter cultures are used (e.g., strains of Levilactobacillus brevis, Limosilactobacillus fermentum, Lactiplantibacillus plantarum, Limosilactobacillus reuteri, or Fructilactobacillus sanfranciscensis), accompanied or not by yeasts, most commonly Saccharomyces cerevisiae, which is often added to the final stage but also can be added along with the LAB. Most strains are selected on the basis of their potential to quickly cause acidification of the dough and/or generate specific flavor compounds. These two properties offer a clear added value, which is reflected in the commercialization of dried SD powders used as a flavor of functional ingredients in bread production (De Vuyst et al., 2017).

Type III SD is remarkably similar to type II, with the difference being in the dehydration or pasteurization of the liquid-stabilized SD (Fig. 1, III). Various

dehydration techniques can be applied, with drum and spray drying being the most common ones. The starter cultures are selected on the basis of their high acidification capacity, ability to produce flavor compounds, and resistance to the drying procedure. The most common species include heterofermentative L. brevis and facultative heterofermentative Pediococcus pentosaceus and L. plantarum strains (Papadimitriou et al., 2019). Type III SD presents several advantages, such as a long shelf life, a smaller volume, and ease of handling for transportation and storage, which makes it more convenient for industrial bakeries, and finally, the production of standardized end-products. Generally, type III SD is used as an acidifier or as a bread improver (Corsetti, 2013).

Type IV SD is initiated with a LAB starter culture, followed by traditional backslopping as in type I SD (Fig. 1, IV) (Luis & Moncayo, 2019). This starter culture-backslopped SD approach is equally characterized by a pattern of three-step succession of LAB communities. However, competition frequently occurs between the added LAB starter culture and the spontaneously growing microorganisms. Differences in stress tolerance may lead to predominance of the autochthonous LAB species and elimination of the LAB starter culture. This may occur because the starter culture is not well adapted to the SD ecosystem and the prevailing conditions (De Vuyst et al., 2017).

Type II and III SD can simplify the production process, but they do not guarantee the same distinctive properties as type I SD; nevertheless, they contain only LAB and require the addition of bakery yeasts as a second step. Recently, liquid SD has been introduced in bakeries as a new technology trend. This type of SD allows the addition of both LAB and yeasts in a single step and thus meets the industrial demand for a more controllable, large-scale SD process (Galli et al., 2019).

2.3. Role of LAB in sourdough fermentation

Sourdough is a very complex ecosystem, where heterofermentative LAB are the dominant organisms and co-exist mostly synergistically with yeasts, which are well adapted to the prevailing acidic environment and can grow to high

concentrations (107 colony forming units (CFU)/g), albeit lower than those of LAB (108 CFU/g) (De Vuyst et al., 2014). More than 90 different LAB species have already been isolated from SD, including obligately and facultatively heterofermentative species and some obligately homofermentative species, as shown in Table 1.

The fermentation process generates mainly acids, alcohols, aldehydes, esters, and ketones; it is the primary route of volatile compound formation in SD and bread crumb (Limbad et al., 2020). The contribution of LAB to the flavor of SD bread is associated with the production of lactic acid (fresh acidity) and acetic acid (sharp acidity), amino acid accumulation (e.g., accumulation of glutamate, which is responsible for the umami taste), generation of 2-acetyl-1-pyrroline as an end metabolite that is responsible for the aroma of the crust and formed through the Maillard reaction of ornithine in the arginine deiminase (ADI) pathway (a pathway of arginine degradation associated with enhanced tolerance to acid environmental stress and with increased ornithine production, which improves the organoleptic characteristics of SD), and/or active peptides generated by glutathione accumulation and glutamyl dipeptide formation, responsible for the kokumi taste (De Vuyst et al., 2017). The conversion of amino acids such as phenylalanine (sweet), isoleucine (acidic), glycine, serine, and alanine (vinegar/sour) to aldehydes and ketones can form additional flavor compounds (Limbad et al., 2020).

Regarding the metabolic pathways of LAB, the ones that influence bread quality are linked to central carbon flux and limited by cofactor availability, which affects the redox potential of the environment and inside the cell. Homo- and heterofermentative LAB differ fundamentally in the reduced cofactors they regenerate, such as nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). Furthermore, the use of co-substrates, such as oxygen or fructose, as electron acceptors by obligate heterofermentative LAB is coupled with an increase in acetate production in doughs. Thus, the different metabolic requirements of homo- and heterofermentative LAB produce other effects

on the redox reactions in SD that influence the quality of the final bread beyond the formation of acetate (Chavan & Chavan, 2011).

The available carbohydrates in wheat flour are maltose, followed by sucrose, glucose, and fructose, along with some trisaccharides such as maltotriose and raffinose. The glucose concentration increases during fermentation because other complex carbohydrates are metabolized by LAB and yeasts; however, yeasts cannot ferment the disaccharide maltose, which is instead fermented by LAB (Chavan & Chavan, 2011). Starting from glucose, homofermentative LAB produce lactic acid through glycolysis, while heterofermentative LAB generate, besides lactic acid, CO2, acetic acid, and/or ethanol (De Vuyst et al., 2017). Moreover, carbohydrate metabolism leads to the development of antimicrobials, flavor compounds, and EPS.

On the other hand, the generation of oligopeptides and amino acids is made possible by endogenous flour proteases that become activated at low pH and reduce the gluten disulfide bonds caused by LAB acidification and glutathione reductase activity (De Vuyst et al., 2017). As described earlier, amino acid conversion contributes to the acid stress response, redox balancing, and flavor formation. Protein acid accumulation due to peptide hydrolysis stimulates SD flavor formation and nutrient enrichment (Gobbetti et al., 2014). Phenolic compounds and lipids are only minor compounds in cereal flours. Nevertheless, some LAB species catalyze the release of bound phenolic compounds through feruloyl esterase, tannase (tannin acyl hydrolase), and glycosyl hydrolases, which hydrolyze esters of ferulic acid, galloyl esters of gallotannins, and flavonoid hexoxides, respectively. Phenolic compounds and flavonoids can be further converted into flavor precursors; these conversions are grounded in specific phenolic acid decarboxylases and cinnamic acid reductases of LAB that are associated with cereal flours (De Vuyst et al., 2017). Sorghum and millet flours contain a high level of polyphenols. Their use is suggested for the metabolism of phenolic compounds by microorganisms, specifically by LAB species capable of degrading these compounds, such as *L. brevis, L. helveticus, L. plantarum,* and *P. pentosaceus* (Gänzle, 2014). Regarding fatty acids, fatty acid hydratases can transform oleic acid and linoleic acid into hydroxy fatty acid chains

(Gänzle, 2014), whereas phytase hydrolysis, which releases inorganic phosphate and makes minerals bioavailable during the SD process, is primarily dependent on endogenous cereal phytases that become activated by acidification; LAB of the SD microbiota have also been shown to possess phytase activity (De Vuyst et al., 2017).

2.4. Role of yeasts in sourdough fermentation

Yeasts, by producing CO_2, act as the primary leavening agents in bread products. Baker's yeasts exhibit good fermentative metabolism and are resistant to many stress factors that are present during breadmaking. The properties of these microorganisms, mainly *S. cerevisiae,* have significant economic and technological value (Chiva et al., 2021). The stability of the SD process depends on cooperation between certain species of LAB and yeasts (Palla et al., 2020). The use of baker's yeast as a leavening agent has been developed as an alternative for elaborating SD in industrial bread production, where time and speed of production are important factors. Nowadays, baker's yeast is sold as various product types with improved shelf-life, osmotolerant properties, retention of activity at low temperatures, and flavor generation. The presentation of yeasts includes liquid cream or small granules, compressed as blocks, and dried yeasts that may be dry active or dry frozen (De Vuyst et al., 2017). The yeast variability in SD is affected by dough hydration, type of cereal, and leavening temperature, among others (Chavan & Chavan, 2011; Urien et al., 2019).

The carbohydrates present in flour are fermented via glycolysis and further pyruvate breakdown to generate CO_2 and ethanol. Ethanol also impacts the dough properties, strengthening the gluten network, but a significant proportion evaporates during baking (Rezaei et al., 2015). Both glycerol and succinate are SD osmoprotectants, reducing pH and influencing dough rheology by improving gas retention and gluten formation (De Vuyst et al., 2021). Besides their leavening ability and influence on the strength of the gluten network, yeasts contribute largely to flavor development in bread. This depends not only on one yeast strain but also on symbiosis with the LAB strains present in the matrix (De Vuyst et al., 2017). Yeasts

can, for instance, generate flavor metabolites via the Ehrlich pathway by converting branched-chain amino acids into higher alcohols and their esters (Pico et al., 2015). Additionally, yeasts produce low levels of organic acids, such as acetic and succinic acids, that contribute to slight acidification of the leavened dough and affect the final flavor (Jayaram et al., 2013). Furthermore, yeasts contribute to antioxidant activity based on the availability of phenols in the flour cereal (Wang et al., 2014). The dephosphorylation of phytate via the action of phytases and the antifungal activities of yeast due to the generation of ethyl acetate lead to the release of phenolic compounds (De Vuyst & Neysens, 2005).

2.5. Presence of postbiotic-like components in sourdough fermentation

In the fermentation process of SD, the microorganisms involved produce a significant quantity of cells and metabolites, which can be in the intracellular or extracellular matrix, and such microorganisms can potentially benefit the consumer, even when present in their non-viable form after baking (Barros et al., 2020). Furthermore, during the baking process, the cell lysis of microorganisms delivers cellular debris that may also have beneficial properties (Sadeghi et al., 2019). Besides whole non-viable microbes, among the examples of postbiotic-like compounds present in SD are SCFAs, secreted proteins/peptides, bacteriocins, secreted biosurfactants, amino acids, flavonoids, EPSs, vitamins, organic acids, and other widely diverse molecules. Other potential postbiotics are residues of the cell debris such as peptidoglycan-derived muropeptides, surface-protruding molecules (pili, fimbriae, flagella), cell surface-associated proteins, cell wall-bound biosurfactants, and cell supernatants (Nataraj et al., 2020). Table 2 presents the main postbiotic components that may occur in foods and the potential health benefits in the host.

Table 2. Health benefits and characteristics of the main postbiotic components.

Postbiotic	Main description	Health benefits in the host	Reference
Biosurfactants	Molecules synthesized during the late log or early stationary phase of the growth cycle. Amphiphilic molecules that are composed of glycolipids, lipopeptides, phospholipids, neutral lipids, polysaccharide-protein complexes, and free fatty acids.	Disruption and prevention of biofilm formation by pathogenic microorganisms. Wetting, foaming, and emulsification properties, that hurdle the pathogen to adhere, establish, and subsequently to communicate in the biofilms.	(Nataraj et al., 2020)
Exopolysaccharides	Extracellular biopolymers synthesized or secreted by microorganisms during the exponential phase. Based on the monosaccharide composition, exopolysaccharides are further classified into homopolysaccharides and heteropolysaccharides.	Biofunctional attributes such as antioxidant, cholesterol-lowering, immunomodulatory effect, anti-aging, gut microbiota modulation, anti-toxic effect, anti-biofilm, and antitumoral at preclinical trials	(Lynch et al., 2018; Nataraj et al., 2020)
Short chain fatty acids	Fatty acids with fewer than six carbon atoms in their chains. The most common are acetate, propionate, formate, and butyrate. LAB synthesize SCFAs from non-digestible carbohydrates. Also, bifidobacteria can synthesize short chain fatty acids, for example acetate and formate.	Management of inflammatory bowel disease and colorectal cancer due to their potentiality to overcome the inflammation and proliferation of cancerous cells.	(Gill et al., 2018; Nataraj et al., 2020)
Teichoic acids	These are amnionic glycopolymers that play key roles in determining the cell shape, regulation of cell division, and other fundamental metabolic aspects of cell physiology. Teichoic acids are generally of two kinds: lipoteichoic acids and wall teichoic acids.	Antibiofilm actions against oral and enteric pathogens, immunomodulatory potential, and decreased leaky gut and inflammation.	(Barros et al., 2020; Nataraj et al., 2020)
Bacteriocins	LAB produce an array of extracellular antimicrobials that inhibit both pathogenic and spoilage causing microorganisms.	Inhibitory potential against various urogenital and antibiotic-resistant pathogens.	(Bartkiene et al., 2020; Nataraj et al., 2020)
Cell-free supernatant	Cell-free supernatant of LAB is a consortium of low molecular weight (hydrogen peroxide, organic acids, carbon dioxide, and di-acetylene) and high molecular weight (bacteriocins) compounds.	Bioliquid-detergent that reduces the adhesion and biofilm formation of pathogens to the various surfaces.	(de Almada et al., 2016; Nataraj et al., 2020)
Peptidoglycan	Peptidoglycan is a linear glycan strand cross-linked by peptides. The strands are constructed by bonding N-acetylglucosamine and N-acetylmuramic acid.	Immunomodulatory, anti-proliferative and anti-tumor effects.	(Nataraj et al., 2020)
Cell surface proteins	Proteins that are found in the plasma membrane or in the cell wall, they can be classified in four categories: proteins anchored to the cytoplasmic membrane, lipoproteins, proteins containing C-terminal motif and non-covalently proteins associated with the cell wall.	Immunomodulatory action, secretion of antibacterial peptides, anti-inflammatory effect, anti-adhesion effect, strengthening the epithelial barrier property, and biosorption of toxic heavy metals.	(Engineering et al., 2014; Nataraj et al., 2020)

The formation of postbiotic-like compounds during SD fermentation has mostly been described for LAB. Many metabolic pathways are activated during fermentation to produce bioactive substances that inhibit pathogenic bacterial growth and prevent bacterial toxin formation. Moreover, LAB produce low-molecular-weight organic components from the metabolism of carbohydrates, amino acids, vitamins, organic acids, and fatty acids. The LAB strain diversity and metabolic capability of generating a variety of bioactive compounds depend on many fermentation parameters, such as pH, time, and temperature. In the case of pH, many differences between SD with a pH of 4.0 and those with a lower pH have been described. When the pH is maintained at 4.0, the presence of lactic acid isomers and ethanol increases the metabolic activity of different LAB and the production of postbiotic-like compounds. By contrast, lower pH values lead to the accumulation of flavor-enhancing volatile compounds. Therefore, optimization of SD pH is a critical determinant of the type of dough obtained, depending on whether the aim is to produce a certain flavor or desired metabolites (Păcularu-Burada et al., 2020a).

Baking is carried out at a temperature up to 200–250 °C and for an average duration of 1 hour (Petrova & Petrov, 2020). Thermal treatment for the generation of postbiotic-like components may be influenced by the type of microorganisms, growth stage, prior exposure to stress, pH value, water activity, and heating mode (conduction, convection, and/or radiation), among other factors. Regarding postbiotics, thermal treatments have been reported to increase cell coarseness and roughness, influencing the immune-modulating properties. The higher the temperature applied, the greater the roughness and degree of coarseness of the cell surface compared to that of viable cells. Furthermore, adhesion is another property affected by the temperature; when the temperature is higher, the adhesion capacity is reduced. These findings are relevant to the development of processes for postbiotic generation because they indicate that each potential microorganism demands a different temperature and time for inactivation (de Almada et al., 2016).

Several studies have reported the possible postbiotic-like components in foods (Table 2); however, very few have detailed the functionality and health benefits

in the context of SD. Therefore, we will focus on specific compounds such as EPSs, antimicrobial molecules, and fatty acids. The most relevant studies addressing this topic are shown in Table 3.

Table 3. Compounds synthetized during sourdough fermentation and in the final bread.

Compounds	Findings	Species	Source	Reference
Exopolysaccharides	Maximum value of exopolysaccharides synthesized in sourdough with approximately 22 g/L.	*Lactobacillus* spp. *Leuconostoc* spp.	Whole wheat flour Sesame seeds	(Păcularu-Burada et al., 2020a)
Antibacterial compounds	Inhibition properties of LAB species against the fifteen pathogenic and opportunistic bacterial strains tested through diameter inhibition zones.	*Lactiplantibacillus plantarum* *Lacticaseibacillus casei* *Latilactobacillus curvatus* *Lacticaseibacillus paracasei* *Loigolactobacillus coryniformis*	Rye wheat flour	(Bartkiene et al., 2020)
Antifungal compounds	Most of the isolated sourdough LAB displayed antifungal activities against seven selected mold strains.	*Lactobacillus* spp. *Leuconostoc* spp. *Enteroccocus* spp. *Pediococcus* spp.	Rye wheat flour	(Bartkiene et al., 2020)
Acidification capacity	Strains of *Lactobacillus* spp. showed the best acidification capacity.	*Lactobacillus* spp.	Whole wheat flour	(Păcularu-Burada et al., 2020b)
Bacteriocins	Five strains were found to produce distinct bacteriocin-like inhibitory substances, but *L. lactis* showed a better inhibitory range.	*Lactiplantibacillus pentosus* *Lactiplantibacillus plantarum*	Rye wheat flour	(Gomaa, 2013)
Fatty acids	*L. hammesii* converts linoleic acid in sourdough and the resulting monohydroxy octadecenoic acid exerts antifungal activity in bread.	*Levilactobacillus hammesii*	Wheat flour	(Black et al., 2013)
Biosurfactants	Lipopeptide biosurfactant of *Bacillus subtilis* significantly improved the appearance of bread and crumb structure, and decreased its susceptibility to microbial contamination during storage.	*Bacillus subtilis*	Wheat flour	(Mnif et al., 2012)

EPSs are often produced during the SD process. The EPS yield can be correlated to many factors, such as the composition of macronutrients and micronutrients of the substrate, temperature, pH, agitation, and the bacterial strains

with defined biochemical properties (Păcularu-Burada et al., 2020b). The EPSs generated by LAB during SD have techno-functional aspects related to their ability to bind water and retain moisture. In the last decade, the use of EPS-producing cultures has attracted the attention of the bakery industry because of the hydrocolloid nature of these polysaccharides, as well as their health benefits, including anticarcinogenic effects (Lynch et al., 2018). Screening of EPS-producing SD strains is limited to wheat and rye fermentations. The SD process offers a convenient means by which the EPS-producing nature of LAB can be exploited to produce baked goods with enhanced quality (Lynch et al., 2018). This is typically achieved by adding a pre-fermented SD starter with defined EPS-producing strains, commonly on a 10% to 40% (w/w) flour basis, to the final bread dough mix (Galle & Arendt, 2014).

EPS from SD provides an opportunity to improve consumers' health. These compounds stimulate carbohydrate fermentation to SCFAs by the intestinal microbiota. For example, dextran is metabolized by gut microbes to acetate, butyrate, and propionate. Propionate has been postulated to have several beneficial effects, such as reducing cholesterol and triglyceride levels, and increasing insulin sensitivity. Furthermore, oligosaccharides can act as soluble receptor analogs of epithelial cell surface carbohydrates and inhibit pathogens or bacterial toxin adhesion to epithelial surfaces, an initial stage of an infective process. Thus, the large variety of oligosaccharides produced by LAB enzymes, also involved in EPS production, makes LAB potential candidates for preventing infection or inflammatory bowel diseases (Galle & Arendt, 2014). Several factors, such as dough yield, fermentation time, pH, sucrose content, and the fermentation substrate, influence the amount of EPS formed *in situ*. It was reported that glucan formation by *L. reuteri* from sucrose was higher in softer doughs, probably because of better diffusion of the substrates and extracellular glycansucrases (Seitter et al., 2019). EPS yield was also improved when sucrose was added stepwise (fed-batch) to the fermenting dough. Furthermore, with the pH adjusted to a constant value of 4.7, the EPS level increased. Interestingly, fermentation with wheat flour, a rye–wheat mixture, or rye bran with 10% sucrose addition showed that EPS production was the most efficient and fastest when rye bran was supplied as a substrate (Di Monaco et al., 2015).

Some examples of LAB metabolites with antimicrobial activity are bacteriocins, organic acids, bacteriocin-like inhibitory substances, and others. Bacteriocins are well-known peptides/proteins synthesized by bacterial ribosomes, and their function is to either kill or inhibit the growth of closely related bacteria. These antimicrobial molecules produced by LAB are usually regulated by environmental conditions and the productive strain growth phase. Bacteriocin production is initiated in the early log phase, suggesting that bacteriocin is a primary metabolite. In the case of other antimicrobial compounds from LAB, it is very important to emphasize that these metabolites are released after 48 h of fermentation (Păcularu-Burada et al., 2020a). The inhibitory effect on pathogenic or spoilage microorganisms varies widely. The effects of some antimicrobial metabolites produced by two LAB (L. plantarum and Lactobacillus delbrueckii) cultivated on wheat dough extracts were studied. The species L. plantarum had a stronger inhibitory effect against Penicillium spp., whereas weaker inhibition was observed against Aspergillus niger. These LAB species were also used to produce SD bread, where good resistance against fungal contamination was observed. Another study with isolated LAB strains from spontaneous rye SD (belonging to species such as Leuconostoc mesenteroides, Latilactobacillus curvatus, and Levilactobacillus brevis) showed no inhibitory effect against Aspergillus spp. and weak inhibition of Penicillium spp. However, it was found that all strains showed satisfactory inhibition of Bacillus cereus (Păcularu-Burada et al., 2020a).

In the case of fatty acids, many LAB can produce SCFA, especially acetate, propionate, and butyrate, with potential therapeutic effects on depression, autism, anxiety, and stress (Gill et al., 2018; Păcularu-Burada et al., 2020b). Additionally, metabolites from the conversion of fatty acids by specific LAB strains may contribute to the prolonged storage life of sourdough bread. Black et al. (2013) demonstrated that *Levilactobacillus hammesii* converts linoleic acid to a monohydroxy octadecenoic acid, preventing fungal spoilage of bread without adversely impacting on the sensory properties. This conversion was observed in SD fermentation supplemented with linoleic acid as a substrate (Black et al., 2013). These conversions from fatty acids have not been fully explored in SD. It remains unknown

whether hydroxy fatty acids produced by LAB could have a specific positive impact on health.

2.6. Effect of heat treatment on the microbial viability

The application of probiotics in bread is unlikely because the high temperature and dehydration that occur during the baking process terminate cell viability. Besides, to ensure the benefits of probiotics, the number of LAB must exceed 10^6 CFU/mL or per gram at the time of consumption (Zhang et al., 2015; 2018). The factors that affect the behavior of probiotics during baking include the temperature, moisture content, and structure of the matrix. Many studies have been performed to determine the effect of thermal and dehydration kinetics on the inactivation of the microorganisms in SD bread during baking. Furthermore, most research mainly studied the impact of the baking conditions, storage, and composition on the technological quality of the baked bread instead of the microbiological quality (Thang et al., 2019).

The kinetics of bread baking is an essential factor in the formation of the crust and the crumb, which give bread its textural and sensory properties. The minimum time required for baking bread is when 98% of the starch in the dough is completely gelatinized, and this time decreases as the baking temperature increases. For example, the baking time for a dough of 60 g at 175 °C is 9 min. Under the same conditions, but at a temperature of 200 °C, the minimum baking time is reduced to 6 min. However, when the temperature increases to 235 °C, the baking time decreases by only 1 min. The viability of microorganisms is affected by the minimum baking time because of the heat and dehydration stresses to which they are exposed. If the baking time is shortened by increasing the baking temperature or reducing the bread size, higher residual viability of LAB species may be obtained after baking (Debonne et al., 2018).

Zhang et al. (2018) evaluated the viability of *L. plantarum* at three different baking temperatures (174, 200, and 235 °C). The results showed that the viable *L. plantarum* counts were reduced by up to 4–5 log CFU/g, while the initial viable count

in the dough was 8.8 log CFU/g (Zhang et al., 2018). Another study by Zhang et al. (2015) evaluated the viability of *L. plantarum* in the same conditions as the previous study. The findings were that the counts of *L. plantarum* also decreased by 4–5 log CFU/g (Zhang et al., 2015). In both studies, the kinetics of the baking process showed that in the first two minutes, the microorganisms were only slightly inactivated, with a reduction of <0.5 log CFU/g; between minutes two and six, the reduction in the bacteria count was exponential, with a decrease of 3 log CFU/g; and in the last two minutes, the kinetic changed into a stationary phase with a maximum decrease of 1 log CFU/g (Zhang et al., 2015, 2018). In the case of yeasts, Debonne et al. (2018) found that the yeast count decreased from an initial value of 9 log CFU/g to 4 log CFU/g with baking at 200 °C for 13 min. Under different conditions, 150 °C for 8 min, the yeast count was reduced by only 2–3 log CFU/g, impacting the product's shelf life. Debonne et al. (2018) observed that the use of SD resulted in negligible detection of spore-forming bacteria, with 3 log CFU/g being the highest recorded value, after heat treatment (150 °C, 8 min).

In vitro metabolic studies on the effect of thermal treatment on the viability of progenitor strains and the functionality of their components already exist. Nachtigall et al. (2021) examined the impact of thermal treatment on the molecular mass of EPS synthetized by *Streptococcus thermophilus*. The EPS solution was analyzed at 60, 80, and 90 °C for 10, 30, and 60 min each and cooled to room temperature in an ice bath. When the EPS was untreated, the average molecular mass was 2.90×10^6 Da. In the case of thermal treatment at 60 and 80°C for up to 60 min, the molecular mass of the EPS was not significantly affected. Similar behavior occurred at 90 °C and 10 min residence time, with no reduction of the molecular mass. However, after 30 min of residence time, the molecular mass decreased significantly to 2.40×10^6 Da, and after 60 min, to 2.40×10^6 Da (Nachtigall et al., 2021). Therefore, a prolongated thermal treatment affects the molecular composition of EPS. Unfortunately, there is scarce information about EPS stability at baking temperatures (160–220 °C). More studies are necessary to determine if the functionality of EPS in bread is compromised by the thermal treatment.

The functionality of antimicrobial compounds has also been analyzed through high temperatures. Păcularu-Burada et al. (2020a) evaluated the functionality of antibacterial and antifungal compounds treated at 60, 80, and 121 °C for 15 min, forming inhibitory ratios or halo zones. The (antimicrobial-compound-producing) LAB were grown in three different types of flour extract: buckwheat, chickpea, and quinoa. Bacillus spp. was used as the pathogen for antibacterial activity, and A. niger, Aspergillus flavus, and Penicillium spp. were the microorganisms assessed for antifungal activity. The stability test showed that the antifungal capacity after the thermal treatments differed depending on the type of flour used. In the case of chickpea flour, neither strain inhibited the three fungal species. For buckwheat treated at 80 °C, various heat-stable compounds with an inhibitory effect against Penicillium spp. were present. The fermented quinoa flour extract showed the highest inhibition ratio of 17% after thermal treatment at 60 °C (15 min). However, at 121 °C, an inhibitory effect against Aspergillus niger was observed with an inhibition ratio of 7.20% (Păcularu-Burada et al., 2020a). This result is in line with those obtained by Varsha et al. (2014), who concluded that antifungal compounds produced during fermentation are resistant to sterilization temperatures and have a high value in the baking industry (Varsha et al., 2014). Regarding antibacterial activity, the flour extracts fermented with both strains showed thermally stable antibacterial compounds that inhibited Bacillus spp. [14]. Similar results were found by Cizeikiene et al. (2013), who concluded that many antibacterial compounds, especially bacteriocins, are resistant to thermal treatment and may have many functions in foods that are exposed to high temperatures, such as baked goods (Cizeikiene et al., 2013). However, experimentation with higher temperatures, up to 220°C, is needed to evaluate the viability of the antifungal and antibacterial activity.

In the case of other compounds (e.g., biosurfactants, cell surface proteins, cell-free supernatants, SCFAs, etc.), there are not sufficient studies to describe their properties and stability after thermal treatment. Therefore, further research is necessary to understand the functionality of these compounds after a baking process using *in vitro* and *in vivo* models.

2.7. Effects of sourdough bread on health

Different studies evaluated the potential health benefits of sourdough bread (SDB) consumption in healthy volunteers and in subjects with metabolic or gastrointestinal diseases. Table 4 summarizes some studies of SDB with different formulations and doses that evaluated the impact of their ingestion in healthy volunteers and patients with pathologies such as irritable bowel syndrome (IBS), among others. The administration of different SDB formulations resulted in reduced glycemic responses compared with a traditional white wheat bread leavened with *S. cerevisiae*, where the content of blood glucose after 120 min of digestion was 125 mmol/L for white bread and 90 mmol/L for SDB (Bondia-Pons et al., 2011; Scazzina et al., 2009). Scazzina et al. (2009) evaluated the influence of SD on starch digestibility in bread. Four experimental formulations made from two wheat flours by two leavening techniques (SD and with *S. cerevisiae*) were analyzed. SDB showed higher resistant starch levels and significantly lower glycemic responses in young, healthy subjects. The reduction in the glycemic response was not related to the starch hydrolysis rate; however, organic acids produced by the SD microbiota might delay gastric emptying without affecting starch accessibility (Scazzina et al., 2009). Likewise, in Bondia-Pons et al. (2010), SD fermented rye bread was tested in healthy subjects. SBD contained a higher level of total fiber and free phenolic acids, a higher starch hydrolysis rate, and a lower postprandial insulin response. SDB altered plasma amino acids and their metabolites. The increase in tryptophan precursors following consumption of SD rye bread might stimulate a higher tryptophan concentration, resulting in lower appetite and food intake. Likewise, the levels of picolinic acid, which catabolizes the tryptophan metabolism associated with pro-inflammatory functions, significantly decreased after SD rye bread intake (Bondia-Pons et al., 2011).

Table 4. Evaluation of the benefits of sourdough bread in human health and disease.

Product	Dose	Model	Findings	Reference
Two SDB (Different fermentation time: 4h and 24h) against yeast fermented bread	One slice of bread (80 g) at three weeks-intervals	Clinical trial (36 healthy volunteers aged 20 to 31 years) Double-blind	Highest fullness perception with 24h fermentation SDB In both SDB: faster gastric emptying, lower glycemic responses, higher concentration of total free amino acids and better digestibility	(Rizzello et al., 2019)
SDB (>12h fermentation) against yeast fermented bread	Six slices of the study bread (150 g)/day for 7 days	Clinical trial (26 patients with IBS aged 18 to 65 years) Double-blind	Higher reduction in ATIs to their monomeric form Lower levels of FODMAPs	(Laatikainen et al., 2017)
Whole grain rye SDB (40h fermentation) against yeast fermented crispbread and unfermented rye crispbread	One slice (59.4 g)	Clinical trial (24 healthy adults aged 18 to 70 years) Single-blinded, cross-over trial	Higher satiety and degradation of *β*-glucans	(Zamaratskaia et al., 2017)
SDB with low FODMAPs against regular rye SDB	3.5–4 slices (105–120 g) of each bread/day in the 1st week 7–8 slices of SDB (210–240 g) per day from 2nd to 4th week	Clinical trial (87 patients with IBS aged 18 to 65 years) Double blind controlled cross-over	Control of IBS symptoms and reduction of gastrointestinal gas accumulation Increase of dietary fiber intake and well acceptance	(Laatikainen et al., 2016)
Wholegrain rye SDB compared to white wheat bread enriched with rye bran, and WB	6-10 slices (25-30 g/slice) In two 4-week test periods	Clinical trial (21 healthy subjects with mild gastrointestinal symptoms aged 38 to 65 years); Cross-over study	Lower postprandial insulin concentration Improvement in the first-phase of insulin secretion Increase in postprandial concentrations of SCFAs	(Lappi et al., 2014)
Wholegrain wheat SDB against WB	6 slices (for women) and 7 (for men) of SDB per day (162.5 g) for 6 weeks	Clinical trial (14 normoglycemic/ normoinsulinemic adults and 14 hyperglycemic/ hyperinsulinemic adults aged 43 to 70 years); Crossover study.	Improvement in glucose iAUC in response to an OGTT within hyperglycemic/ hyperinsulinemic subjects	(MacKay et al., 2012)

Endosperm rye SDB against standard WB	One portion (50 g) at intervals of 1-2 weeks	*In vitro* Starch and protein hydrolysis Clinical trial (16 healthy subjects aged 23 ± 3.7 years)	Higher level of total fiber, phenolic acids, and starch hydrolysis rate Lower postprandial insulin response Beneficial changes in plasma amino acids and their metabolites	(Bondia-Pons et al., 2011)
Wholemeal wheat SDB (19.5h fermentation) against: WB, wholemeal wheat, and wholemeal wheat + xylanase	One slice with crust (50 g) of the test breads	Clinical trial (11 insulin resistant subjects, aged 40 to 65 years)	Lowest postprandial glucose and insulin responses	(Lappi et al., 2010)
Two SDB against yeast fermented wholemeal bread and yeast fermented WB	One slice (50 g)	*In vitro* Starch hydrolysis Clinical trial (8 healthy volunteers aged 23 to 25 years)	Significantly lower glycemic responses in SDB Resistant starch levels were higher in the SDB	(Scazzina et al., 2009)
Wholewheat SDB (3h fermentation) against whole wheat barley bread and WB	One slice (50 g)	Clinical trial (10 overweight male subjects) Single blind, crossover	Lower overall glucose and GLP-1 responses Lower glucose iAUC	(Najjar et al., 2008)
SDB (24h fermentation) with 4 flours against yeast fermented 4 flours bread	A portion of 80 g for 2 days	Clinical trial (17 celiac sprue patients) Double-blind	Not significantly different intestinal permeability values from the baseline values in 13 of the 17 patients	(Di Cagno et al., 2004)

SDB: Sourdough bread; WB: white bread; IBS: irritable bowel syndrome; ATIs: alpha-amylase/trypsin inhibitors; FODMAPs: Fermentable, Oligo-, Di-, Mono- saccharides and Polyols; SCFAs: Short Chain Fatty Acids; OGTT: oral glucose tolerance test; iAUC: incremental area under the curve; GLP-1: glucagon-like p

Moreover, SDB elicited a weaker insulin response in overweight human males, as well as insulin-resistant, and hyperglycemic/hyperinsulinemic patients (Lappi et al., 2010; MacKay et al., 2012; Najjar et al., 2008). Najjar et al. (2008) analyzed the effect of SD-fermented whole wheat bread in overweight male patients. SDB was associated with the least disturbance in carbohydrate homeostasis, as well as lower overall glucose and glucagon-like peptide-1 (GLP-1) responses, indicating that its consumption could promote the prevention and management of metabolic disorders associated with type 2 diabetes (Najjar et al., 2008). Lappi et al. (2010) evaluated the postprandial insulin response to five different bread formulations in insulin-resistant patients. SDB (19.5 h of fermentation) showed the lowest postprandial glucose and insulin responses. The proteolysis in SD may be the contributory factor (Lappi et al., 2010). Finally, MacKay et al. (2012) reported that SDB showed an improvement in incremental area under the glucose curve in response to an oral glucose tolerance test (OGTT) in patients with hyperglycemia/hyperinsulinemia, which represents a potential to positively influence postprandial glucose responses in people at risk of cardiovascular disease (MacKay et al., 2012).

Moreover, SDB can increase the postprandial concentrations of SCFAs (Lappi et al., 2014). SD has been shown to change the nutritional quality and health effects of grain ingredients (Lappi et al., 2014). Lappi et al. (2014) compared the impact of three bread formulations (SD rye bread, white bread with rye bran, and white bread) on glucose metabolism and SCFA plasma levels in healthy subjects with mild gastrointestinal symptoms. SD rye bread lowered the postprandial insulin concentration and improved first-phase insulin secretion. The improvement in first-phase insulin secretion and the reduction of hyperinsulinemia in the later postprandial phase may prevent alterations in glucose metabolism. The consumption of both rye bran formulations improved subjects' gastrointestinal quality of life. Additionally, SD rye bran bread increased postprandial concentrations of butyrate and propionate (Lappi et al., 2014).

SDB is also considered to have beneficial effects on postprandial satiety (Rizzello et al., 2019; Zamaratskaia et al., 2017). Rizzello et al. (2019) and Zamaratskaia et al. (2017) tested the impact of SDB in healthy subjects and obtained the highest satiety response; however, only Rizzello et al. (2019) showed a lower glycemic response. Wholegrain SD rye bread led to the highest satiety (Zamaratskaia et al., 2017). Wholegrain rye bread is linked to a reduced molecular weight of arabinoxylans and *β*-glucans. Fibers with high solubility and low molecular weight might increase their fermentability and satiety. Additionally, organic acids produced in SD could affect satiety (Zamaratskaia et al., 2017). Rizzello et al. (2019) evaluated two SDBs with different fermentation times (4 h and 24 h) in healthy subjects. SDB fermented for 24 h obtained the highest fullness perception in the shortest time. Both SDBs showed faster gastric emptying, a lower glycemic response, a higher total free amino acid concentration, and better digestibility than yeast-fermented bread (Rizzello et al., 2019).

Additionally, SDB has the potential benefit of improving some enteropathies such as celiac sprue (CS) or irritable bowel syndrome (IBS) (Di Cagno et al., 2004; Laatikainen et al., 2016; Laatikainen et al., 2017). Di Cagno et al. (2004) evaluated SDB formulations for tolerance by CS patients, while Laatikainen et al. (2016) and Laatikainen et al. (2017) focused on specific characteristics of SD that would benefit IBS patients. Di Cagno et al. (2004) included selected SD lactobacilli for their ability to hydrolyze albumin, globulin, and gliadin fractions during SD fermentation in SDB formulated with four flours (wheat, oat, millet, and buckwheat flour). The result was improved tolerance of SDB with 30% wheat flour and no significant alterations in the intestinal permeability values in 13 of the 17 patients (Di Cagno et al., 2004). In the case of IBS patients, Fermentable, Oligo, Di-, and Mono- saccharides, and Polyols (FODMAPs) are considered triggers of IBS symptoms. Low-FODMAP SDB was administered and evaluated against regular SDB. Low-FODMAPs SDB improved IBS symptoms and reduced gastrointestinal gas accumulation, presenting lower fermentation in the colon, flatulence, abdominal pain, and stomach rumbling; it also increased dietary fiber intake and was well accepted by patients (Laatikainen et al., 2016). Likewise, SDB presented a greater reduction in alpha-amylase/trypsin

inhibitors (ATIs) to their monomeric form and showed lower levels of FODMAPs than yeast-fermented bread (Laatikainen et al., 2017).. ATIs and other non-gluten proteins are associated with a pro-inflammatory effect on intestinal epithelial cells, which could cause gastrointestinal symptoms (Laatikainen et al., 2017). SDB applications have been demonstrated to improve health and even to aid in the management of symptoms of sensitive pathologies such as CS and IBS. However, further studies are needed to characterize and elucidate the mechanisms of action of these benefits and to perform additional clinical trials considering other diseases and a more diverse demographic.

2.8. Chapter conclusions and future perspectives

SD allows the production of a final dough with properties that depend on temperature, pH, time, and the microbiological consortium of LAB and yeasts. Postbiotics have become novel trends in SD because of their potential beneficial health properties. Recent studies are attempting to characterize the different compounds and cell debris present in SD. Among the examples are SCFAs, EPSs, biosurfactants, bacteriocins, cell-free supernatants, and cell surface proteins, and the main health benefits are anti-immunomodulatory, antioxidant, and antimicrobial effects. Nowadays, the SD bread-making process presents various challenges in areas such as ingredient selection, SD starters, fermentation time, the baking process, and microbiota characterization, among others. Starters have a key role in the physical characteristics of SDB, modifying raw bread dough and cooked bread products (extensibility, elasticity, viscosity, and the organoleptic properties of the final bread) (Fujimoto et al., 2019; Landis et al., 2021; Reese et al., 2020).

Moreover, the potential health benefits promote consumer preference for foods prepared with LAB. This increasing need for the formulation of functional food products containing LAB starter cultures from sources such as kefir, yogurt, or kombucha, which have reported health benefits, has led to the application of different consortiums such as kefir grains as mixed starters for SD breadmaking to evaluate the functional and physical properties of the final bread (Limbad et al., 2020; Plessas

et al., 2020a). There are numerous consortiums with potential health benefits that need to be assessed. Another research opportunity is related to ingredients. Organic farming and products are gaining increasing consumer demand due to their association with sustainable production. Recent studies tested the impact of flour selection by evaluating new organic flour options created to meet consumers' needs (Pontonio et al., 2021; Urien et al., 2019). The use of organic flours may offer better functional characteristics, such as a higher total free amino acid content, than non-organic flour (Pontonio et al., 2021). Likewise, researchers are analyzing the effect of ingredients and the environment on the SD microbiota. Factors such as the bakers' skin microbiota may have an important role in the composition of bacteria and fungi in starters (Reese et al., 2020). Also, cereal fermentation may potentially improve nutritional quality and health effects; there is a wide variety of cereals and pseudocereals that can be combined and evaluated to obtain different profiles for fiber content and potential postbiotic-like components (Koistinen et al., 2018; Păcularu-Burada et al., 2020a). However, a noticeable challenge is to reach a consensus for applying standardized culture-based techniques to characterize SD microbial diversity to minimize the variability and biases among studies and define criteria for evaluation of SD starters and their bread products.

More studies are needed with a defined microbiological consortium and ingredients, to generate a SD with specific nutrients and health benefits; therefore, it is imperative that the different postbiotic forms present in the final dough be characterized, their yield measured, and their potential health properties probed by in vitro and in vivo studies.

CHAPTER 3. ISOLATION, IDENTIFICATION, AND BIOTECHNOLOGICAL PROPERTIES OF LACTIC ACID BACTERIA AND YEASTS FROM A SPONTANEOUS SOURDOUGH

3.1. Introduction

In recent years, sourdough (SD) fermentation has acquired more popularity due to its beneficial effects on flavor, shelf-life, texture, and nutrition/health properties. In fact, extensive efforts have been made to study the species diversity and identification of LAB and yeasts found in SD fermentation. However, there is more research addressing the identification and characterization of lactic acid bacteria (LAB) than of yeast (Vrancken et al., 2010). Spontaneous SD inoculated only with flour and water is fermented by a specific cereal matrix-associated microbiota. In the initial phase of the fermentation, yeast and bacterial species belonging to the family Enterobacteriaceae are prevalent, which are later replaced in the backslapping by more acid-tolerant LAB species until a number of different lactobacilli prevail (Llamas-Arriba et al., 2021).

The microbial composition of a SD varies depending on the type of flour and water used, the region or country, processing environments and fermentation parameters such as temperature, time of fermentation, number of backslappings and type of SD which is made. In the case of a wholewheat SD, the geographical conditions where the wheat is cultivated seems to harbor diverse LAB and yeasts, which affect the SD microbiota composition (Gobbetti et al., 2016). In Mexico, the most common species of LAB and yeasts found in wholewheat SD are *Companilactobacillus paralimentarius, Pediococcus pentosaceus, Limosilactobacillus fermentum, Limosilactobacillus pontis, Leuconostoc mesenteroides, Lactobacillus delbrueckii, Saccharomyces cerevisiae, Candida glabrata, Candida humilis,* among others (De Vuyst et al., 2014).

Nowadays, the increasing demand for healthy food boosted studies of the SD microbiota, with the objective of using potential functional starters for the production of baked goods with enhanced nutritional properties. Most works are focused on the

metabolism of LAB to produce metabolites that increase the functional and nutritional features of SD, especially for the ability to synthesize essential amino acids, vitamins, and bioactive compounds (polyphenols, organic acids, peptides, and amino acids derivatives) (Palla et al., 2020). Furthermore, LAB strains are capable of generating enzymes, for example proteases, phytases, and lipases, to promote the degradation of antinutritional factors such as phytic acid and raffinose (Gobbetti et al., 2014). In the case of yeasts, an increased number of studies have highlighted the key role of yeast metabolism in producing different vitamins, and antioxidant compounds, and degrading antinutritional factors such as phytates. In particular *S. cerevisiae* has shown phytase activity and antioxidant properties (Palla et al., 2019).

Moreover, wholegrain flours have a high content of bioactive compounds released by LAB and yeasts during cereal fermentation, particularly phenolic acid compounds, the main ones responsible for the antioxidant activity of cereal grains, flours and baked goods (Adom & Liu, 2002). Additionally, anthocyanins and carotenoids, phytochemicals with strong antioxidant activity, are also attractive among wholegrain products derived from cereal fermentation of wheat, rice, maize, and oat (Giordano et al., 2017).

Therefore, the main objective of this chapter was the isolation and identification of LAB and yeasts from a spontaneous wholewheat SD and the evaluation of their biotechnological properties to obtain autochthonous starter cultures useful for the development of novel SD products. For comparative purposes, isolated microorganisms from raspberry and commercial probiotic strains (*Lactobacillus acidophilus* LA3, *Lacticaseibacillus rhamnosus* GG, and *Lactiplantibacillus plantarum* 299v) were also included in this study.

3.2. Methodology

3.2.1. Isolation and identification of LAB and yeast from a wholewheat sourdough

3.2.1.1. Elaboration of a wholewheat sourdough

A sourdough was made from a mixture of 65 g of a commercial whole wheat flour (Tres Estrellas, México) and 55 mL of tap water with a dough yield of 185 (dough yield=dough weight×100/flour weight). The sourdough was incubated at room temperature for 72 h. Each 24 h, the sourdough was fed with 8 g of whole wheat flour and 5 mL of tap water.

3.2.1.2. Isolation of LAB and yeasts

The isolation of LAB and yeasts were made in petri plates with De Man, Rogosa y Sharpe (MRS Difco™) agar and potato and dextrose agar (PDA MCD LAB™), respectively. Briefly, 1 g of wholewheat SD was suspended with 9 mL of sterile saline solution (0.85% NaCl) and homogenized using a vortex (VortexGENE™) for 5 min. Decimal dilutions were made and plated in MRS agar and PDA. The incubation conditions for the yeasts were 30 °C in aerobiosis for 48 h. In the case of LAB, the plates were incubated in aerobiosis and anaerobiosis at 37 °C for 48 h. Different colonies were isolated, depending on the morphology and color. The yeasts were inoculated in Nutritive Broth and the LAB colonies were grown in MRS broth. The conditions of incubation were 30°C, aerobiosis for yeast, and anaerobiosis for LAB. Gram stain and catalase tests were also conducted for each isolated colony. A total of 25 isolates of LAB and yeasts were selected and cryopreserved at -80 °C in glycerol (40% v/v) at a rate of 1:1 glycerol-medium. Additionally, microorganisms from raspberry were isolated and identified. One gram of fresh raspberry was processed as described above. Finally, three isolates were selected and cryopreserved. The identification of the LAB and yeast isolates was conducted by the Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) technique.

3.2.1.3. Identification of LAB and yeasts by MALDI-TOF

For bacteria and yeast colonies, an isolated colony was transferred to a tailless steel plate by triplicate to minimize random effect, following the “Extended Direct Transfer Method” procedure (Bruker Daltonics GmbH). To provide external calibration of the spectra, the Bruker bacterial test standard (BTS 255343 from *Escherichia coli* DH5α, Bruker Daltonics) was included according to the instructions of the manufacturer. Mass spectra were generated with the method “MBT_FC.par” in a Microflex LT equipment (Bruker Daltonics GmbH). Utilizing MALDI BIOTYPER 3.1 software, the spectra obtained were compared with reference spectra from the BDAL database (Bruker Daltonics GmbH). Some spectra from MALDI-TOF mass analysis are shown in Figure 1-A (Annexes). The software estimated a score between 0 and 3 to evaluate the similarity between the sample and reference spectrum. Therefore, scores between 2.300 and 3.000 represented a high identification reliability at the species level; scores of 2.000 to 2.299 provided a high reliability of identification at the genus level and a probable species; scores between 1.700 and 1.999 represented a probable genus identification, and scores below o equal 1.699 showed and unreliable identification. All the identified isolates (8 yeasts and 20 LAB) along with three probiotic strains were used in the further studies.

3.2.2. Biotechnological properties of LAB and yeast isolates

Sterile flour extract (SFE) was used as liquid broth to assess the biotechnological properties of the LAB and yeast isolates. SFE was prepared according to the protocol of Sáez et al. (2018) with some modifications. Wholewheat flour was suspended at 40% (w/v) in tap water and stirred for 15 min at room temperature. Flour from the suspension was removed by centrifugation (4000 rpm, 10 min, 4 °C). The supernatant was sterilized at 121 °C /15 psi, for 20 min. Firstly, LAB and yeasts were grown in MRS and Yeast Extract Peptone Dextrose (YEPD), respectively, overnight at 30 °C. Cells were harvested by centrifugation (4,000 rpm, 10 min, 4 °C), washed in sterile saline solution (0.85% w/v), and then inoculated in SFE (2% w/v) for 24 h at 30 °C. After that, absorbance (560 nm) and pH were

determined. Furthermore, the following assays were performed after the growth of LAB and yeasts in SFE during 24 h.

3.2.2.1. Proteolytic activity

Proteolytic activity of LAB and yeast isolates was measured by the spectrophotometry assay reported by Church et al. (1983). Briefly, LAB and yeast isolates in SFE were deproteinized with 0.75 mol/L of trichloroacetic acid (1:2). Then supernatants were incubated with o-phthaldialdehyde (o-PA) solution (50 mL sodium tetraborate 100 mmol/L, 5 mL sodium dodecyl sulfate 20% (w/v), 2 mL o-Pa 40 mg/mL dissolved in methanol, 0.2 mL β-mercaptoetanol for 10 min at room temperature before reading their optical density at 340 nm (OD_{340}). The results were expressed as mmol/L of free amino acids referring to a standard curve of L-leucine.

3.2.2.2. Amylolytic activity

The amylolytic activity was assayed by the ability of LAB and yeast isolates to hydrolyze starch in agar according to the protocol of Sáez et al. (2018). SFE cultures of LAB and yeasts were streaked on MRS agar plates containing 1% (w/v) of starch instead of glucose, the plates we incubated at 30 °C for 48 h, and then submerged with 4% (w/v) of iodine solution during 15 min. Amylase production was evidenced by the appearance of a clear zone around the colonies.

3.2.2.2. Total protein content

Protein content was assayed in cell free extracts (CFE) by colorimetric method. CFE were obtained as previously reported Sáez et al. 2018. LAB and yeast cultures in SFE were centrifuged (10,000 rpm, 10 min, 4 °C), washed twice with 10 mM of sodium phosphate buffer (pH 6.0) and resuspended in 0.5 mL. Cells were disrupted with 500 mg glass beads (0.10 – 0.11 mm, Sigma Aldrich) in a bead beater (FastPrep 24^{tm}) for three cycles at 6 m/sec, 30 sec. Cell debris, glass beads and unbroken cells were removed by centrifugation (10,000 rpm, 5 min, 4 °C), the supernatant was recovered and used as CFE. Protein concentration was determined by the Bradford procedure (1976). Briefly, 300 µL of the supernatant was mixed with

3 mL of Bradford reaction kit, incubated for 10 min at room temperature and determined at 595 nm. The results were expressed as mg/mL of protein referring to a standard curve of albumin.

3.2.2.3. α-Galactosidase activity

α-Galactosidase activity was assessed according to the method reported by LeBlanc et al. (2004). 45 µL of CFE were mixed with 15 µL 10 mM *p*-nitrophenyl-α-D-galactopyranoside (pNPG) and incubated for 15 min at 37 °C. 900 µL of 0.25 M Na_2CO_3 were added to stop de reaction. Absorbance at 405 was measured. One unit (U) of α-galactosidase was defined as the amount of enzyme that release 1.0 µmol/min of *p*-nitrophenol.

3.2.2.3. Presence of extracellular proteases

The presence of extracellular proteases was determined by the procedure of Vermelho et al. (1996). Active cultures are streaked on agar plates containing 1% (w/v) gelatin as substrate and incubated at 30°C for 48 h. LAB and yeast cultures were streaked on the surface of agar plates After growth, plates were flooded with 0.25% (w/v) of Coomassie blue in methanol-acetic acid-water 5:1:4 (v/v/v) for 1 h, and then destained with methanol-acetic acid-water at the same concentration. Presence of extracellular proteases was evidenced by a clear zone around the colonies.

3.2.2.4. Endopeptidase activity

Endopeptidase activity was monitored based on the protocol of Rizzello et al. (2015) by using N-succinyl L-phenyl-alanine-p-NA and N-glutaryl L-phenyl-alanine-p-NA as substrates. The assay mixture contained 900 µL of 2.0 mM substrate in 0.05 M potassium phosphate buffer at pH 7.0, and 100 µL of CFE The reaction was incubated at 30 °C for 1 h and the absorbance was measured at 410 nm. The data was compared to a standard curve of *p*-nitroaniline. One enzymatic unity (U) was defined as the amount of enzyme required to liberate 1µmol/min of *p*-nitroaniline under assay conditions.

3.2.2.5. Gallate decarboxylase activity

Gallate decarboxylase activity was assessed according to the method of Osawa et al. (2000). Active LAB and yeast cultures were inoculated at 1 % (v/v) in MRS broth supplemented with 100 mmol/l of gallic acid and incubated in anaerobiosis at 37°C for 72 h. The growth cultures were alkalinized with 2 mM of $NaHCO_3$ solution at pH 8.6 and incubated aerobically at 37 °C for 1 h. Dark yellow to dark brown coloration of the medium was taken as positive result for gallate decarboxylase activity.

3.2.3. Functional properties of LAB and yeast isolates in a wholewheat sourdough

3.2.3.1. Leavening capacity of the microorganisms

Leavening capacity was evaluated based on the method of Palla et al. (2020). LAB and yeast isolates were cultivated in MRS and YEPD broth, respectively, overnight at 30 °C. Cells were harvested by centrifugation (4,000 rpm, 10 min, 4 °C), and washed in sterile saline solution (0.85% w/v). Biomass between 6.0-7.0 Log CFU/mL was used as starter for dough fermentation. Each microorganism was inoculated at 2% (v/w) in doughs prepared with 6.5 g of wholewheat flour and 7.5 mL of tap water, obtaining a dough yield of 220. Mix was done manually using a vortex for 3 min. The dough containing each microorganism was fermented at 30 °C for 24 h. Additionally, a spontaneous wholewheat SD was made as control dough for comparative purposes. The pH of the doughs was determined after 24 h of fermentation by a pH meter (LAQUAact^{tm}). The increase in volume (mL) was monitored after 24 h of incubation and leavening performance was calculated as the increase in volume (mL) during the fermentation.

3.2.3.2. Total phenolic content

The analysis of total phenolic content was conducted on the methanolic extracts (ME) of the fermented doughs using the Folin–Ciocalteu technique. In order to obtain the ME, 1 g of each fermented dough was mixed with 3 mL of methanol. The mixture was centrifuged at 4,000 rpm for 5 min at 4 °C. The ME was transferred

into test tubes and the sediments were mixed with 4 mL of ethanol and centrifuged at the previous condition. This step was repeated two times, obtaining 10 mL of ME as the final volume. The Folin–Ciocalteu reaction contained 50 µL of ME, 250 µL of Folin-Ciocalteu reagent and 450 µL of distilled water. After 10 min of incubation at room temperature in darkness, 1.25 mL of a 20% (w/v) sodium carbonate solution was added. The mixture was incubated under previous conditions for 30 min and the absorbance was measured at 765 nm. The concentration of total phenolics was calculated as gallic acid equivalents (GAE) with a calibration curve.

3.2.3.3. Phytase activity

To measure phytase activity, first water/salt-soluble extracts (WSE) of the fermented doughs were prepared according to Weiss et al. (1993). Briefly, 100 mg of sample were extracted with 1 mL of Tris-HCl buffer (50 mM, pH 8.8) for 1 h a 4 °C, with vortexing intervals each 15 min. The samples were centrifuged (13,500 rpm for 20 min), and the supernatants with the WSE were carefully removed. Phytase activity was evaluated by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) as stated by Palla et al. (2020). The assay contained 400 µL of WSE, and 200 µL of 1.5 mM *p*-NPP in 0.2 M of Na-acetate, pH 5.2. The mixture was incubated for 10 min at 45 °C and the reaction was stopped by adding 600 µL of NaOH 0.1 M. The *p*-nitrophenol released was measured by absorbance at 405 nm. One unit (U) of activity was defined as the amount of phytase required to liberate 1 µmol/min of *p*-nitrophenol under assay conditions.

3.2.4. Statistical Analysis

The results were expressed as the mean ± standard deviation of triplicates for the characterization of the LAB and yeast isolates. Significant differences ($P < 0.05$) between treatments were assessed by Tukey's test after an analysis of variance (one-way ANOVA) in the Minitab® Statistic Program for Windows.

A Cluster analysis was made to evaluate the similarity between the LAB and yeast isolates in terms of their biotechnological properties, proteolytic activities,

leavening capacity, total phenolic compounds and phytase activities. This is a technique for distinguishing and grouping similar and near objects within a data set into clusters based on their characteristics. The cluster may be identified using the linkage distance Dlink/Dmax, which denotes the quotient between the linkage distances for a particular case divided by the maximal linkage distance, and the quotient was multiplied by 100 to standardize the linkage distance presented on the x-axis (Hurtado-Romero et al., 2021). A Principal Component Analysis (PCA) was performed for biotechnological properties, proteolytic activities, leavening capacity, total phenolic compounds and phytase activities of the LAB and yeast isolates, with the purpose of evaluating differences between treatment units. PCA is a frequently used technique in multivariate data analysis to decrease the dimensionality of large databases and maximize the variance by calculating uncorrelated variables as linear functions of those in the original database. This technique allows the identification of parameters that best describe data set variations, reducing the dimension variables (Casillas-García et al., 2021), thus, developing microbial consortiums that reflects the biotechnological properties with an acceptable proportion of the total variation. Furthermore, a Mixed Model analysis was also performed with the objective of evaluating qualitative data coming from amylolytic activity, presence of extracellular proteases and gallate decarboxylase activity along with the quantitative data of the biotechnological properties. Mixed models or multilevel models are used when the data have a sort of hierarchal form, time series, repeated measures, and blocked experiments (Zuur et al., 2007). The Cluster analysis, PCA analysis, and Mixed Data analysis were performed with the software R-4 2.0 for Windows.

3.3. Results and Discussion

3.3.1. Identification of LAB and yeasts by MALDI-TOF

Spontaneous SD usually develops a stable yeast community of only one or two species, which is supported by the low diversity of yeasts during this study. The average yeast population in our wholewheat SD was $2.8x10^8$ CFU/g. Seven species-level cultures were identified by MALDI-TOF as shown in Table 5, of which six correspond to *C. glabrata* and one to *S. cerevisiae*. Yeast scores ranged from 2.00 to 2.17 (genus level, probable species). According to the literature (Vrancken et al., 2010), yeast microbiota in controlled laboratory SD fermentations has revealed the dominance of *S. cerevisiae, C. humilis, C. glabrata* and *W. anomalus*. Compared with a bakery SD, *S. cerevisiae* is the dominant specie, because the extensive use of commercial *S. cerevisiae* in many baked goods; likewise, *W. anomalus* is another specie that is present in artesian SD, but in fewer percentage than *S. cerevisiae* (Vrancken et al., 2010). In the case of México, the most common yeast species found in wholegrains (maize or wheat) are *C. glabrata* and *S. cerevisiae* (Moroni et al., 2011), which may explain the high presence of these two microorganisms in our spontaneous wholewheat SD.

With respect to the LAB community, mostly heterofermentative LAB occur in controlled laboratory SD fermentations. The average LAB population in our wholewheat SD was $3.5x10^8$ CFU/g in aerobiosis and $4.5x10^8$ CFU/g in anaerobiosis. Eighteen species-level cultures were identified by MALDI-TOF as presented in Table 5, of which ten belong to *P. pentosaceus,* six to *C. paralimentarius*, and one to *L. curvatus.* LAB scores ranged from 1.73 to 2.43 (majority in genus level, probable species). *P. pentosaceus* and *C. paralimentarius* are a very found species in controlled SD incubated at room temperature or 30 °C (Gobbetti et al., 2016). In the case of *L. curvatus* is more rarely isolated from a controlled SD and is most common to find in spontaneous SD, particularly in rye SD (Weckx et al., 2010). LAB community of Mexican spontaneous SD and Mexican cereal is represented by a great amount of lactobacilli, and a few pediococci and weisellas. Generally, the most common identified LAB species in a Mexican SD are

L. crispatus, L. fermentum, L. gallinarum, L. graminis, L. plantarum, L. sakei, L. vaginalis, P. pentosaceus, W. cibaria and *C. paralimentarius* (De Vuyst et al., 2014), which may justify the high presence of *P. pentosaceus* and *C. paralimentarius* in our spontaneous wholewheat SD.

For comparative purposes, isolation, and identification of microorganisms were made from raspberry. Three isolates were selected in MRS plates and identified by MALDI-TOF. The results (Table 5) showed the identification of two *P. pentosaceus*, and one yeast species known as *Hanseniaspora opuntiae.*

Table 5. Isolation and identification of LAB and yeast microorganisms by MALDI-TOF.

Assigned code	Gram test[1]	Catalase test[2]	MALDI-TOF Result[3]	MALDI-TOF score[4]
Yeast				
BIOTEC020	N/A	Positive	*Candida glabrata*	2.059
BIOTEC021	N/A	Positive	*Candida glabrata*	2.052
BIOTEC022	N/A	Positive	*Candida glabrata*	2.070
BIOTEC023	N/A	Positive	*Candida glabrata*	2.037
BIOTEC024	N/A	Positive	*Candida glabrata*	2.172
BIOTEC025	N/A	Positive	*Candida glabrata*	2.090
BIOTEC026	N/A	Positive	*Saccharomyces cerevisiae*	2.032
Lactic Acid Bacteria				
BIOTEC027	Positive	Negative	*Pediococcus pentosaceus*	2.126
BIOTEC028	Positive	Negative	*Pediococcus pentosaceus*	2.018
BIOTEC029	Positive	Negative	*Companilactobacillus paralimentarius*	2.262
BIOTEC030	Positive	Negative	*Pediococcus pentosaceus*	2.295
BIOTEC031	Positive	Negative	*Companilactobacillus paralimentarius*	2.343
BIOTEC032	Positive	Negative	*Pediococcus pentosaceus*	2.151
BIOTEC033	Positive	Negative	*Companilactobacillus paralimentarius*	1.727
BIOTEC034	Positive	Negative	*Pediococcus pentosaceus*	2.125
BIOTEC035	Positive	Negative	*Companilactobacillus paralimentarius*	2.434
BIOTEC036	Positive	Negative	*Latilactobacillus curvatus*	2.350
BIOTEC037	Positive	Negative	*Companilactobacillus paralimentarius*	2.288
BIOTEC038	Positive	Negative	*Pediococcus pentosaceus*	2.281
BIOTEC039	Positive	Negative	*Pediococcus pentosaceus*	2.246
BIOTEC040	Positive	Negative	*Companilactobacillus paralimentarius*	2.205
BIOTEC041	Positive	Negative	*Pediococcus pentosaceus*	1.978
BIOTEC042	Positive	Negative	*Pediococcus pentosaceus*	2.194
BIOTEC043	Positive	Negative	*Companilactobacillus paralimentarius*	2.099
BIOTEC044	Positive	Negative	*Pediococcus pentosaceus*	2.201
Lactic Acid Bacteria from Raspberry				
BIOTEC045	Positive	Positive	*Hanseniaspora opuntiae*	2.094
BIOTEC046	Positive	Negative	*Pediococcus pentosaceus*	2.147
BIOTEC047	Positive	Negative	*Pediococcus pentosaceus*	2.142

[1]Gram test was expressed as + (isolates stained purple) or – (isolates stained pink-red).
[2]Catalase test was expressed as + (reaction to hydrogen peroxide) or – (not reaction to hydrogen peroxide).
[3]MALDI-TOF results show the species and genus of the LAB and yeast isolates.
[4]MALDI-TOF score represents the identification reliability at genus and species level.
Scores between 2.300 and 3.000 represented a high identification reliability at species level.
Scores of 2.000 to 2.299 provided a high identification reliability at genus level and a probable species.
Scores between 1.700 and 1.999 represented a probable genus identification.

3.3.2. Growth, acidification, and carbohydrate metabolism of LAB and yeast isolates in wholewheat SFE

All the yeast and LAB isolates were capable of growing in wholewheat SFE, since the absorbance of the biomass was around 0.90 to 1.30 (Table 6). LAB microorganisms were able to grow higher than yeasts because they have a fastly lag phase and a higher exponential period. In particular, isolates *P. pentosaceus* BIOTEC027, *P. pentosaceus* BIOTEC028, *C. paralimentarius* BIOTEC029, *P. pentosaceus* BIOTEC030 and *C. paralimentarius* BIOTEC031 had a higher growth in comparison with the other isolates, with an absorbance of the biomass in a range between 1.28 to 1.31. The pH values (Table 6) indicated a higher acidification process for the LAB compared with the yeasts, where pH values of LAB were in a range of 4.52 – 4.78 while yeast species were in a pH range of 5.08 – 5.29.

None of the LAB and yeast isolates showed α-galactosidase activity and only four isolates (*P. pentosaceus* BIOTEC042, *P. pentosaceus* BIOTEC044, *P. pentosaceus* BIOTEC046 and *P. pentosaceus* BIOTEC047) developed the ability to hydrolyze starch (Table 6). Therefore, most of the species metabolized readily usable sugars such as sucrose, glucose and other monosaccharides already present in wholewheat SFE (Sáez et al., 2018). The α-Galactosidase that hydrolyzes α(1→ 6) linked sugars like stachyose, raffinose and melibiose, has been described in LAB, bifidobacteria and fungi (LeBlanc et al., 2004b).

3.3.3. Gallate decarboxylase activity

Tannins are antinutritional factors present in most cereals since they inhibit the digestive enzymes and affects the digestion of vitamins and minerals. Gallate decarboxylase is a relevant enzyme in the remotion of tannins and the release of bioactive phenolic compounds. The mechanism of action of this enzyme is the decarboxylation of gallic acid to pyrogallol, which is considered a bioactive compound. In this study, all the isolates assayed showed gallate decarboxylase activity, with the exception of *C. paralimentarius* BIOTEC040 and *P. pentosaceus* BIOTEC041 (Table 6). Osawa et al. (2000) already reported the metabolism of

gallate decarboxylase for the *Enterococcus* genus. For LAB species, *L. pentosus* and *L. plantarum* were described as positive for gallate decarboxylase activity by Muñoz et al. (2017), and *Weisella* species isolated from different bean varieties in the research of Sáez et al. (2017). In the case of the isolates assayed for the present study; none has been reported for gallate decarboxylase activity previously.

Table 6. Properties of LAB and yeast isolated from wholewheat sourdough

Isolate	pH SFE[1]	OD[560 2]	α-galactosidase activity[3]	Amylolytic activity[4]	Gallate decarboxylase activity[5]
BIOTEC020	5.18 ± 0.05^{ABC}	0.97 ± 0.01^{FGH}	n.d.	-	+
BIOTEC021	5.08 ± 0.04^{C}	0.93 ± 0.01^{GH}	n.d.	-	+
BIOTEC022	5.21 ± 0.08^{AB}	0.91 ± 0.02^{H}	n.d.	-	+
BIOTEC023	5.29 ± 0.06^{A}	0.90 ± 0.09^{H}	n.d.	-	+
BIOTEC024	5.21 ± 0.07^{AB}	1.01 ± 0.02^{EFG}	n.d.	-	+
BIOTEC025	5.20 ± 0.07^{ABC}	0.87 ± 0.02^{H}	n.d.	-	+
BIOTEC026	5.13 ± 0.07^{BC}	0.88 ± 0.02^{H}	n.d.	-	+
BIOTEC027	4.78 ± 0.06^{DEF}	1.29 ± 0.01^{A}	n.d.	-	+
BIOTEC028	4.70 ± 0.04^{EFG}	1.28 ± 0.01^{A}	n.d.	-	+
BIOTEC029	4.58 ± 0.05^{GHIJ}	1.31 ± 0.03^{A}	n.d.	-	+
BIOTEC030	4.62 ± 0.04^{GHIJ}	1.28 ± 0.02^{A}	n.d.	-	+
BIOTEC031	4.58 ± 0.06^{GHIJ}	1.31 ± 0.02^{A}	n.d.	-	+
BIOTEC032	4.80 ± 0.05^{DE}	1.11 ± 0.03^{BCDE}	n.d.	-	+
BIOTEC033	4.71 ± 0.07^{EFG}	1.08 ± 0.03^{BCDE}	n.d.	-	+
BIOTEC034	4.70 ± 0.08^{EFG}	1.07 ± 0.03^{BCDEF}	n.d.	-	+
BIOTEC035	4.67 ± 0.04^{FGHI}	1.13 ± 0.01^{BCD}	n.d.	-	+
BIOTEC036	4.80 ± 0.07^{DE}	1.16 ± 0.04^{B}	n.d.	-	+
BIOTEC037	4.60 ± 0.03^{GHIJ}	1.14 ± 0.03^{AB}	n.d.	-	+
BIOTEC038	4.86 ± 0.04^{D}	1.06 ± 0.02^{BCDEF}	n.d.	-	+
BIOTEC039	4.69 ± 0.06^{EFGH}	1.10 ± 0.02^{BCDE}	n.d.	-	+
BIOTEC040	4.70 ± 0.06^{EFG}	1.06 ± 0.02^{CDEF}	n.d.	-	-
BIOTEC041	4.70 ± 0.08^{EFG}	1.10 ± 0.01^{AB}	n.d.	-	-
BIOTEC042	4.70 ± 0.05^{EFG}	1.10 ± 0.02^{BCDE}	n.d.	+	+
BIOTEC043	4.70 ± 0.06^{EFG}	1.09 ± 0.03^{BCED}	n.d.	-	+
BIOTEC044	4.65 ± 0.05^{FGHIJ}	1.08 ± 0.03^{BCDE}	n.d.	+	+
BIOTEC045	5.20 ± 0.07^{ABC}	0.91 ± 0.03^{GH}	n.d.	-	+
BIOTEC046	4.60 ± 0.04^{GHIJ}	1.07 ± 0.02^{BCDEF}	n.d.	+	+
BIOTEC047	4.66 ± 0.06^{FGHI}	1.12 ± 0.02^{BCD}	n.d.	+	+
299V	4.57 ± 0.05^{HI}	1.04 ± 0.02^{DEF}	n.d.	-	+
LA3	4.52 ± 0.04^{IJ}	1.08 ± 0.03^{BCDE}	n.d.	-	+
GG	4.54 ± 0.05^{J}	1.07 ± 0.02^{BCDEF}	n.d.	-	+

The values measured are means of triplicate measurements ± standard deviation. Means with different letters in the same column indicate significant differences (P ≤ 0.05).
n.d.: Not detected.
[1]pH values of wholewheat SFE after 24 h of growth.
[2]Absorbance of bacterial biomass after 24 h of growth in wholewheat SFE.
[3]α-galactosidase activity was not detected in any species of LAB and yeast.
[4]Amylolytic activity was expressed as + (halo presence) or – (no halo around streak).
[5]Gallate decarboxylase activity was expressed as + (dak yellow to brown color development) or – (light brown).

3.3.4. Proteolytic activities of LAB and yeast isolates

Proteolysis is a relevant enzymatic process during SD fermentation, since improves the nutritional value, digestibility, and bioactivity of the proteins, peptides, and amino acids. FAA are released during primary proteolysis, where LAB and yeast generate proteolytic enzymes. Because cereals have a high protein content, proteolytic enzymes of LAB and yeast become relevant for the release of bioactive peptides and essential AA that promote human well-being and the flavor development of fermented products, such as SD baked bread (Sáez et al., 2018). Proteolytic activities of the species were determined indirectly by the quantification of FAA released during fermentation, and directly by assessing extracellular proteases and endopeptidase activities. LAB and yeast isolates were able to generate a wide range of proteases and consequently FAA (Table 7). Particularly two yeasts, *C. glabrata* BIOTEC025 and *S. cerevisiae* BIOTEC026, showed a significant ($P < 0.05$) proteolytic activity with a FFA concentration of 4.48±0.11 mmol/L and 4.69±0.40 mmol/L respectively. All LAB species had similar results, since the concentration of FAA were in a range of 2.79 – 3.47 mmol/L.

The majority of LAB and yeast isolates displayed extracellular protease activity (Table 7). Only five isolates of LAB, *P. pentosaceus* BIOTEC032, *C. paralimentarius* BIOTEC035, *C. paralimentarius* BIOTEC037, *C. paralimentarius* BIOTEC043 and *P. pentosaceus* BIOTEC044, were negative for this test. There are studies that corroborate the activity of extracellular proteases for LAB; in particular *P. pentosaceus, L. plantarum and P. acidilactici* demonstrated the ability of generating extracellular proteinase activity by using skim milk agar hydrolysis assay (Lim et al., 2019). Furthermore, *L. acidophilus, Bifidobacterium spp., L. casei* and *S. thermophilus* were also reported for the presence of extracellular proteases (Donkor et al., 2007). Yeast species have also been identified for extracellular proteinase activity; *C. albicans, C. glabrata, Y. lipolyitica,* and *S. cerevisiae* are species that have been described for producing extracellular proteases (Ogrydziak, 1993).

Results of endopeptidase activity on Succ-Phe-pNA (Table 7) showed that six LAB (*P. pentosaceus* BIOTEC030, *C. paralimentarius* BIOTEC031, *P. pentosaceus* BIOTEC041, *C. paralimentarius* BIOTEC043, *L. plantarum* 299V and *L. rhamnosus* GG) and one yeast (*C. glabrata* BIOTE023) exhibited significant ($P < 0.05$) enzymatic activity. *P. pentosaceus* BIOTEC041 displayed the highest enzymatic activity between all the LAB and yeast with a value of 2.37 ± 0.05 U/mg, and *C. glabrata* BIOTE023 presented an enzymatic activity of 1.29 ± 0.04 U/mg, the best enzymatic activity for the yeast.

For endopeptidase activity on glut-Phe-pNA (Table 7), the results revealed that seven LAB (*P. pentosaceus* BIOTEC030, *C. paralimentarius* BIOTEC031, *P. pentosaceus* BIOTEC041, *C. paralimentarius* BIOTEC043, *P. pentosaceus* BIOTEC046, *L. plantarum* 299V and *L. rhamnosus* GG) and one yeast (*C. glabrata* BIOTEC023) showed significant ($P < 0.05$) enzymatic activity. The highest enzymatic activity between all the isolates was displayed by *P. pentosaceus* BIOTEC041 with a value of 2.62 ± 0.06 U/mg, and the highest enzymatic activity for yeast isolates was 0.98 ± 0.03 U/mg of *C. glabrata* BIOTEC023.

Sáez et al. (2018) also determined the endopeptidase activity of LAB on chickpea SFE, the results denoted that 3 LAB strains (*Enterococcus mundtii* CRL 2192, *Enterococcus mundtii* CRL 2196 and *Lactococcus garviae* CRL 2199) had higher endopeptidase activity on Glu-Phe-pNA than our work, with values of 7.23 ± 0.13, 3.48 ± 0.3 and 6.19 ± 0.25 U/mg respectively. In the case of endopeptidase activity on Succ-Phe-pNA, a very low activity was recorded for all the species assayed compared to this study, with a maximum value of 0.54 ± 0.01 U/mg for *E. mundtii* CRL 2196. Another study showed the ability of *P. pentosaceus* for generating peptidase activity on fermented faba bean flour, obtaining a result of 2.472 ± 0.067 U/mg; other species that present lowest enzymatic activities were *W. koreensis, W. cibaria, Leuc. mesenteroides* and *L. sakei* (Verni et al., 2017).

Table 7. Proteolytic activities of LAB and yeast isolates.

Isolate	Endopeptidase activity[1]		FAA (mmol/L)[2]	Total proteins (mg)[3]	Extracellular proteases[4]
	Succ-Phe-pNA (U/mg)	Glut-Phe-pNA (U/mg)			
BIOTEC020	0.44 ± 0.02^{HIJK}	0.43 ± 0.03^{CDEF}	3.37 ± 0.08^{BC}	0.27 ± 0.03^{BCDEF}	+
BIOTEC021	0.56 ± 0.04^{GHI}	n.d.	3.28 ± 0.08^{BCD}	0.31 ± 0.01^{B}	+
BIOTEC022	0.36 ± 0.02^{KLM}	0.50 ± 0.05^{HIJ}	3.49 ± 0.12^{BC}	0.26 ± 0.00^{CDEFGHI}	+
BIOTEC023	1.29 ± 0.04^{E}	0.98 ± 0.03^{E}	3.30 ± 0.05^{BCD}	0.27 ± 0.01^{BCDEF}	+
BIOTEC024	0.41 ± 0.03^{IJKL}	0.40 ± 0.05^{BCDEF}	3.55 ± 0.08^{BC}	0.26 ± 0.01^{DEFGHI}	+
BIOTEC025	0.40 ± 0.06^{JKL}	0.44 ± 0.03^{IJKL}	4.48 ± 0.11^{A}	0.22 ± 0.00^{GHIJ}	+
BIOTEC026	0.44 ± 0.07^{HIJK}	0.67 ± 0.03^{FGH}	4.69 ± 0.40^{A}	0.28 ± 0.01^{BCDE}	+
BIOTEC027	0.58 ± 0.01^{GH}	0.47 ± 0.04^{IJK}	3.61 ± 0.20^{B}	0.26 ± 0.00^{BCDEFG}	+
BIOTEC028	0.14 ± 0.01^{NOPQ}	0.14 ± 0.02^{MN}	3.25 ± 0.08^{BCD}	0.25 ± 0.01^{DEFGHI}	+
BIOTEC029	n.d.	0.05 ± 0.02^{N}	3.27 ± 0.03^{BCD}	0.22 ± 0.00^{GHIJ}	+
BIOTEC030	1.84 ± 0.07^{B}	1.87 ± 0.04^{C}	3.47 ± 0.13^{BC}	0.30 ± 0.01^{BC}	+
BIOTEC031	1.84 ± 0.13^{B}	1.67 ± 0.03^{D}	3.21 ± 0.24^{BCD}	0.25 ± 0.01^{DEFGHI}	+
BIOTEC032	0.10 ± 0.01^{OPQ}	0.06 ± 0.04^{N}	3.03 ± 0.08^{CD}	0.21 ± 0.01^{IJ}	-
BIOTEC033	0.90 ± 0.01^{F}	0.76 ± 0.09^{F}	2.79 ± 0.09^{D}	0.23 ± 0.01^{FGHIJ}	+
BIOTEC034	0.02 ± 0.02^{Q}	0.04 ± 0.04^{N}	3.10 ± 0.11^{BCD}	0.26 ± 0.01^{CDEFGH}	+
BIOTEC035	n.d.	n.d.	3.19 ± 0.02^{BCD}	0.19 ± 0.01^{CDEFGH}	-
BIOTEC036	n.d.	n.d.	2.79 ± 0.16^{D}	0.27 ± 0.03^{BCDEF}	+
BIOTEC037	n.d.	0.12 ± 0.01^{MN}	3.44 ± 0.05^{BC}	0.24 ± 0.00^{EFGHIJ}	-
BIOTEC038	0.28 ± 0.03^{LMN}	0.29 ± 0.07^{KLM}	3.52 ± 0.14^{BC}	0.29 ± 0.02^{BCD}	+
BIOTEC039	0.54 ± 0.09^{GHIJ}	0.57 ± 0.06^{GHI}	3.16 ± 0.11^{BCD}	0.21 ± 0.02^{IJ}	+
BIOTEC040	0.05 ± 0.00^{PQ}	0.09 ± 0.01^{N}	3.62 ± 0.05^{B}	0.24 ± 0.00^{EFGHI}	+
BIOTEC041	2.37 ± 0.05^{A}	2.62 ± 0.06^{A}	3.41 ± 0.22^{BC}	0.25 ± 0.01^{DEFGHI}	+
BIOTEC042	0.61 ± 0.08^{G}	0.72 ± 0.04^{FG}	3.19 ± 0.32^{BCD}	0.21 ± 0.01^{IJ}	+
BIOTEC043	1.92 ± 0.02^{B}	2.18 ± 0.05^{B}	3.15 ± 0.03^{BCD}	0.26 ± 0.01^{CDEFG}	-
BIOTEC044	0.02 ± 0.02^{Q}	0.08 ± 0.01^{N}	2.99 ± 0.20^{CD}	0.23 ± 0.01^{FGHIJ}	-
BIOTEC045	n.d.	n.d.	3.08 ± 0.04^{BCD}	0.27 ± 0.03^{BCDEF}	+
BIOTEC046	0.56 ± 0.03^{GHI}	0.97 ± 0.02^{E}	3.37±0.24^{BC}	0.40 ± 0.01^{A}	+
BIOTEC047	0.21 ± 0.02^{NOP}	0.34 ± 0.05^{JKL}	3.37±0.10^{BC}	0.21 ± 0.00 ^{HIJ}	+
299V	1.45 ± 0.03^{D}	1.53 ± 0.19^{D}	3.23±0.43^{BCD}	0.39 ± 0.01^{A}	+
LA3	0.24 ± 0.04^{MNO}	0.28 ± 0.02^{LM}	3.47±0.29^{BC}	0.25 ± 0.02^{DEFGHI}	+
GG	1.63 ± 0.03^{C}	1.69 ± 0.06^{D}	3.02±0.08^{CD}	0.29 ± 0.01^{BCD}	+

The values measured are means of triplicate measurements ± standard deviation. Means with different letters in the same column indicate significant differences (P ≤ 0.05).

n.d.: Not detected

[1]Endopeptidase activities were expressed as U/mg released from each protein substrate.

[2]Indirect proteolytic activity determined by FAA quantification (millimols/L) in wholewheat SFE after 24 h of LAB and yeast growth.

[3]Total protein was expressed as mg from each isolate.

[4]Extracellular proteases were detected by hydrolysis of gelatin and expressed as + (halo presence) or – (no halo around streak).

3.3.5. Leavening capacity of the LAB and yeast isolates

Leavening capacity was evaluated as the increase in volume (mL) after 24 h of wholewheat SD fermentation. Yeast isolates presented a higher leavening capacity compared with LAB isolates (Table 8). *S. cerevisiae* BIOTEC026 showed the maximum leavening capacity, with a result of 9.17 ± 0.72 mL. Moreover, *C. glabrata* BIOTEC021 and *C. glabrata* BIOTEC026 obtained similar results for the leavening ability, with values of 8.75 ± 1.25 mL and 8.33 ± 1.44 mL, respectively. The LAB isolates showed a very low leavening capacity, since *P. pentosaceus* BIOTEC032, exhibited the maximum increasing volume with 4.17 ± 0.72 mL and *C. paralimentarius* BIOTEC035 the minimum increasing volume of 1.25 ± 0.00 mL.

Palla et al. (2020) determined the leavening capacity of *S. cerevisiae* in yellow grain wheat SD and blue grain wheat SD. The results indicated a leavening capacity of 12 mL and 9.6 mL, respectively, after 24 hrs of fermentation. Another yeast specie evaluated was *Pichia fermentans*, which showed an increasing volume of 6.0 mL for yellow grain wheat and 7.2 mL for blue grain wheat after 24 h of fermentation. Therefore, the main role of yeast in bread-making is their ability of producing gas, specially CO_2, to ensure a uniform dough leavening; whereas LAB are important for the acidification of the dough and generation of flavors (Palla et al., 2020). In the baking industry, *S. cerevisiae* is the species of choice due to its high fermentation capacity, indeed, it is characterized by a rapid consumption of sugars and fast CO_2 production, which are two of the most important qualities for dough leavening (Zhou et al., 2017).

After 24 h, the pH of the wholewheat SD for each isolate was monitored (Table 8). LAB isolates demonstrated the ability to acidify the doughs more than yeast, since their pH was in a range of 3.57 – 3.71, whereas pH from yeasts was between 3.76 – 3.98. Moreover, the control wholewheat SD control obtained a pH value of 4.52 ± 0.10. Thus, it is corroborated that a SD with a starter culture of LAB acidify more than a yeast SD or a spontaneous SD which it is fermented with the flour and environmental microbiota.

3.3.6. Total phenolic content

Capacity of LAB and yeast isolates to release phenolic compounds was determined after 24 h of wholewheat SD fermentation (Table 8). The assay showed the ability of most LAB isolates to release more phenolic compounds that yeast isolates. The best LAB isolates to release phenolic compounds were *P. pentosaceus* BIOTEC039 (549.55 ± 18.35 µg/g), *P. pentosaceus* BIOTEC046 (575.41 ± 20.90 µg/g) and *L. plantarum* 299V (547.84 ± 66.92 µg/g). In the case of the yeasts, *H. opuntiae* displayed the highest phenolic content (472.35 ± 2.30 µg/g), and the spontaneous SD control showed a phenolic content of 220.63 ± 3.28 µg/g Hence, the use of LAB and yeast isolates as stater cultures exhibited the capacity to increase the concentration of phenolic compounds compared to a spontaneous wholewheat SD.

Rizzello et al. (2016) evaluated the released phenolic compounds in quinoa SD fermented with *L. plantarum* and *L. rossiae* versus a spontaneous quinoa SD. In the case of *L. plantarum* and *L. rossiae* as stater cultures, a concentration of 2.75 mg of gallic acid equivalents/g was obtained, and for *the spontaneous quinoa* SD a value of 1.39 mg of gallic acid equivalents/g was found. *S. cerevisiae* has also been assayed for total phenolic compounds in yellow grain wheat SD and blue grain wheat SD by the research of Palla et al. (2020). The results indicated a phenolic concentration of 0.80 ± 0.40 mg of gallic acid equivalents/g for yellow grain wheat SD and 0.90 ± 0.30 mg of gallic acid equivalents/g for blue grain wheat SD. LAB acidification improves the extraction of phenolic compounds when selected starters cultures are used. Esterase activities, able to hydrolyze complex phenolic compounds and their glycosylated form into their corresponding phenolic acids during SD fermentation has been widely described for LAB (Rizzello et al., 2016). Furthermore, yeasts have an important role in increasing the polyphenol content in cereal products by releasing bound and conjugated phenolic acids to free forms after cell wall degradation processes (Palla et al., 2020). Therefore, SD fermentation using starter cultures could increase the concentration of total phenolic compounds compared with spontaneous SD or traditional leavened bread.

3.3.7. Phytase activity

The ability of degrading phytic acid by phytase enzyme of LAB and yeast isolates in wholewheat SD was evaluated after 24 h of fermentation (Table 8). In general, LAB isolates showed a higher phytase activity compared with yeast isolates and spontaneous wholewheat SD. The highest enzymatic activity was displayed by *P. pentosaceus* BIOTEC044 with a result of 15.63 ± 0.03 U. *C. glabrata* BIOTEC021 and *S. cerevisiae* BIOTEC026 showed the best enzymatic activity, with values of 6.68 ± 0.16 U and 6.58 ± 0.14 U, respectively, while the control sample (spontaneous wholewheat SD) exhibited a phytase activity of 3.92 ± 0.07 U. Hence, the use of LAB and yeasts as starter cultures for SD fermentation improve the nutritional feature of cereals by decreasing phytic acid content in comparison with a spontaneous SD.

Rizzello et al. (2016) assayed phytase activity in quinoa SD fermented with *L. plantarum* and *L. rossiae* as starter cultures and compared with a spontaneous quinoa SD. The phytase activity using starter cultures was of 4.76 ± 0.55 U and for the spontaneous SD of 1.73 ± 0.72 U. *S. cerevisiae* was also evaluated for phytase activity in grain yellow wheat SD and blue grain yellow wheat SD (Palla et al. ,2020). In yellow grain wheat SD, a phytase activity of 8.00 ± 0.05 U was found and for blue grain wheat SD, a value of 15.90 ± 0.10 U was obtained. Phytase catalyzes the hydrolysis of phytic acid into myoinositol and phosphoric acid, making available phosphate and leading to non-metal chelator compound that causes the availability of minerals. Besides, the drop of pH caused by LAB starters during fermentation is suitable to activate flour endogenous phytases (Rizzello et al., 2016). Another important situation to highlight is the type of flour used for SD fermentation, since the phytase activity is strongly affected by the quantity of endogenous phytases of the cereal flours (Palla et al., 2020). Consequently, the use of LAB and yeast starters may increase the phytase activity of a SD, by their ability to generate phytases and by activating endogenous cereal flour phytases during SD fermentation.

Table 8. Biotechnological properties of LAB and yeast in a wholewheat sourdough.

Isolate	Sourdough pH[1]	Leavening capacity (mL)[2]	Phytase activity (U)[3]	Total phenolics (µg/g)[4]
BIOTEC020	3.97 ± 0.30^{B}	6.42 ± 0.29^{BCD}	5.15 ± 0.06^{KLM}	273.69 ± 33.19^{LM}
BIOTEC021	3.83 ± 0.06^{BCDE}	8.75 ± 1.25^{AB}	6.68 ± 0.16^{IJ}	378.20 ± 17.28^{GHIJK}
BIOTEC022	3.82 ± 0.02^{BCDEF}	7.17 ± 1.77^{ABC}	4.14 ± 0.10^{NO}	376.85 ± 44.86^{GHIJK}
BIOTEC023	3.76 ± 0.06^{BCDEFG}	7.08 ± 0.72^{ABC}	3.37 ± 0.04^{O}	394.69 ± 6.52^{FGHIJK}
BIOTEC024	3.81 ± 0.07^{BCDEF}	6.58 ± 0.80^{BCD}	4.57 ± 0.11^{LMN}	359.01 ± 42.61^{IJK}
BIOTEC025	3.86 ± 0.14^{BCD}	8.33 ± 1.44^{AB}	4.48 ± 014^{MN}	410.45 ± 7.30^{EFGHIJ}
BIOTEC026	3.85 ± 0.10^{BCD}	9.17 ± 0.72^{A}	6.58 ± 0.14^{IJ}	330.811 ± 5.96^{JKL}
BIOTEC027	3.67 ± 0.06^{DEFG}	1.25 ± 0.00^{H}	14.67 ± 0.29^{B}	433.78 ± 7.99^{DEFGHI}
BIOTEC028	3.68 ± 0.02^{CDEFG}	1.25 ± 0.00^{H}	14.89 ± 0.10^{AB}	435.86 ± 24.06^{DEFGHI}
BIOTEC029	3.57 ± 0.01^{G}	2.08 ± 0.72^{FGH}	12.38 ± 0.16^{EFG}	405.50 ± 6.08^{EFGHIJ}
BIOTEC030	3.57 ± 0.01^{G}	1.67 ± 0.72^{GH}	4.53 ± 0.25^{LMN}	502.43 ± 44.19^{ABCD}
BIOTEC031	3.59 ± 0.03^{FG}	2.92 ± 1.44^{FGH}	9.81 ± 0.14^{H}	469.73 ± 40.44^{BCDEF}
BIOTEC032	3.65 ± 0.11^{DEFG}	4.17 ± 0.72^{DEF}	12.64 ± 0.40^{DEF}	317.12 ± 27.44^{KL}
BIOTEC033	3.61 ± 0.02^{EFG}	3.33 ± 0.72^{EFGH}	11.64 ± 0.23^{G}	454.78 ± 9.58^{CDEFG}
BIOTEC034	3.60 ± 0.01^{FG}	1.67 ± 0.72 ^{GH}	12.96 ± 0.35^{CDE}	386.22 ± 6.39^{GHIJK}
BIOTEC035	3.60 ± 0.01^{EFG}	1.25 ± 0.00^{H}	9.79 ± 0.12^{H}	364.14 ± 17.92^{HIJK}
BIOTEC036	3.70 ± 0.14^{CDEFG}	1.67 ± 0.72^{GH}	10.52 ± 0.02^{H}	520.00 ± 13.81^{ABC}
BIOTEC037	3.62 ± 0.03^{EFG}	1.42 ± 0.29^{GH}	12.24 ± 0.14^{EFG}	483.51 ± 44.02^{BCDE}
BIOTEC038	3.69 ± 0.08^{CDEFG}	1.25 ± 0.00^{H}	13.58 ± 0.40^{C}	504.41 ± 9.22^{ABCD}
BIOTEC039	3.66 ± 0.06^{DEFG}	1.25 ± 0.00^{B}	14.46 ± 0.33^{B}	549.55 ± 18.35^{AB}
BIOTEC040	3.61 ± 0.02^{EFG}	1.67 ± 0.72 ^{H}	13.42 ± 0.20^{CD}	482.79 ± 29.49^{BCDE}
BIOTEC041	3.71 ± 0.09^{CDEFG}	3.33 ± 0.72 ^{EFGH}	11.69 ± 0.15^{G}	473.87 ± 14.88^{BCDEF}
BIOTEC042	3.67 ± 0.06^{DEFG}	1.25 ± 0.00^{H}	11.80 ± 0.07^{FG}	376.67 ± 13.31^{GHIJK}
BIOTEC043	3.62 ± 0.01^{EFG}	1.67 ± 0.72 ^{GH}	5.96 ± 0.16^{JK}	444.14 ± 13.50^{CDEFGH}
BIOTEC044	3.60 ± 0.02^{EFG}	1.67 ± 0.72 ^{GH}	15.63 ± 0.03^{A}	384.60 ± 11.10^{GHIJK}
BIOTEC045	3.90 ± 0.08^{BC}	1.67 ± 0.72 ^{GH}	4.68 ± 0.18^{LMN}	472.35 ± 2.30^{BCDEF}
BIOTEC046	3.64 ± 0.04^{DEFG}	2.92 ± 0.72 ^{FGH}	14.62 ± 0.49^{B}	575.41 ± 20.90^{A}
BIOTEC047	3.65 ± 0.04^{DEFG}	3.75 ± 0.00 ^{EFG}	13.42 ± 0.18^{CD}	318.11 ± 8.27^{KL}
299V	3.57 ± 0.02^{G}	1.25 ± 0.00^{H}	7.17 ± 0.08^{I}	547.84 ± 66.92^{AB}
LA3	3.57 ± 0.04^{G}	1.25 ± 0.00^{H}	6.69 ± 0.15^{IJ}	395.68 ± 20.16^{FGHIJK}
GG	3.58 ± 0.04^{G}	1.67 ± 0.72^{GH}	5.37 ± 0.59^{KL}	420.18 ± 24.15^{EFGHI}
Control	4.52 ± 0.10^{A}	5.42 ± 0.72^{CDE}	3.92 ± 0.07^{NO}	220.63 ± 3.28^{M}

The values measured are means of triplicate measurements ± standard deviation. Means with different letters in the same column indicate significant differences ($P \leq 0.05$).

[1]pH values of wholewheat SD after 24 h of fermentation.

[2]Leavening capacity of each specie measured as increase of volume (mL) after 24 h of fermentation.

[3]Phytase activity was expressed as U.

[4]Total phenolics were expressed as mg of gallic acid equivalents/g.

3.3.8. Cluster and PCA Analysis

All data from biotechnological and functional properties were used as input variables to run a cluster analysis and divide in homogenous groups. Cluster dendrogram is shown in Figure 2. Two main clusters can be identified, one with the yeast isolates, and the second group is made up with the LAB isolates. Furthermore, it can be noted that the two main clusters are divided into seven sub-clusters which are grouped according to the results of the biotechnological properties for each LAB and yeast isolate. Thus, these clusters may support the selection of some microorganisms to define a single or coculture starter in the formulation of a SD bread.

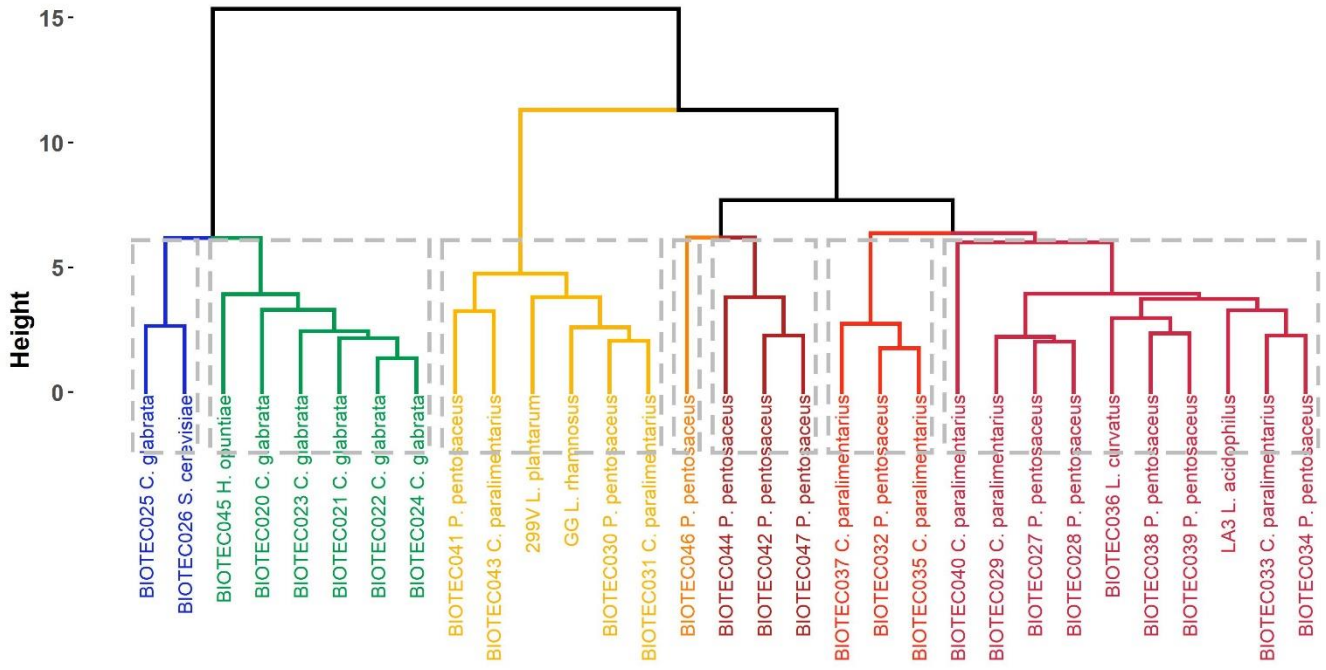

Figure 2. Cluster analysis of wholewheat SD isolates and commercial probiotics. The differences were computed as dissimilarities, and all measured data from different assays performed were used as input variables.

A PCA was performed for all properties of LAB and yeast isolates (Figure 3). It can be observed that leavening capacity, FAA, pH of SFE and pH of SD were grouped on the dimension 1 axis, being leavening capacity and pH of SFE the parameters with the biggest contributions. Total protein content and endopeptidase activity on Glut-Phe-pNA and Succ-Phe-pNA were correlated positively on dimension 2, and endopeptidase activity on Glut-Phe-pNA and Succ-Phe-pNA represented the biggest contribution to this dimension. Growth of the microorganisms (OD_{560nm}) represented a significant contribution to dimension 1 and a completely negative correlation to leavening capacity. Phytase activity had an important contribution on both dimensions, and presented a negative correlation with leavening capacity. Lastly, total phenolic compounds showed a small contribution to both dimensions and does not present any correlation with the other parameters. Since leavening capacity, pH of SFE, phytase activity, OD_{560nm} and proteolytic activities are the parameters that represent the biggest proportion of the total variation of the system, then, these parameters should be considered for designing microbial consortiums (single or coculture starter) in the formulation of SD breads with enhanced biotechnological properties.

Furthermore, a mixed model analysis was realized to evaluate the contribution of qualitive and quantitative parameters to the data (Figure 4). It corroborates the results from the Figure 3, since endopeptidase activity has the greatest contribution to dimension 2, leavening capacity and pH of SFE on the dimension 1 and phytase activity on both dimensions. However, it is important to note the small contribution to the total variation of the qualitative assays (amylolytic activity, gallate decarboxylase activity and presence of extracellular proteases). Therefore, the qualitative assays may not be considered in the design of LAB and yeast clusters for the elaboration of SD breads.

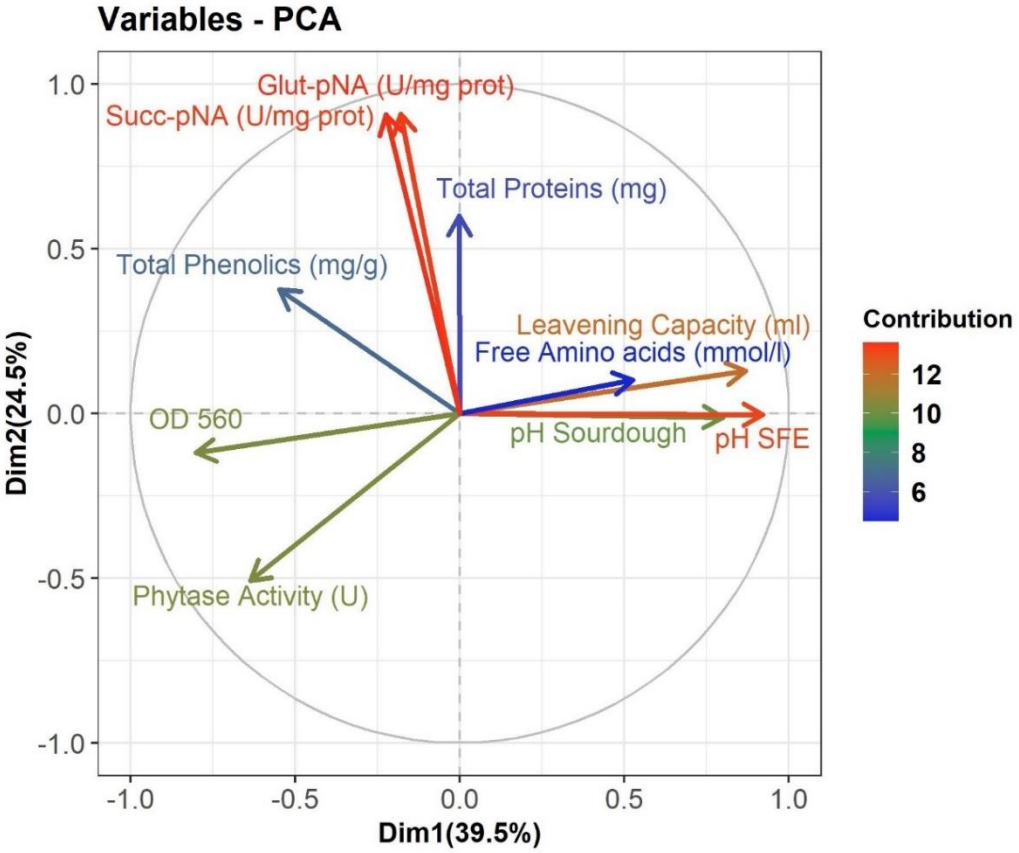

Figure 3. Bioplot for biotechnological and functional properties of LAB and yeast isolates.

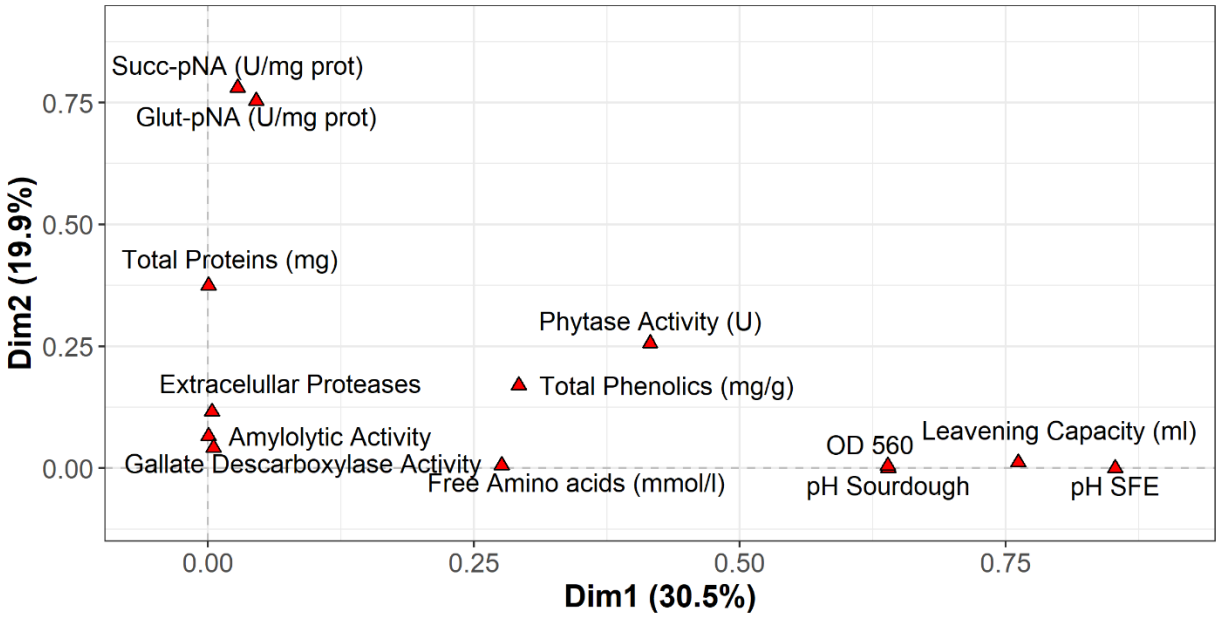

Figure 4. Mixed analysis for evaluating the contribution of qualitive and quantitative parameters to the data.

The interactions of LAB and yeast isolates were evaluated in a matrix plot according to the results of the biotechnological and functional properties (Figure 5). Depending on the score, a good or bad interaction between two isolates is determined. Hence, a score of 6 represents the best interaction, a score of 5 – 4 denotes a good interaction, a score of 4 – 2 indicates a bad interaction, and a score 2 – 0 implies the worst interaction. The majority of the interactions between LAB and yeast isolates were in the range of 4 – 6, whereas the interaction between the same yeast isolates or LAB isolates were in the range of 2 – 0. Therefore, a matrix plot simulating the interaction between two isolated microorganisms may help to create microbial consortiums for the design of SD breads with specific biotechnology properties.

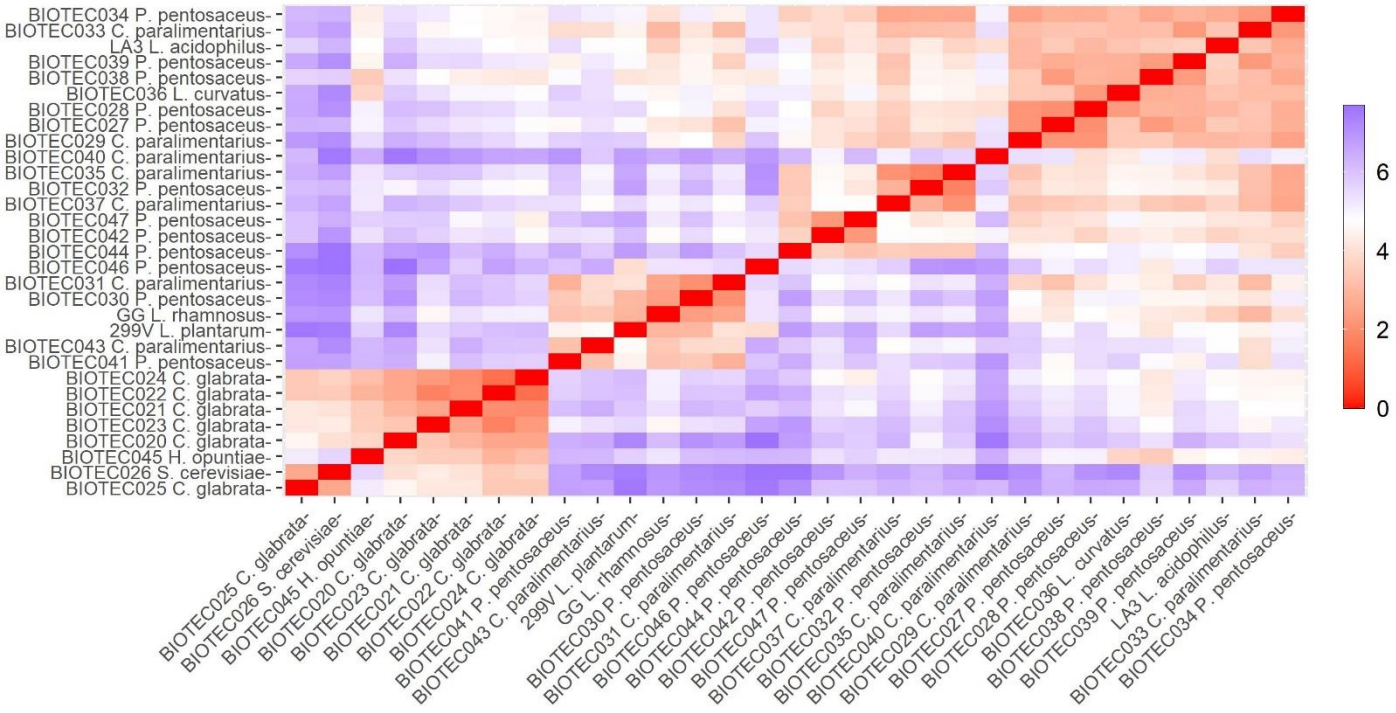

Figure 5. Matrix plot that shows the interaction of each LAB and yeast isolate with the others.

3.4. Chapter conclusions

LAB and yeast microorganisms were isolated and identified from a spontaneous wholewheat SD and raspberry. The main identified species were *Candida glabrata, Saccharomyces cerevisiae, Pediococcus pentosaceus, Companilactobacillus paralimentarius* and *Latilactobacillus curvatus*. Biotechnological properties, proteolytic activities, leavening capacity, total phenolic compounds and phytase activities were assayed for the 25 isolated microorganisms and 3 commercial probiotic strains. LAB and yeasts were capable of releasing FAA by the action of proteases, furthermore, most of the isolates presented the ability of generating extracellular proteases. Gallate decarboxylase activity was shown in most of the LAB and yeast isolates. In specific, a few LAB isolates showed endopeptidase activity. Yeast isolates exhibited a high leavening capacity in wholewheat SD, particularly *S. cerevisiae* BIOTEC026. The ability to release phenolic compounds was displayed by LAB and yeast isolates used as stater cultures in wholewheat SD versus a spontaneous SD. LAB isolates showed a high phytase activity in a wholewheat SD compared with yeast isolates and spontaneous SD. Therefore, the use of selected LAB and yeast isolates as starter cultures improves the properties of a SD compared with a spontaneous fermented SD. Moreover, the results from statistical analysis may help to generate microbial consortiums of LAB and yeasts with the purpose of developing SD bread with enhanced technological and healthy properties.

CHAPTER 4. DEVELOPMENT OF FUNCTIONAL BREADS USING DEFINED MICROBIAL STARTERS AND SOURDOUGH

4.1. Introduction

Bread is one of the most consumed bakery products worldwide with an average per capita consumption of 24.5 kg in 2020 and an expected market growth annually by 2.8% (Garzon et al., 2021). Furthermore, there is strong research interest in bakery products as vehicle for delivering functional components that can enhance the nutritional profile and health properties of the final product. (Fekri et al., 2020; Garzon et al., 2021). Those functional components include fibers, antioxidant phytochemicals, minerals; and additionally the so-called postbiotic-like components which refer to a group of inanimate microorganisms and/or their components such as cell debris, short chain fatty acids (SCFAs), bacteriocins, biosurfactants, secreted proteins/peptides, amino acids, flavonoids, and exopolysaccharides, among other molecules (Salminen et al., 2021)

In recent years the consumer preference for SD bread instead of a baker yeast leavened bread has increased. Moreover, the addition of lactic acid bacteria (LAB) and yeast starter cultures to SD fermentation have shown positive effects in both the dough and the resulting bread by extending the shelf life through the inhibition of spoilage fungi and bacteria, increasing the loaf volume, increasing resistant starch formation in bread, delaying staling, reducing antinutritional factors, improving the bread flavor and enhancing the nutritional properties (Tamani et al., 2013).

To guarantee the biotechnological and health properties of baking products while allowing the standardization of the process, a defined microbial consortium with LAB and yeast and their interactions should be considered. These interactions between different species of microorganisms in the same environment are complicated and, in some cases, are vital to improve their fermentation performances. For example, *Saccharomyces cerevisiae* could produce beneficial factors to stimulate LAB growth during SD fermentation and vice versa. However,

these microorganisms might also influence or inhibit the cell growth of each other. In the case of LAB, there are three major factors through which could influence yeast cell growth. The first one is living space occupancy, especially when the living space of yeast was compressed by the aggressive growth of LAB. The second is the competition of LAB and yeast for essential nutriments for cell growth and product conversion. At least and more importantly, LAB can secrete metabolites such as lactic or acetic acids to inhibit yeast growth (Ding et al., 2021).

In this chapter microbial consortiums of LAB and yeasts were generated and used as starter cultures for wheat SD and dough fermentations. These consortiums were designed based on the Cluster and PCA analysis of the previous chapter. For example, with the matrix plot of chapter 3 (Figure 5), the interactions between LAB and yeast isolates can be projected and a decision may be taken for creating a microbial consortium (single or coculture starters). Furthermore, the PCA analysis (Figure 4) showed that the leavening capacity is one of the main contributory factors for the variability of the system, hence, the LAB and yeast isolates with the highest leaving capacity should be considered as starter cultures (single or coculture) for bread formulation.

Thus, the main objective of this chapter was the development of functional wheat breads through the combined use of a defined microbial consortium and the SD fermentation technique. The impact on the biotechnological, physicochemical, sensorial and postbiotic properties was evaluated. The research was divided into different phases: (1) a preliminary trial to select from all isolates characterized in Chapter 3, the best microbial candidates to be used as starter culture; and (2) use of the most promising starter cultures and SD for bread-making.

4.2. Methodology

4.2.1 Preliminary assays

Preliminary assays were performed to evaluate the interactions between LAB and yeast isolates for the design of the final microbial consortiums. Based on the PCA analysis was observed that the leavening capacity is one of the main contributory factors for the variability of the system. Thus, *S. cerevisiae* BIOTEC026 and *Candida glabrata* BIOTEC021 were the selected yeast isolates, whereas *Pediococcus pentosaceus* BIOTEC032 and *Companilactobacillus paralimentarius* BIOTEC033 were the selected LAB isolates. Furthermore, *Hanseniaspora opuntiae* BIOTEC045 and *Latilactobacillus curvatus* BIOTEC036 were also select for the assays, with the objective of having 3 different species of LAB and yeast, and the possible properties it could bring to the bread. For the bread formulation (Tables 9 and 10), 6 treatments were designed based on the microbial consortium used as starter cultures for the dough fermentation, and the addition of SD for 3 treatments. The microbial consortium for each treatment was based in the combination of the 3 yeast isolates with 1 LAB isolate as the differential factor (see Tables 9 and 10). The properties evaluated were the dough pH, and the LAB and yeast counts after overnight dough fermentation. The methodology for breadmaking and pH and microbial counts is described in section 4.2.2.

Table 9. Bread formulation for the preliminary studies.

Ingredients	**1**	**2**	**3**
Wheat flour (g)	100	100	100
Water (g/100 g of wheat)	68	68	68
Sugar (g 100 g of wheat)	5	5	5
Salt (g/100 g- of wheat)	1.8	1.8	1.8
Corn oil (g /100 g- of wheat)	5	5	5
Baker yeast (g /00 g- of wheat)	0.5	0.5	0.5
Microbial inoculum (log CFU/g)			
Yeats (*S. cerevisiae, C. glabrata* and *H. opuntiae*)	5	5	5
C. paralimentarius	7	-	-
L. curvatus	-	7	-
P. pentosaceus	-	-	7

Table 10. SD bread formulation for the preliminary studies.

Ingredients	1 SD	2 SD	3 SD
Wheat flour (g)	100	100	100
Water (g/100 g of wheat)	63.42	63.42	63.42
Sugar (g/100 g of wheat)	5	5	5
Salt (g/100 g of wheat)	1.8	1.8	1.8
Corn oil (g/100 g of wheat)	5	5	5
Baker yeast (g/100 g of wheat)	0.5	0.5	0.5
Wheat SD			
Wheat flour (g 100 g^{-1} of wheat)	15	14.29	14.29
Water (g 100 g^{-1} of wheat)	15	14.29	14.29
Microbial inoculum in wheat SD (log CFU/g)			
Yeats (*S. cerevisiae, C. glabrata* and *H. opuntiae*)	5	5	5
C. paralimentarius	7	-	-
L. curvatus	-	7	-
P. pentosaceus	-	-	7

4.2.2 Breadmaking process

4.2.2.1 Preparation of LAB and yeast starter cultures

LAB and yeast isolates (*P. pentosaceus* BIOTEC035*, S. cerevisiea* BIOTEC026*, C. glabrata* BIOTEC021 and *H. opuntiae* BIOTEC045) were cultivated in MRS and YEPD broth, respectively, overnight at 30 °C. Cells were harvested by centrifugation (4,000 rpm, 10 min, 4 °C), and washed twice in sterile saline solution (0.85% w/v). A biomass of 7.0 and 5.0 Log CFU/g of dough for LAB and yeasts, respectively, were used as starters for dough fermentation.

4.2.2.2 Wheat sourdough elaboration

Four wheat SDs (Control, Yeast, Bac+Yeast and Bacteria) were prepared by mixing 50 g of wheat flour and 50 mL of tap water during 3 min, obtaining a dough yield of 200 (dough yield=dough weight×100/flour weight). LAB and yeast starter cultures were inoculated at the concentration previously mentioned. SDs were fermented at 30 °C for 24 h.

4.2.2.3 Breadmaking procedure

Traditional breads were formulated with a basic recipe based on wheat flour: 68% water, 10 % sucrose, 10% oil, 2% baker yeast, 1.8% salt; and LAB and yeast starter cultures were inoculated at the concentration previously mentioned. Four types of traditional bread (Table 11) were produced as follows: Control, Yeast

Bac+Yeast, and Bacteria. SD bread were prepared with the same recipe but adding 15% of wheat sourdough. Four types of SD bread (Table 12) were elaborated as follows: Control SD, Yeast SD Bac+Yeast SD and Bacteria SD. The process for generating and baking the doughs was the following (Figure 6). First, the water and wheat flour were mixed, and the resulting dough was allowed to rest for 1 h at room temperature to activate the of endogenous enzymes (autolysis process). After that, the dough was mixed and kneaded with the rest of the ingredients (KitchenAid^{tm} ST. Josheph, Michigan USA) up to reach the optimum consistency. The dough was incubated at 30 °C for 2 h, and every 30 min was kneaded by hand to promote the expansion of the dough and the formation of the gluten strands. After 2 h, the dough was placed in the fridge at 4 °C overnight to promote a slow fermentation process. The next day, the dough was rested 30 min at 30 °C after which, it was shaped manually, placed into metallic pan, and allowed to ferment for 1.5 h at 30 °C or till the volume double itself. Finally, fermented dough was baked at 200 °C for 5 min and 180 °C for 25 min, and then cooled down at room temperature for 1 h.

Table 11. Recipe for traditional bread formulation.

Ingredients	Control	Yeast	Bac+Yeast	Bacteria
Wheat flour (g)	100	100	100	100
Water (g /100 g of wheat)	68	68	68	68
Sugar (g/100 g of wheat)	5	5	5	5
Salt (g/100 g of wheat)	1.8	1.8	1.8	1.8
Safflower oil (g/100 g of wheat)	5	5	5	5
Baker yeast (g/100 g of wheat)	0.75	0.75	0.75	0.75
Microbial inoculum (log CFU/g)				
Yeasts (*S. cerevisiae, C. glabrata* and *H. opuntiae*)	-	5	5	-
LAB (*P. pentosaceus*)	-	-	7	7

Table 12. Recipe for SD bread formulation.

Ingredients	Control SD	Yeast SD	Bac+Yeast SD	Bacteria SD
Wheat flour (g)	100	100	100	100
Water (g /100 g of wheat)	63.42	63.42	63.42	63.42
Sugar (g/100 g of wheat)	5	5	5	5
Salt (g/100 g of wheat)	1.8	1.8	1.8	1.8
Safflower oil (g/100 g of wheat)	5	5	5	5
Baker yeast (g/100 g of wheat)	0.75	0.75	0.75	0.75
Wheat SD				
Wheat flour (g/100 g of wheat)	15	15	15	15
Water (g/100 g of wheat)	15	15	15	15
Microbial inoculum in wheat SD (log CFU/g)				
Yeasts (*S. cerevisiae, C. glabrata* and *H. opuntiae*)	-	5	5	-
LAB (*P. pentosaceus*)	-	-	7	7

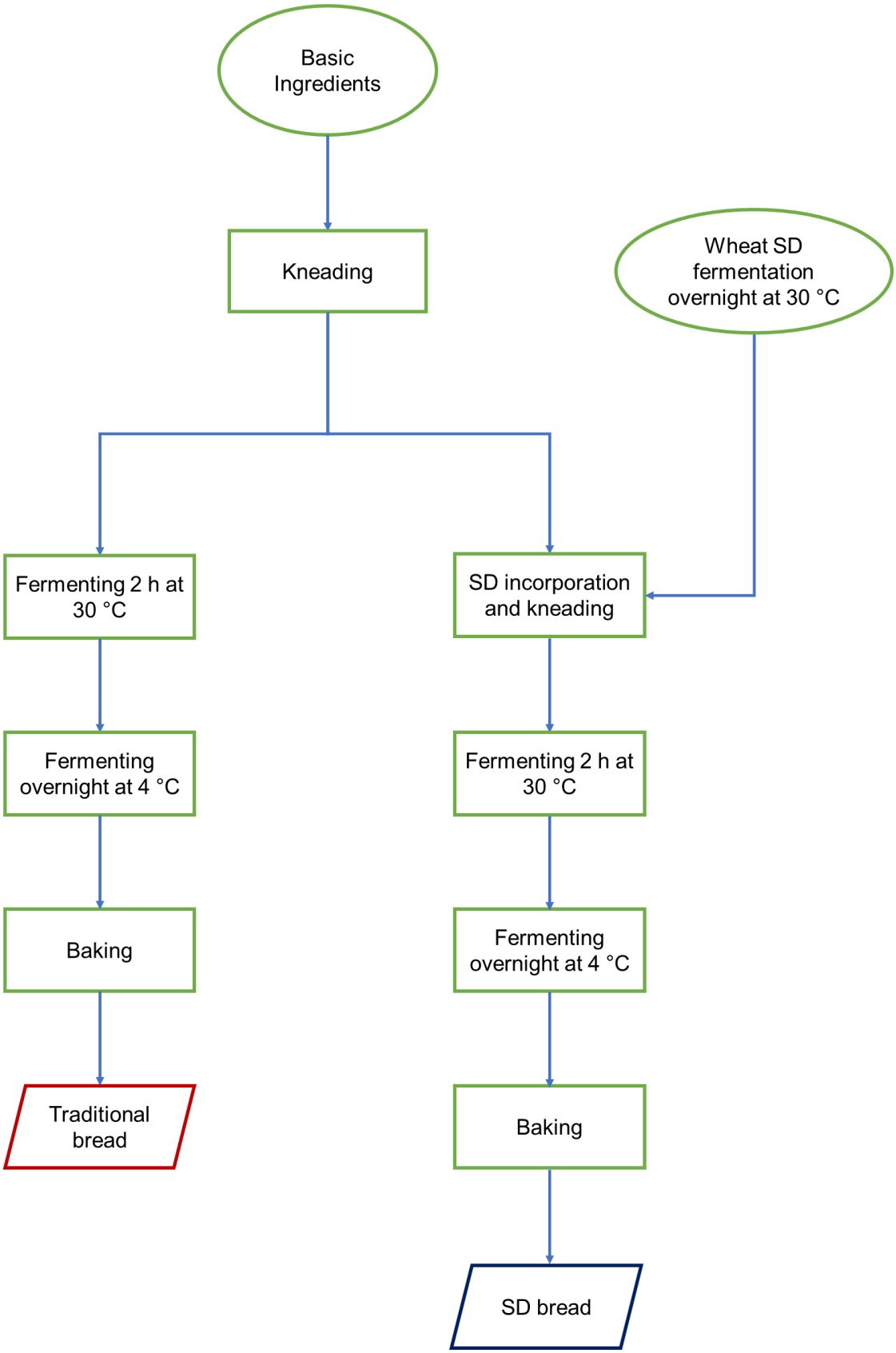

Figure 6. Flowchart of traditional bread and SD bread-making.

4.2.3. Microbiological and acidification features

4.2.3.1. Enumeration of LAB and yeasts

LAB and yeast cell counts were made in petri plates with MRS agar and PDA, respectively. One gram of dough sample was suspended with 9 mL of sterile saline solution (0.85% NaCl) and homogenized in vortex (VortexGENE™) during 5 min. Decimal dilutions were made and 10 µL of these suspensions were plated in MRS agar and PDA. The incubation conditions for the yeasts were 30 °C in aerobiosis for 48 h. In the case of LAB, plates were incubated in anaerobiosis at 37 °C for 48 h. Plate counts were performed in duplicate and expressed as CFU/g of dough.

4.2.3.2. pH and total titratable acidity

The pH of the fermented dough and baked bread were determined using a pH meter (LAQUAact™). Total titratable acidity (TTA) was measured according to the protocol of (Fekri et al., 2020) in the dough and baked bread samples. Briefly, 10 g of sample was mixed with 90 mL of distilled water and was titrated with 0.1 N NaOH to a final pH of 8.5. TTA was expressed as the consumed volume of NaOH (mL).

4.2.4. Postbiotic properties

The postbiotic-like properties measured for the traditional and SD bread were the following: total phenolic content, phytase activity, antioxidant activity and presence of exopolysaccharides (EPS).

4.2.4.1 Total phenolic content

The analysis of total phenolic content was conducted on the methanolic extracts (ME) of the fermented doughs and the baked bread samples, using the Folin–Ciocalteu technique. To obtain the ME, 1 g of each fermented dough was mixed with 3 mL of methanol. The mixture was centrifuged at 4,000 rpm for 5 min at 4 °C. ME was transferred into test tubes and the sediments were mixed with 4 mL of ethanol and centrifuged at the previous condition. This step was repeated two times, obtaining 10 mL of ME as final volume.

The Folin–Ciocalteu reaction contained 50 µL of ME, 250 µL of Folin-Ciocalteu reagent and 450 µL of distilled water. After 10 min of incubation at room temperature in darkness, 1.25 mL of a 20% (w/v) sodium carbonate solution was added. The mixture was incubated at the previous conditions for 30 min and the absorbance was measured at 765 nm. The concentration of total phenolics was calculated as gallic acid equivalents (GAE) with a calibration curve.

4.2.4.2 Antioxidant activity

Two assays were applied to evaluate the antioxidant activity based on antiradical activities: radical scavenging capacity activity (DPPH) and radical cation scavenging activity (ABTS) according to the methods of Frías-Moreno et al. (2021) and Rajurkar & Hande (2011), with some modifications. ABTS solution was prepared by mixing 7 mM of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with 140 mM potassium persulfate solution. ABTS solution was rested for 16 h in darkness to complete the reaction. The solution was diluted to a concentration that would give 0.7 ± 0.02 at 734 nm. The ABTS activity was measured by mixing 34 µL of ME with 1044 µL of ABTS solution. The mixture was incubated 6 min in darkness and then the absorbance was measured at 734 nm. DPPH solution was prepared 30 min prior the analysis by mixing 1.5 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with 60 mL of ethanol. DPPH activity was determined by mixing 140 µL of ME with 910 µL of DPPH solution and allowed to rest 5 min before reading the absorbance at 516 nm. Results were reported in µg of ascorbic acid equivalents (AAE)/g of bread.

4.2.4.3. Phytase activity

To measure phytase activity, first water/salt-soluble extracts (WSE) of the fermented doughs were prepared according to Weiss et al. (1993). Briefly, 100 mg of sample were extracted with 1 mL of Tris-HCl buffer (50 mM, pH 8.8) during 1 h a 4 °C, with vortexing intervals each 15 min. The samples were centrifuged (13,500 rpm for 20 min), and the supernatants with the WSE were carefully removed. Phytase activity was evaluated by monitoring the rate of hydrolysis of *p*-nitrophenyl

phosphate (*p*-NPP) as stated by Palla et al. (2020). The assay contained 400 µL of WSE, and 200 µL of 1.5 mM *p*-NPP in 0.2 M of Na-acetate, pH 5.2. The mixture was incubated for 10 min at 45 °C and the reaction was stopped by adding 600 µL of NaOH 0.1 M. The *p*-nitrophenol released was measured by absorbance at 405 nm. One unit (U) of activity was defined as the amount of phytase required to liberate 1 µmol/min of *p*-nitrophenol under assay conditions.

4.2.4.4 Isolation and quantification of exopolysaccharides

The isolation of exopolysaccharides (EPS) from fermented dough and baked bread samples were made according to the protocol of Zisu & Shah (2003) with some modifications. Samples (100 mg) were mixed with 1 mL of 20% (w/v) trichloroacetic acid solution overnight at 4 °C. Then, the mixture was centrifuged (10,000 rpm, 20 min) to remove the precipitated proteins and biomass. The supernatant was neutralized to pH 6.8 with 4 M NaOH and boiled for 30 min in a water bath and recentrifuged (10,000 rpm, 20 min) to discard the remaining insoluble proteins. One mL of ethanol (96% v/v) was added to the supernatant to precipitate EPS from the solution at 4 °C overnight. The pellet with the crude EPS was recovered by centrifugation (10,000 rpm, 20 min) and resuspended in 1 mL of ethanol (96% v/v) to remove remain contaminants. The suspension with the EPS was centrifuged at 10,000 rpm, 20 min. The recovered pellet was suspended in 1 mL of distilled water and homogenized in vortex (VortexGENE^{TM}) for 3 min. EPS were quantified using the phenol sulphuric method of Dubois et al. (1951). Briefly, 1 mL of crude EPS were mixed with 0.05 mL of 80% (w/v) of phenol solution. Subsequently, 5 mL of 96% (v/v) of sulphuric acid was added rapidly and the reaction stood for 10 min. Then, the tubes were shaken and placed for 20 min in a water bath at 30 °C. The absorbance of the mixtures was measured at 490 nm and the concentration of EPS was expressed as mg of glucose equivalents with a calibration curve.

4.2.5. Physical analysis

4.2.5.1. Specific volume

The specific volume of the breads was determined with the protocol of Fekri et al.(2020) with some modifications. A sample of 20 ± 0.5 g of bread loaf was weighted with an electronic balance and its volume was measured based on the chia seed displacement. Specific volume was determined as cm^3/g by dividing the volume of the bread loaf by its weight.

4.2.5.2. Colorimetric assay

The color evaluation for the 8 bread formulations were assayed based to the methodology of Alcázar-Valle et al. (2021). The color of the crust and the crumb were determined using the CIE L*a*b scale spectrophotometry (CM-5, Konica Minolta Sensing Americas, Ramsey, NJ, USA).

4.2.5.3. Determination of penetration force on the crust

Penetration force of the crust was calculated after 2 h of bread baking according to the method of Altamirano-Fortoul & Rosell (2011). Texturometer TA XT PLUS (Godalming, Surrey GU7 1YL, UK) equipped with a 5 kg load cell was used and a cylindrical stainless-steel probe of 5 mm diameter was attached to the crosshead. The sample was punched in the middle of the crust at 2 cm distance at 40 mm/s cross-speed to simulate the bitting with the front teeth. Average and standard deviation values were reported.

4.2.5.4. Texture profile analysis

The texture profile analysis was performed after 2 h of bread baking based in the method of López-Alarcón et al. -(2019). Texturometer TA XT PLUS (Godalming, Surrey GU7 1YL, UK) equipped with a 5 kg load cell was used and a cylindrical stainless-steel probe of 36 mm diameter (P/100) was attached to the crosshead. The instrument test parameters were the following: pre-test speed at 1.7 mm s^{-1}; crosshead speed at 1.0 mm s^{-1}; post-test speed at 1.7 mm s^{-1} and compression was

set to 40%. Bread loaves were sliced to 15 mm thickness. The textural quality properties measured were hardness, elasticity, cohesiveness, resilience, and chewiness. Average and standard deviation values were reported.

4.2.6. Sensory evaluation

According to functional properties evaluated, SD breads were selected to perform a sensory evaluation based on the methodology of Fekri et al. (2020). A 9-point hedonic scale (0 undesirable to 9 excellent) was used by rating appearance, odor, taste, texture, and overall acceptability by 43 panelists (24 females and 19 males, age 18 – 30, students of Tecnológico de Monterrey Campus Guadalajara). The four SD breads were evaluated and coded with aleatory numbers, with the purpose that the panelist did not know what treat was tasting. Panelists were also asked to base their decision…

4.2.7. Statistical analysis

The results were expressed as the mean ± standard deviation. Significant differences ($P < 0.05$) between treatments were assessed by Tukey's test after an analysis of variance (one-way ANOVA) in Minitab Statistic Program for Windows.

4.3. Results and Discussion

4.3.1 Preliminary assays and selection of the microorganisms

The cell counts for the wheat SD (Table 13) showed that LAB presented one logarithmic scale (8 log CFU/g) higher than the yeasts (7 log CFU/g). In the case of the yeast, treatments 1 and 3 showed the highest yeast concentration, whereas in the LAB the highest content were denoted by treatment 2 and 3 . For the acidification capacity of LAB and yeast isolates, the treatment 2 exhibited the highest acidification capacity for the wheat SD with a pH value of 3.41 ± 0.02.

Table 13. LAB and yeast concentration (CFU/g) and pH wheat SD combinations (preliminary assays).

Combination	Yeast (CFU/g)[1]	LAB (CFU/g)[2]	Dough pH[3]
1[X]	$7.88 \pm 1.95 \times 10^{7A}$	$4.88 \pm 0.83 \times 10^{8B}$	3.54 ± 0.01^{B}
2[Y]	$4.25 \pm 1.16 \times 10^{7B}$	$8.38 \pm 1.92 \times 10^{8A}$	3.41 ± 0.02^{C}
3[Z]	$6.50 \pm 0.76 \times 10^{7A}$	$7.38 \pm 1.60 \times 10^{8A}$	3.61 ± 0.01^{A}

Means with different letters in the same column indicate significant differences (P ≤ 0.05).
Results were expressed as mean ± standard deviation.
[X]Combination 1 with *C. paralimentarius*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[Y]Combination 2 with *L. curvatus*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[z]Combination 3 with *P. pentosaceus*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[1]Yeast cell counts were expressed as CFU/g of SD after overnight fermentation.
[2]LAB cell counts were expressed as CFU/g of SD after overnight fermentation.
[3]pH values of SD after overnight fermentation.

The cell counts of the final wheat doughs before baking (Table 14) showed that the concentration of LAB and yeasts are in the same logarithmic scale with an average of 7 log CFU/g, except for the 2 SD and 3 SD treatments, which presented a LAB concentration of $1.41 \pm 0.26 \times 10^8$ and $2.34 \pm 0.33 \times 10^8$ UFC/g, respectively. Additionally, in the case of the dough acidification, the 1 SD treatment showed the lowest pH value of 4.56 ± 0.02.

Table 14. LAB and yeast concentration (CFU/g) and pH for bread combinations (preliminary assays).

Combination	Yeast (CFU/g)[1]	LAB (CFU/g)[2]	Dough pH[3]
1[X]	$1.04 \pm 0.28 \times 10^{7C}$	$2.38 \pm 0.56 \times 10^{7C}$	5.48 ± 0.04^{A}
2[Y]	$1.02 \pm 0.17 \times 10^{7C}$	$2.63 \pm 0.77 \times 10^{7C}$	5.19 ± 0.01^{B}
3[Z]	$1.21 \pm 0.30 \times 10^{7C}$	$5.38 \pm 1.30 \times 10^{7C}$	5.12 ± 0.04^{BC}
1 SD[U]	$3.50 \pm 0.76 \times 10^{7A}$	$2.43 \pm 0.23 \times 10^{8A}$	4.56 ± 0.02^{E}
2 SD[V]	$2.87 \pm 0.83 \times 10^{7AB}$	$1.41 \pm 0.26 \times 10^{8B}$	5.07 ± 0.01^{C}
3 SD[W]	$2.62 \pm 0.74 \times 10^{7B}$	$2.34 \pm 0.33 \times 10^{8A}$	4.86 ± 0.04^{D}

Means with different letters in the same column indicate significant differences ($P \leq 0.05$).
Results were expressed as mean ± standard deviation.
[X]Combination 1 with *C. paralimentarius*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[Y]Combination 2 with *L. curvatus*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[Z]Combination 3 with *P. pentosaceus*, *S. cerevisiae*, *C. glabrata* and *H. opuntiae* as starter cultures.
[U]Combination 1 SD with *C. paralimentarius*, *S. cerevisiae, C. glabrata* and *H. opuntiae* as starter cultures.
[V]Combination 2 SD with *L. curvatus, S. cerevisiae, C. glabrata* and *H. opuntiae* as starter cultures in in
[W]Combination 3 SD with *P. pentosaceus, S. cerevisiae, C. glabrata* and *H. opuntiae* as starter cultures.
[1]Yeast cell counts were expressed as CFU/g of dough after overnight fermentation.
[2]LAB cell counts were expressed as CFU/g of dough after overnight fermentation.
[3]pH values of dough after overnight fermentation.

The selection of the yeast isolates for the doughs and SD was based in their leavening capacity*. S. cerevisiae* BIOTEC026 and *C. glabrata* BIOTEC021 were selected as starters for the doughs since they presented the highest leavening capacity between all yeast isolates. In the case of *H. opuntiae* BIOTEC045, it was chosen for the possible properties it could bring to the bread, as it is a yeast isolated from another vegetable matrix (raspberry). For the selection of the LAB isolates, the leavening capacity was an important criteria, although the interactions in the matrix plot (Figure 5) of the LAB isolates with the 3 selected yeasts was the most determinant factor. The selected LAB isolate was *P. pentosaceus* BIOTEC032, because it denoted the highest leavening capacity for all the LAB isolates. Furthermore, in the matrix plot it can be observed that it presents a good interaction (4-6) with *S. cerevisiae* BIOTEC026, *C. glabrata* BIOTEC021 and *H. opuntiae* BIOTEC045. Finally, the preliminary assays showed that the dough 3 SD, which contained *P. pentosaceus* BIOTEC032 as starter culture, exhibited the highest LAB cell counts ($2.34 \pm 0.33 \times 10^{8}$ UFC/g) in comparison to the other 5 treatments, and its pH was the second lowest for all the doughs, with a value of 4.86 ± 0.04.

4.3.2. Microbial counts and acidification

The cell counts for the wheat SD (Table 15) showed that Yeast SD presented the highest content of yeast cells with $1.41 \pm 0.26 \times 10^{8}$ CFU/g. In the case of LAB,

Bacteria and Bac+Yeast treatments exhibited the highest cell counts with 5.38 ± 1.06 x 10^8 and 1.08 ± 0.21 x 10^9 CFU/g, respectively. Moreover, in the Bacteria SD, the cell counts of yeast were very low (3.50 ± 0.92 x 10^6) compared with the other treatments, which denotes the inhibition of the LAB to the flour endogenous yeast, since Bacteria SD was not inoculated with any yeast isolate. Galli et al. (2019) determined the concentration of LAB and yeast from a wheat SD, using *S. cerevisiae* and 5 LAB species (*L. sanfranciscensis , L. rossiae, L. farciminis, L. plantarum* and *L. brevis*). The cell counts showed a similar results to the Bact+Yeast SD and Bacteria SD of this work, since the concentration of LAB and yeast were in 9 log CFU/g and 7 log CFU/g, respectively.

Table 15. LAB and yeast counts (CFU/g), pH and titratable acidity (TTA) (mL) for wheat SD combinations.

Combination	Yeast (CFU/g)[1]	LAB (CFU/g)[2]	SD pH[3]	SD TTA(mL)[4]
Control	1.15 ± 0.24 x 10^{8B}	1.01 ± 0.28 x 10^{8C}	4.79 ± 0.01^{B}	7.18 ± 0.10^{D}
Yeast	1.41 ± 0.26 x 10^{8A}	1.18 ± 0.21 x 10^{8C}	5.19 ± 0.02^{A}	7.48 ± 0.10^{C}
Bac+Yeast	2.04 ± 0.37 x 10^{7C}	5.38 ± 1.06 x 10^{8B}	3.51 ± 0.01^{C}	8.48 ± 0.10^{B}
Bacteria	3.50 ± 0.92 x 10^{6C}	1.08 ± 0.21 x 10^{9A}	3.43 ± 0.01^{D}	9.13 ± 0.10^{A}

Results were expressed as mean ± standard deviation.
Means with different letters in the same column indicate significant differences (P ≤ 0.05).
[1]Yeast counts were expressed as CFU/g of SD after overnight fermentation.
[2]LAB counts were expressed as CFU/g of SD after overnight fermentation.
[3]pH values of SD after overnight fermentation.
[4]TTA values of bread after overnight fermentation expressed as mL of NaOH.

The enumeration of LAB and yeasts in the final doughs (Table 16) showed that the yeast counts for the 8 dough treatments were in the range of 7 log CFU/g, being the Control dough, the highest cell counts with a value of 8.38 ± 0.92 x 10^7 CFU/g. For the LAB, the doughs without addition of wheat SD presented a concentration of LAB in the same range than the yeasts (7 log CFU/g). In the case of the doughs with addition of wheat SD, the treatments Bac+Yeast SD and Bacteria SD showed a concentration of LAB of 8 log CFU/g, which means that the use of LAB as starter culture in a wheat SD increase the concentration of LAB in the final dough. Gül, et al. (2005) assessed the microbial composition of a wheat dough with addition of a spontaneous wheat SD. The yeast cell counts indicated an average concentration of 8 log CFU/g, a higher result compared to the yeast concentration of our study. Whereas LAB counts were an average concentration of 7 log CFU/g, a

very similar result to the LAB concentration of this study, with exception of the Bac+Yeast SD and Bacteria SD bread.

Table 16. LAB and yeast concentration (CFU/g), pH and TTA (ml) for traditional bread and SD bread combinations.

Combination	Yeast (CFU/g)[1]	LAB (CFU/g)[2]	Dough pH[3]	Bread pH[4]	Dough TTA (mL)[5]	Bread TTA (mL)[6]
Control	$8.38 \pm 0.92 \times 10^{7A}$	$8.63 \pm 1.19 \times 10^{7C}$	5.21 ± 0.04^{AB}	5.31 ± 0.02^{AB}	5.68 ± 0.10^{E}	5.40 ± 0.16^{C}
Yeast	$6.50 \pm 0.93 \times 10^{7AB}$	$8.75 \pm 0.88 \times 10^{7C}$	5.17 ± 0.01^{B}	5.32 ± 0.04^{A}	5.90 ± 0.08^{DE}	5.58 ± 0.10^{BC}
Bac+Yeast	$4.75 \pm 1.16 \times 10^{7B}$	$8.63 \pm 0.74 \times 10^{7C}$	5.16 ± 0.03^{B}	5.28 ± 0.02^{AB}	6.18 ± 0.13^{CD}	5.60 ± 0.29^{BC}
Bacteria	$2.50 \pm 0.53 \times 10^{7C}$	$3.13 \pm 0.64 \times 10^{7F}$	5.13 ± 0.06^{B}	5.18 ± 0.03^{C}	6.33 ± 0.17^{C}	5.90 ± 0.18^{B}
Control SD	$7.63 \pm 1.92 \times 10^{7A}$	$5.38 \pm 0.92 \times 10^{7D}$	5.22 ± 0.05^{AB}	5.26 ± 0.02^{ABC}	6.23 ± 0.17^{C}	6.03 ± 0.25^{B}
Yeast SD	$4.75 \pm 1.58 \times 10^{7B}$	$1.04 \pm 0.20 \times 10^{7E}$	5.27 ± 0.02^{A}	5.23 ± 0.03^{BC}	5.88 ± 0.10^{DE}	5.63 ± 0.17^{BC}
Bac+Yeast SD	$4.88 \pm 1.36 \times 10^{7B}$	$1.10 \pm 0.09 \times 10^{8B}$	4.47 ± 0.02^{D}	4.63 ± 0.04^{D}	7.33 ± 0.17^{B}	7.12 ± 0.30^{A}
Bacteria SD	$7.13 \pm 1.55 \times 10^{7A}$	$2.61 \pm 0.15 \times 10^{8A}$	4.66 ± 0.02^{C}	4.67 ± 0.06^{D}	7.80 ± 0.08^{A}	7.60 ± 0.14^{A}

Results were expressed as mean ± standard deviation. Means with different letters in the same column indicate significant differences ($P \leq 0.05$).
[1]Yeast counts were expressed as CFU/g of dough after overnight fermentation.
[2]LAB counts were expressed as CFU/g of dough after overnight fermentation.
[3]pH values of doughs after overnight fermentation.
[4]pH values of bread after baking process.
[5]TTA values of doughs after overnight fermentation expressed as mL of NaOH.
[6]TTA values of bread after baking process expressed as mL of NaOH.

The pH and TTA measurements exhibited the ability of *P. pentosaceus* BIOTEC032 for acidifying the SD, since Bacteria treatment obtained a pH value of 4.43 ± 0.01 and a TTA of 9.13 ± 0.10 ml of NaOH. This acidification process of *P. pentosaceus* BIOTEC032 in the SD may be correlated with the low quantity of yeasts in the Bacteria treatment, because the production of organic acids by the LAB plays a key role in the growth inhibition of the autochthonous yeast populations from the flour (Ding et al., 2021). The pH and TTA of the doughs and final breads showed the same tendency as the SDs, where Bac+Yeast SD and Bacteria SD treatments displayed higher acidification properties than the other combinations. The pH values for the doughs and final breads of Bac+Yeast SD and Bacteria SD treatments were in a range of 4.47 ± 0.02 - 4.66 ± 0.02 4.63 ± 0.04 and 4.63 ± 0.04 - 4.67 ± 0.06, respectively. In the case of TTA, the results for the doughs were between 7.33 ± 0.17 - 7.80 ± 0.08 and 7.12 ± 0.30 - 7.60 ± 0.14 ml of NaOH, respectively.

Fekri et al. (2020) evaluated the acidification capacity of *P. pentosaceus* in a wholewheat SD bread. The results showed a lower pH in the final bread than in our study, with a value of 3.41 ± 1.01. In the case of TTA, the use of *P. pentosaceus* exhibited a higher TTA result than this study, with value of 13.79 ± 2.09 mL of NaOH

in the bread. Another research (Gül, Süleyman, et al., 2005) examined the ability of *L. plantarum* to acidify a wheat dough with a pH of 3.95 ± 0.59, a lower result than in this research; and TTA of 7.5 mL of NaOH, being a similar result than in our Bact+Yeast SD and Bacteria SD treatments.

4.3.3 Evaluation of postbiotic potential

The results of the postbiotic-like properties for the traditional and SD bread are presented in the next sections.

4.3.3.1. Phytase activity

Phytase activity plays a key role during dough fermentation through the degradation of phytic acid, and increasing the bioavailability of minerals, free amino acids, and vitamins (Gobbetti et al., 2019). Phytase activity was assayed for the 8 treatments, and the assay was performed in the dough before the baking process. The results showed a higher enzymatic activity for the SD breads than the traditional breads (Figure 7). Bacteria SD treatment exhibited the highest enzymatic activity with a value of 7.26 ± 0.17 U. In this respect, a SD bread with the use of *P. pentosaceus* BIOTEC032 as starter culture would contain lower phytic acid than a traditional bread; thus, the bioavailability of nutrients, such as minerals would be favored in a SD bread.

Nielsen et al. (2007) studied the capability of a rye bread SD for increasing the phytase activity. Their results showed a maximum phytase activity of 3.30 ± 0.2 U, which it is lower compared with the phytase activity of Bacteria SD or Bact+Yeast SD treatments. Furthermore, it was observed that phytase activity was reduced to 10 – 15% in an unfermented dough compared when a SD is added(Nielsen et al., 2007). Therefore, with the results obtained in our research, it can be confirmed the ability of SD fermentation to increase the phytase activity in the final doughs.

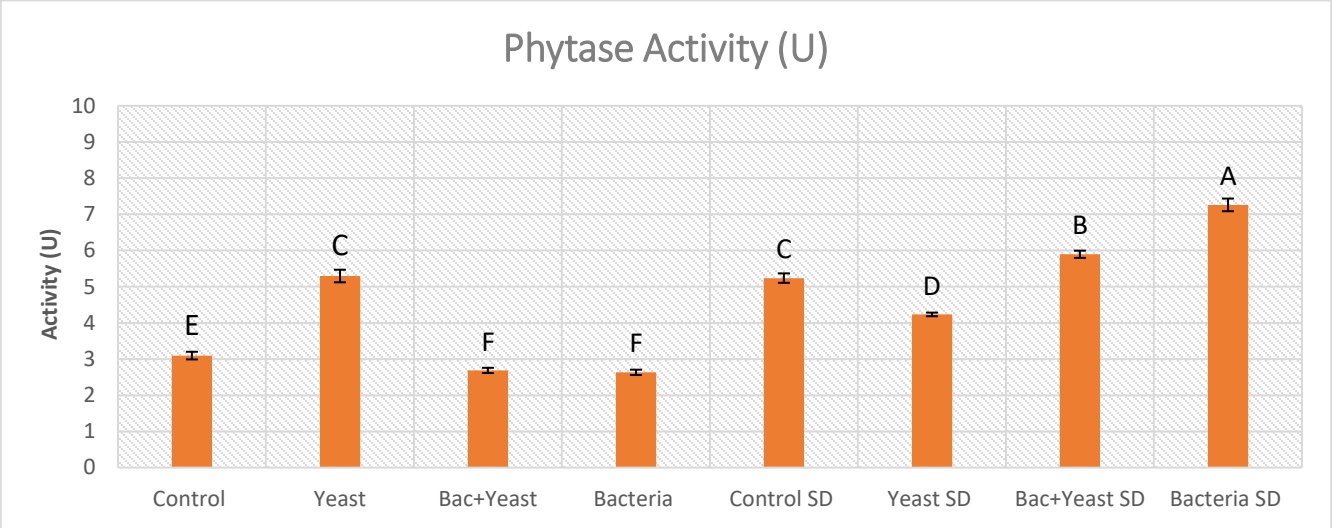

Figure 7. Phytase activity for the different combinations expressed as enzymatic units (U). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

4.3.3.2 Total phenolic content

The total phenolic content was measured in the dough before baking and in the final bread. In the case of the doughs, the addition of SD increased the phenolic content compared to the dough without SD addition (Figure 8a). Yeast SD, Bact+Yeast SD and Bacteria SD were the treatments with the highest phenolic content in a range of 315.05 ± 4.96 - 342.70 ± 8.26 µg of gallic acid equivalents/g. These results are in line with those reported previously (Garzon et al., 2021), in which the use of SD with LAB and yeast starter cultures increases the phenolic compounds in a final bread compared with traditional breads.

In the case of the final breads, a reduction in the phenolic content was observed, although, the SD breads exhibited a greater phenolic content that traditional breads (Figure 8b). Particularly Bac+Yeast SD treatment showed the highest phenolic content with 300.81 ± 28.29 µg of gallic acid equivalents/g. Fekri et al. (2020) evaluated the total phenolic compounds in both dough and final bread for a wholewheat SD bread using *P. pentosaceus* as starter culture. The results also showed a reduction in the phenolic content after the baking process. However, their total phenolic compounds were lower compared with this study, obtaining values of 69.2 ± 17.1 µg of gallic acid equivalents/g for the dough, and 65.4 ± 12.3 µg of gallic

acid equivalents/g for the bread. Therefore, it is very important to consider that there is a reduction in the total phenolic content after the baking process due to the thermal treatment.

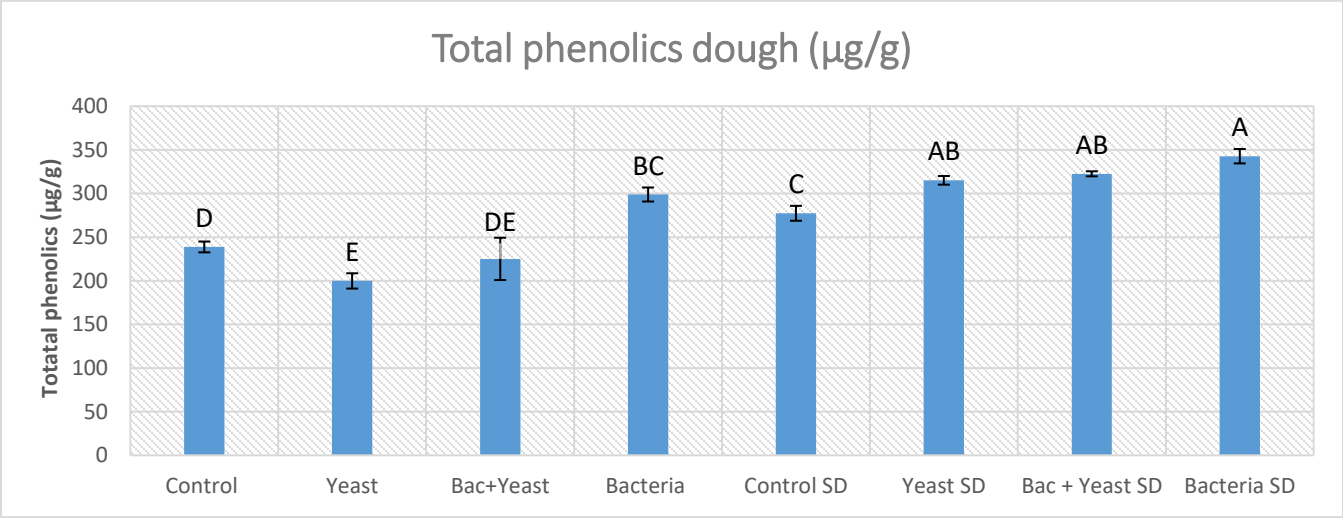

Figure 8a. Total phenolics for dough combinations (µg of gallic acid equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

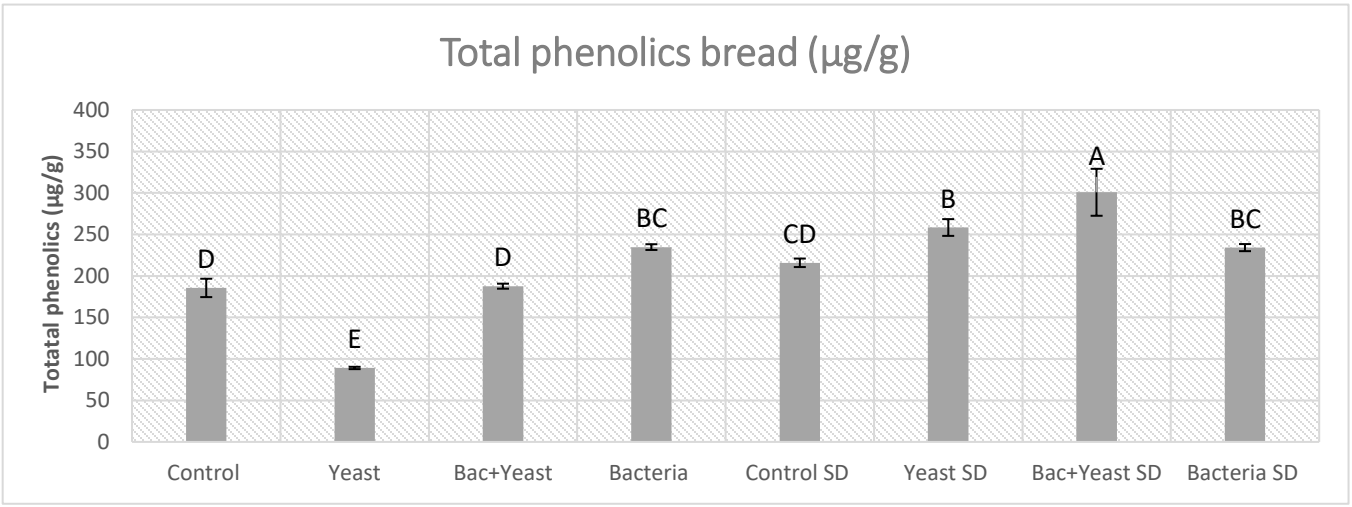

Figure 8b. Total phenolics for bread combinations (µg of gallic acid equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

4.3.3.3. Antioxidant activity

Antioxidant activity in ABTS and DPPH was determined in the final breads for the 8 treatments (Figures 9 and 10). For ABTS, Yeast, Bac+Yeast, Bacteria and Control SD did not show antioxidant activity, and the treatment Bac+Yeast SD displayed the highest ABTS activity with 291.10 ± 52.52 µg of acid ascorbic equivalents/g. In the case of DPPH, the SD breads exhibited a greater antioxidant activity, particularly Yeast SD, Bac+Yeast SD and Bacteria SD combinations, obtaining a result in a range between 447.29 ± 57.70 - 565.42 ± 42.30 µg of acid ascorbic equivalents/g. Similar to the total phenolic content, it can be observed that the use of SD with LAB and yeast starter cultures may enhance the antioxidant capacity of wheat bread.

Garzon et al. (2021) also determined the antioxidant activity of a wheat SD bread. The results of ABTS activity showed a lower value than the maximum values of the present study, with 170.09 ± 10.01 µg of acid ascorbic equivalents/g. Similar trend for DPPH, where the antioxidant activity was of 72.17 ± 6.98 µg of acid ascorbic equivalents/g, a lower result than the maximum values of this study. It is very important to highlight that the type of fermentation and the LAB and yeast starter cultures play an important role in the antioxidant capacity of a bread activity (Garzon et al., 2021). Additionally, factors such as the synergistic/antagonist effects between phenolic and other bioactive compounds have an important contribution on the overall antioxidant activity of the final bread. Hence, fermentation not only affects the amount of bioactive compounds, but also their profile since other compounds may be synthetized resulting in an alteration of the antioxidant activity (Garzon et al., 2021).

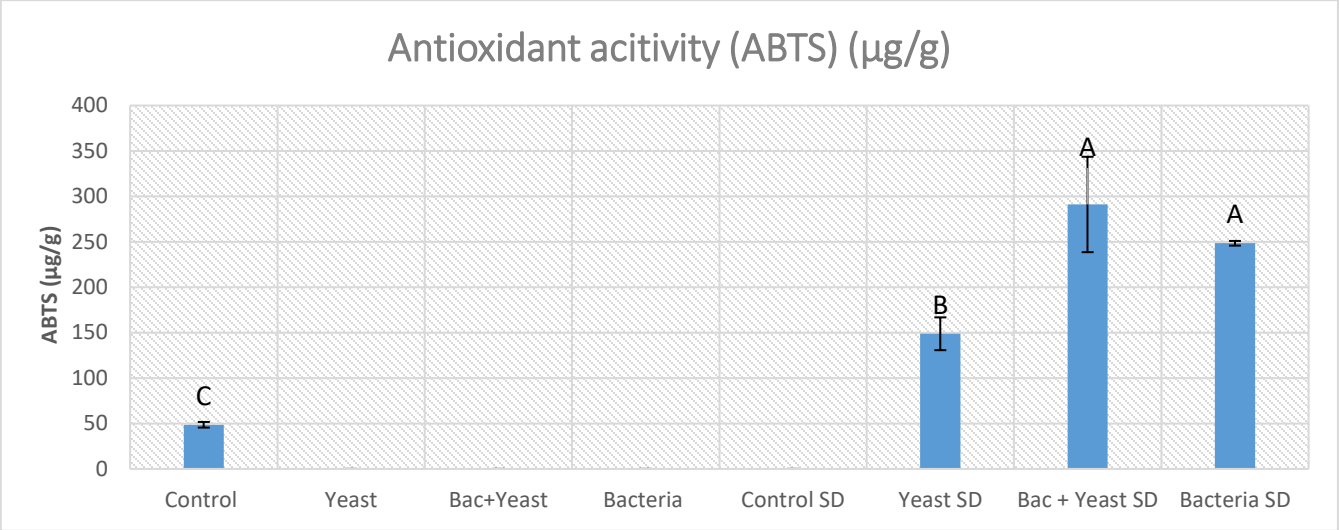

Figure 9. Antioxidant activity (ABTS) for bread combinations (µg of ascorbic acid equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

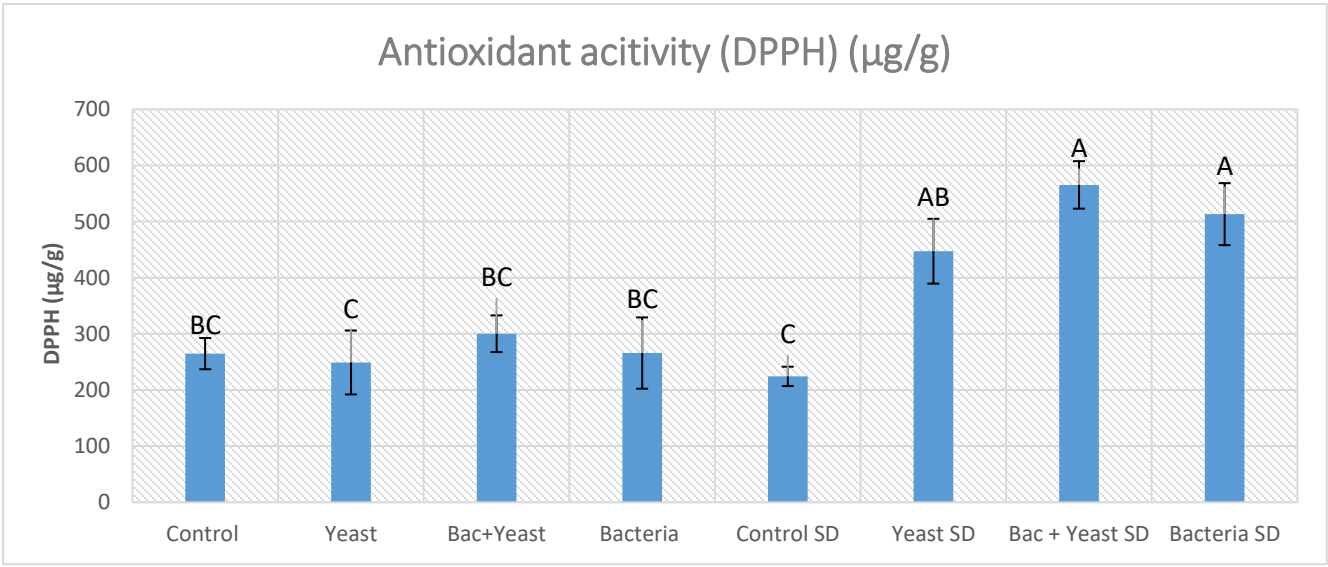

Figure 90. Antioxidant activity (DPPH) for bread combinations (µg of ascorbic acid equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

4.3.3.4. Presence of EPS

The amount of EPS was determined for the dough before the baking process and in the final bread (Figures 11a and 11b). Bacteria SD was the treatment that generated most EPS in both, dough, and final bread, with values of 3.40 ± 0.02 and 1.41 ± 0.03 mg of glucose equivalents/g, respectively. Similar to the phenolic

content, it can be observed that the amount of EPS decreased after the thermal treatment of baking for the 8 formulations. Furthermore, it is likely that yeasts did not contribute to the synthesis of EPS, since the result showed the lowest EPS production for Yeast and Yeast SD treatments for the doughs and in the breads.

Păcularu-Burada et al., (2020a) evaluated the biosynthesis of EPS by *Pediococcus* spp. in quinoa and buckwheat flour extracts. The results demonstrated the capacity of *Pediococcus* spp. to generate EPS in a concentration of 18.26 - 21.03 mg of glucose equivalents/g, which are higher results compare to our study. It is important to emphasize that fermentation conditions (inoculum, fermentation time temperature) affect the yield rates of EPS production, as an example, the carbon-nitrogen ratio on the growth medium has a great impact on the amount and of the EPS (Zhang et al., 2019).

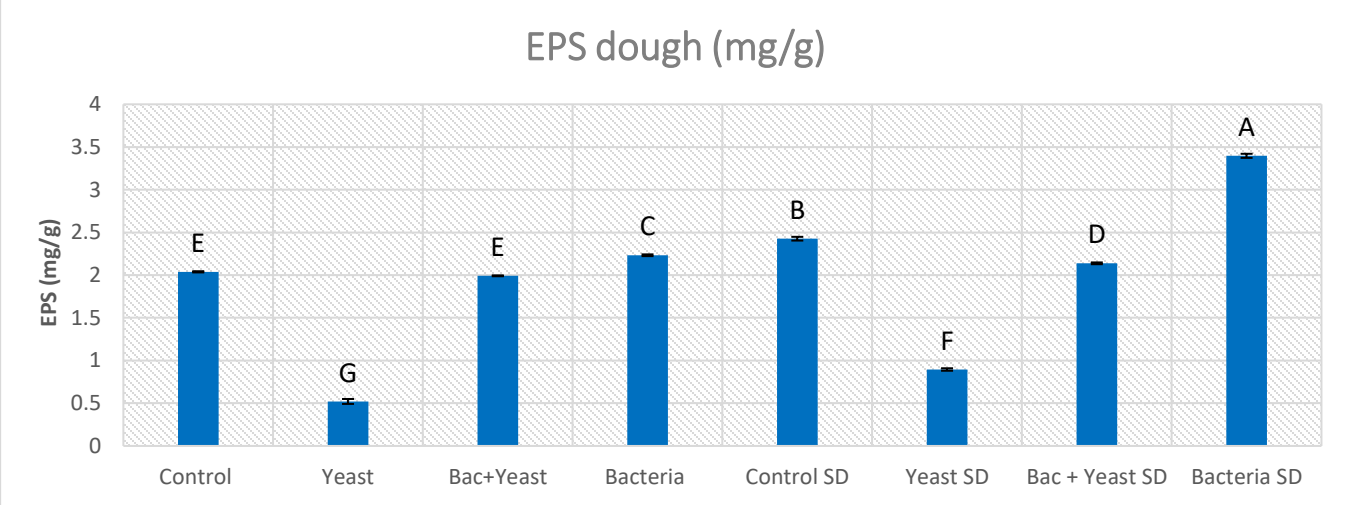

Figure 11a. Exopolysaccharides for dough combinations (mg of glucose equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences (P ≤ 0.05).

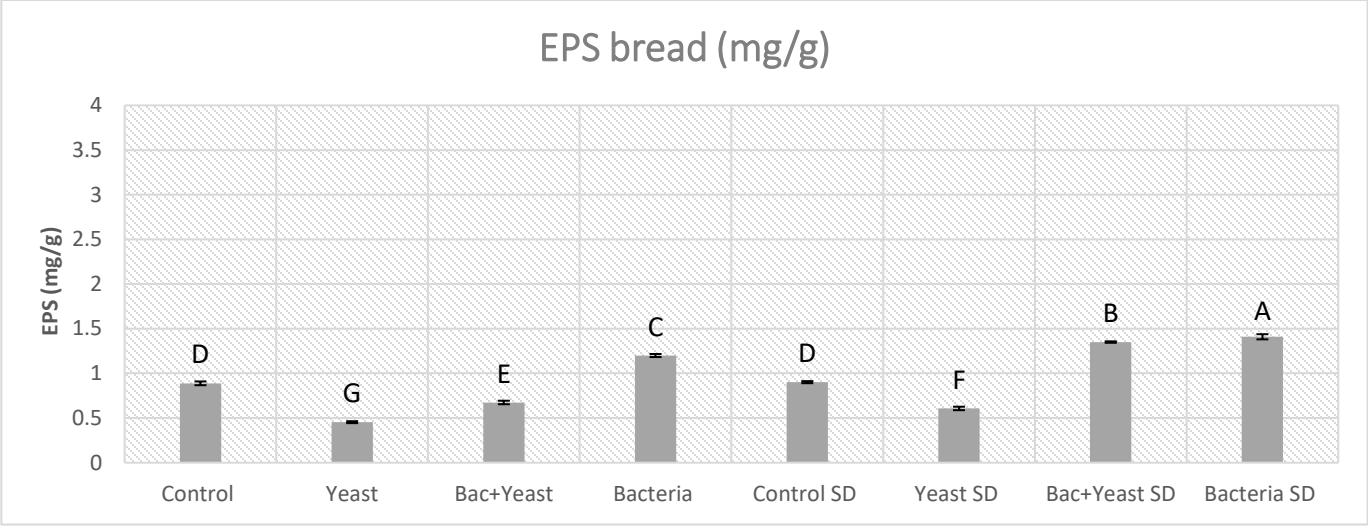

Figure 11b. Exopolysaccharides for bread combinations (mg of glucose equivalents/g). The values measured are means of triplicate measurements ± standard deviation. Means with different letters indicate significant differences ($P \leq 0.05$).

4.3.4. Physical properties of the breads

The physical properties of bread including specific volume, penetration force, color and TPA were assessed for the 8 treatments (Table 17). In the case of specific volume, the Yeast and Yeast SD breads showed the highest leavening ability with values of 3.62 ± 0.14 and 3.13 ± 0.07 cm^3/g respectively. These results showed that a bread inoculated only with yeast isolates and baker yeast increase more its final volume, and the breads inoculated with LAB reduce the leavening ability by means of the inhibition of the baker yeast and endogenous flour yeasts (Ding et al., 2021). Fekri et al. (2020) evaluated the specific volume of a whole wheat fermented with *S. cerevisiea,* a similar result was found than in our study, obtaining a value of 3.00 ± 0.50 cm^3/g. Furthermore, Olojede et al. (2020) determined the specific volume of a sorghum SD bread with the use of *P. pentosaceus* as starter culture, the result showed a specific volume of 2.21 ± 0.17 cm^3/g, which it is a similar result compared with the specific volume of Bac+Yeast SD (2.69 ± 0.11 cm^3/g) and Bacteria SD (2.43 ± 0.26 cm^3/g) breads of this study

The penetration assay evaluated the force that is needed to break the bread crust by simulation the bitting with the front teeth (Table 17). The results showed significant differences between the treatments ($P < 0.05$), with Yeast, Bac+Yeast SD and Bacteria SD treatments exhibiting the highest penetration forces, and

consequently the hardness of bread crusts. Olojede et al. (2020) assessed the penetration of sorghum SD bread with the use of *P. pentosaceus* as starter culture and a Control sorghum bread with *S. cerevisiae*. In the case of *P. pentosaceus* bread, a higher result was obtained compared with this study, with a penetration force of 31.67 ± 4.55 N. For the control bread with *S. cerevisiae,* a similar result was found compared with the Yeats bread of our study, with a penetration force of 11.08 ± 1.59 N.

For the color of the bread crust (Figure 12 and Table 17), the results showed slight significant differences between the 8 bread treatments. In general, the *L, a* and *b* parameters indicated that the crust for the 8 breads were in the color region of dark brown. The color of the crumb also showed slight significant differences between the 8 treatments; the *L, a* and *b* parameters denoted that the crumb for all the treatments was in the light yellow to white region. Garzon et al. (2021) also assessed of wheat bread and wheat SD bread. For the crust, a dark yellow to brown color was identified, presenting a little difference with the color profile for the crust of this study. On the other hand, a lighter crumb, around light yellow to white, was detected, obtaining a very similar color profile to crumb of our study.

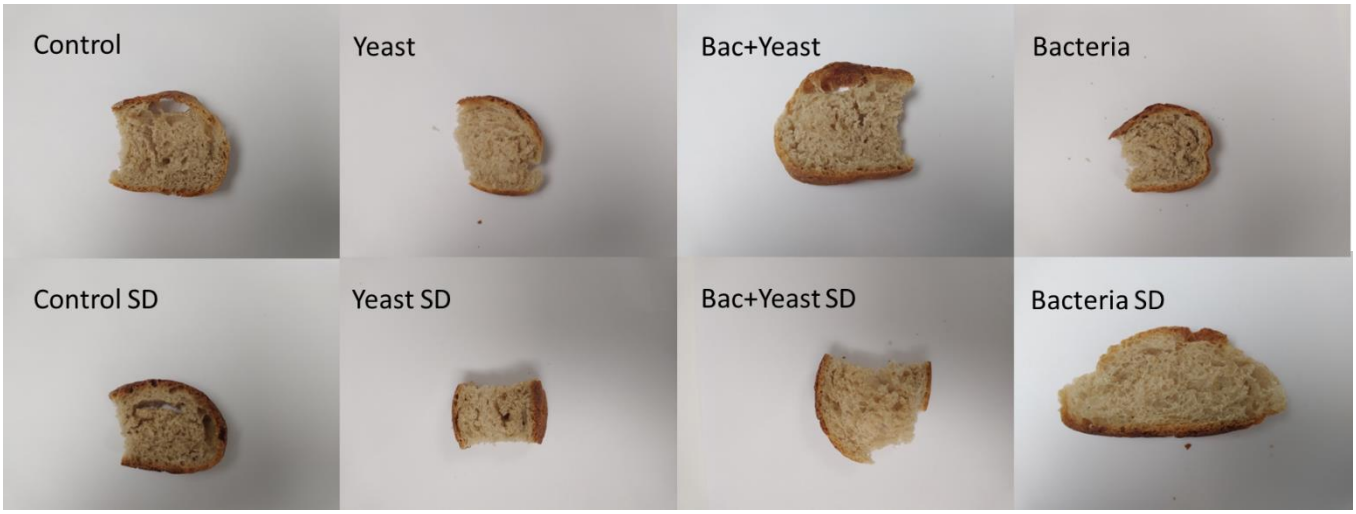

Figure 12. Appearance of the 8 bread treatments (see Tables 11 and 12 for description of each bread).

The TPA analysis (Table 17) exhibited that SD breads presented a high significant hardness than traditional breads. Particularly Bacteria SD treatment displayed the highest hardness (10.20 ± 0.90 N) between the 8 types of bread. The results of springiness, cohesiveness and resilience showed slight significant differences for the 8 bread treatments. The gumminess denoted a similar behavior as the hardness, where Bacteria SD bread obtained the highest value (7.18 ± 0.48), and Control and Yeast treatments presented the lowest values with 1.80 ± 0.24 and 1.84 ± 0.39, respectively. Finally, the chewiness also exhibited the performance of hardness and gumminess, Control and Yeast breads obtained the lowest values for the assay with 1.69 ± 0.21 and 1.72 ± 0.36 N, respectively, whereas Bacteria SD treatment exhibited the highest chewiness between the all the treatments with 6.12 ± 0.31 N. It is important to underline that bread samples formulated with LAB as starter cultures showed high hardness, gumminess, and chewiness, in particular for the SD breads (Bac+Yeast SD and Bacteria SD). A possible explanation for this phenomenon may be related to the LAB protease activity, causing the hydrolysis of various gluten residues, and generating a more stable but less elastic emulsion. In contrast, breads formulated with yeast starter cultures or baker yeast present greater extensibility and elasticity (Fekri et al., 2020).

Table 17. Physical characteristics of traditional bread and SD bread combinations.

Technological properties	Control	Yeast	Bact+Yeast	Bacteria	Control SD	Yeast SD	Bact+Yeast SD	Bacteria SD
Spec. vol. (cm^3/g)	2.94 ± 0.24^{BC}	3.62 ± 0.14^{A}	2.62 ± 0.22^{CDE}	2.50 ± 0.18^{DE}	2.86 ± 0.19^{BCD}	3.13 ± 0.07^{B}	2.69 ± 0.11^{CDE}	2.43 ± 0.26^{E}
Penetration (N)	9.27 ± 0.55^{AB}	10.22 ± 2.02^{A}	7.36 ± 0.49^{B}	7.68 ± 1.09^{B}	6.90 ± 0.92^{B}	7.55 ± 1.36^{B}	11.43 ± 1.75^{A}	11.17 ± 1.11^{A}
Color of crust								
L	41.04 ± 2.37^{B}	44.85 ± 5.15^{B}	51.98 ± 2.65^{A}	55.04 ± 2.90^{A}	44.56 ± 0.91^{B}	41.28 ± 2.53^{B}	41.04 ± 2.30^{B}	55.88 ± 3.24^{A}
a	13.31 ± 0.75^{ABC}	12.23 ± 1.06^{BC}	14.05 ± 0.66^{AB}	11.37 ± 1.82^{C}	14.37 ± 0.08^{AB}	15.34 ± 0.84^{A}	12.63 ± 1.34^{BC}	11.77 ± 1.30^{BC}
B	23.19 ± 1.27^{A}	21.01 ± 3.35^{A}	25.26 ± 1.58^{A}	23.10 ± 1.66^{A}	22.74 ± 1.21^{A}	23.57 ± 2.76^{A}	21.48 ± 1.76^{A}	25.19 ± 2.13^{A}
Color of crumb								
L	65.70 ± 0.34^{D}	67.40 ± 0.67^{ABC}	68.73 ± 0.42^{A}	66.22 ± 0.62^{CD}	66.64 ± 0.92^{BCD}	68.14 ± 0.06^{AB}	66.45 ± 0.10^{CD}	68.28 ± 0.97^{AB}
a	0.67 ± 0.24^{D}	0.86 ± 0.14^{CD}	1.63 ± 0.17^{B}	1.56 ± 0.16^{B}	1.37 ± 0.31^{BC}	2.35 ± 0.30^{A}	2.41 ± 0.30^{A}	1.50 ± 0.10^{B}
B	20.44 ± 0.32^{AB}	20.16 ± 0.19^{B}	20.17 ± 0.15^{B}	19.68 ± 0.61^{B}	20.44 ± 0.36^{AB}	21.25 ± 0.47^{A}	20.22 ± 0.43^{A}	20.36 ± 0.25^{AB}
TPA analysis								
Hardness (N)	2.47 ± 0.34^{E}	2.46 ± 0.56^{E}	3.73 ± 0.58^{DE}	5.13 ± 0.88^{C}	4.54 ± 0.82^{CD}	5.023 ± 1.00^{CD}	6.90 ± 0.91^{B}	10.20 ± 0.90^{A}
Springiness	0.94 ± 0.01^{A}	0.94 ± 0.01^{A}	0.88 ± 0.02^{B}	0.86 ± 0.01^{BC}	0.83 ± 0.05^{C}	0.88 ± 0.03^{B}	0.87 ± 0.02^{BC}	0.85 ± 0.03^{BC}
Cohesiveness	0.73 ± 0.01^{AB}	0.75 ± 0.01^{A}	0.70 ± 0.03^{BC}	0.66 ± 0.02^{C}	0.74 ± 0.02^{BC}	0.69 ± 0.01^{AB}	0.72 ± 0.05^{AB}	0.71 ± 0.03^{ABC}
Gumminess	1.80 ± 0.24^{E}	1.84 ± 0.39^{E}	2.60 ± 0.35^{DE}	3.37 ± 0.58^{CD}	3.14 ± 0.53^{CD}	3.69 ± 0.66^{C}	4.97 ± 0.62^{B}	7.18 ± 0.48^{A}
Resilience	0.37 ± 0.01^{A}	0.38 ± 0.01^{A}	0.32 ± 0.03^{CD}	0.28 ± 0.02^{D}	0.33 ± 0.02^{BCD}	0.36 ± 0.02^{AB}	0.36 ± 0.03^{AB}	0.34 ± 0.02^{ABC}
Chewiness (N)	1.69 ± 0.21^{E}	1.72 ± 0.36^{E}	2.30 ± 0.29^{DE}	2.90 ± 0.52^{CD}	2.59 ± 0.39^{CD}	3.22 ± 0.48^{C}	4.32 ± 0.59^{B}	6.12 ± 0.31^{A}

Means with different letters in the same row indicate significant differences ($P \leq 0.05$). Results were expressed as mean ± standard deviation.

4.3.5. Sensory analysis

SD breads were selected based on the results of pH, TTA, postbiotics properties and physical characteristics. The sensory analysis did not reveal any significance difference (P ≤ 0.05) between the 4 treatments (Control SD, Yeast SD, Bac+Yeast SD and Bacteria SD) for all the parameters evaluated: appearance, odor, texture, taste, and overall acceptability (Table 18). Thus, the panelists found the SD breads evaluated to be very similar.

Table 18. Sensory evaluation of SB bread combinations (n=43)

Parameter	Control SD	Yeast SD	Bac+Yeast SD	Bacteria SD
Appearance	7.14 ± 2.09	7.07 ± 1.97	7.30 ± 1.82	7.37 ± 1.97
Odor	6.40 ± 2.19	6.58 ± 2.10	6.88 ± 2.00	7.26 ± 2.03
Texture	7.00 ± 2.17	6.77 ± 2.15	6.79 ± 2.01	7.14 ± 1.92
Taste	6.95 ± 2.00	6.74 ± 2.01	7.16 ± 1.84	6.74 ± 2.19
Overall acceptability	7.00 ± 1.92	6.72 ± 2.05	6.72 ± 1.95	7.19 ± 2.04

Means in the same row did not reveal statistically significant differences (P ≤ 0.05). Results were expressed as mean ± standard deviation.

Fekri et al. (2020) performed a sensory analysis of whole wheat SD using different LAB as starter cultures. The highest overall acceptability belonged to the bread fermented with *P. pentosaceus,* whereas the bread with *S. cerevisiae* as starter culture was the preferred in terms of texture. Nowadays, many bakeries use the SD technology to enhance the quality of baked product. For example, researchers observed that a bread prepared with LAB as starter culture, specifically *L. plantarum* and *P. pentosaceus*, exhibited a high acceptability in a panel of consumers (Fekri et al., 2020). However, this acceptability may vary depending on the starter cultures involved, especially because of the LAB activity.

4.4. Chapter conclusions

Different types of wheat breads were formulated with the use of LAB and yeast starter cultures (*P. pentosaceus* BIOTEC032*, C. glabrata* BIOTEC021*, S. cerevisiea* BIOTEC026 *and H. opuntiae* BIOTEC045) in combination or not with SD fermentation. Four types of bread were elaborated with a traditional recipe, whereas the others were made with the addition of SD. Cell counts of LAB and yeasts, pH, TTA, biotechnological and postbiotics properties, physical characteristics and

sensory evaluation were performed for the 8 bread treatments. The cell counts of yeasts for all the breads were in a concentration of 7 log CFU/g. In the case of LAB, the cell counts were kept in the same concentration as the yeasts, however, in the Bac+Yeast SD and Bacteria SD breads the LAB increased to 8 log CFU/g. For acidity, the SD breads showed as expected a higher acidification capacity than the traditional breads, particularly, for Bacteria SD bread. Related to the postbiotic potential (linked with phytase activity, total phenolic compounds, antioxidant activity and EPS), Bac+Yeast SD and Bacteria SD treatments exhibited the best results compared with the rest of treatments. The physical characteristics showed that Yeast bread presented the highest specific volume and the lowest values for hardness, chewiness, and gumminess, whereas Bac+Yeast SD and Bacteria SD exhibited the highest value for hardness. In the case of the color of the breads, there are not statistically significant differences ($P \leq 0.05$) on the color of the crust and crumb for the 8 bread formulations. The sensory analysis did not show any significant difference between the 4 SD breads.

Overall, the use of a defined consortium of LAB and yeast as starter culture for SD bread production enhances the nutritional, biotechnological and potential postbiotic properties of the baking breads. However, more studies are needed to evaluate the generation of other postbiotic-like components with health benefits: biosurfactants, bacteriocins, short-chain fatty acids, bioactive peptides, among others. Moreover, it is necessary to assess the effect of the thermal treatment during the baking process in the stability and functionality of these compounds.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

1. LAB and yeast microorganisms were isolated from a spontaneous wholewheat SD and raspberry, and a reliable identification at genus and specie levels was performed through MALDI-TOF MS analysis. The main identified species were *Candida glabrata, Saccharomyces cerevisiae, Pediococcus pentosaceus, Companilactobacillus paralimentarius* and *Latilactobacillus curvatus*.

2. Biotechnological and functional properties were evaluated for 23 isolated microorganisms and 3 commercial probiotics. Proteolytic activity was observed in both LAB and yeast isolates. The presence of extracellular proteases and gallate decarboxylase activity was presented in the majority of the isolates. Yeast isolates exhibited a better leveling capacity compared with LAB isolates, particularly *S. cerevisiae* BIOTEC026. In the case of phytase activity, LAB isolates displayed a higher enzymatic activity compared to yeast isolates and spontaneous SD. Moreover, LAB and yeast isolates showed a higher ability to release phenolic compounds than a spontaneous wholewheat SD. Thus, the use of LAB and yeast isolates as starter cultures for SD proves greater biotechnological and functional properties, compared to a spontaneous fermented SD.

3. The use of PCA and Cluster Analysis including the results of the biotechnological and functional properties allowed the selection of *P. pentosaceus* BIOTEC032*, C. glabrata* BIOTEC021*, S. cerevisiae* BIOTEC026 and *H. opuntiae* BIOTEC045 as the best candidates to be used as starter cultures in the formulation of functional wheat breads.

4. Different types of wheat bread were elaborated with the use of the selected LAB and yeast isolates in combination or not with SD fermentation. SD breads with *P. pentosaceus* BIOTEC032 (Bac+Yeast SD and Bacteria SD) showed the best results for the postbiotic properties (total phenol content, phytase activity, antioxidant activity and presence of exopolysaccharides), and also for the microbiological and acidification features. Traditional bread with yeast cultures (Yeast bread) presented the highest specific volume, and it was the softness bread, whereas Bac+Yeast SD

and Bacteria SD treatments were the hardest breads according to the TPA. Differences in color and sensory acceptance between the 8 treatments was not detect with a statically significant difference (P < 005). Therefore, the use of defined consortiums of LAB and yeasts as starter cultures for SD bread-making improve the biotechnological and potential postbiotic properties of bread.

Future research to complement the results obtained in this thesis are the following:

- To design new microbial consortiums with the LAB and yeast and assess their postbiotic potential.
- To evaluate the generation of other postbiotic-like components such as antimicrobial compounds, biosurfactants, bacteriocins, short-chain fatty acids, bioactive peptides, among others.
- To assess the effect of the thermal treatment during the baking process in the stability and functionality of the postbiotic-like components.
- To monitor the stability and health benefits of postbiotic-like components through an *in vitro* gastrointestinal digestion.

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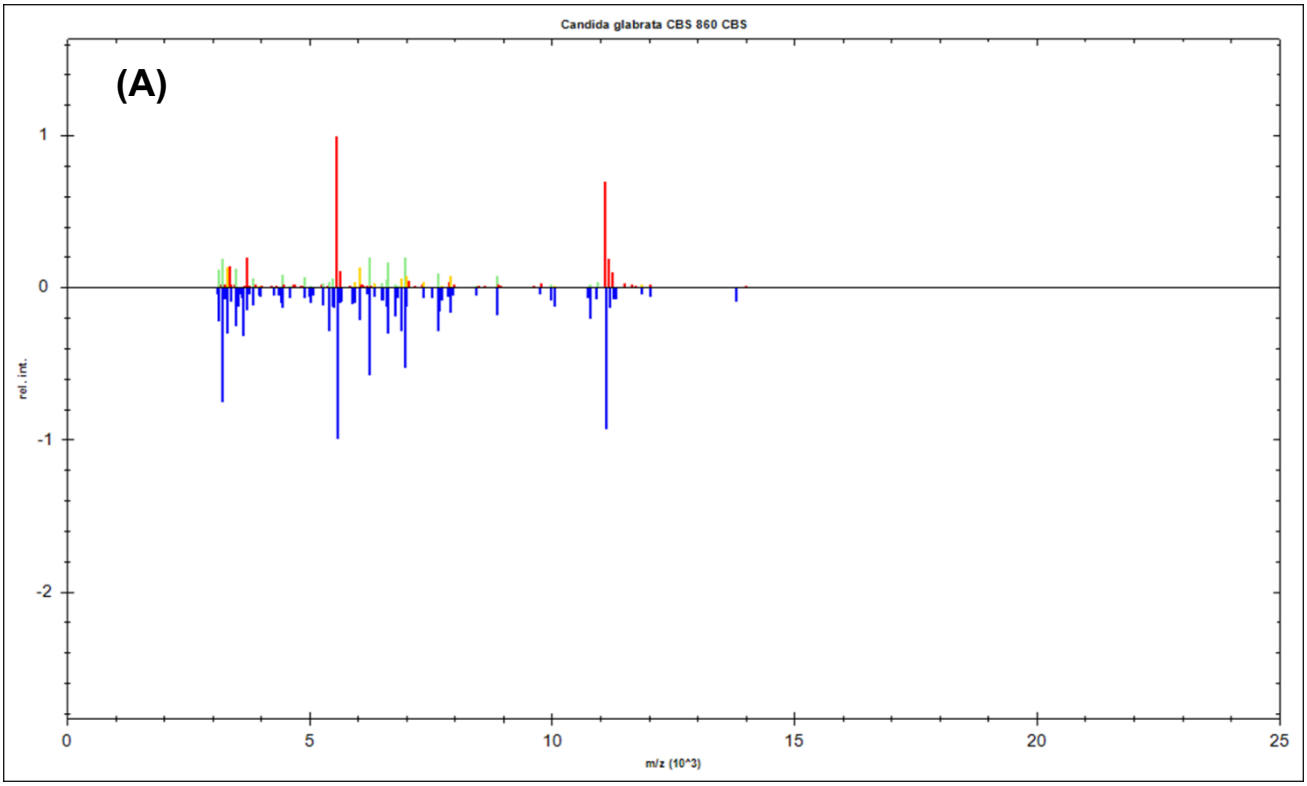
ANNEXES



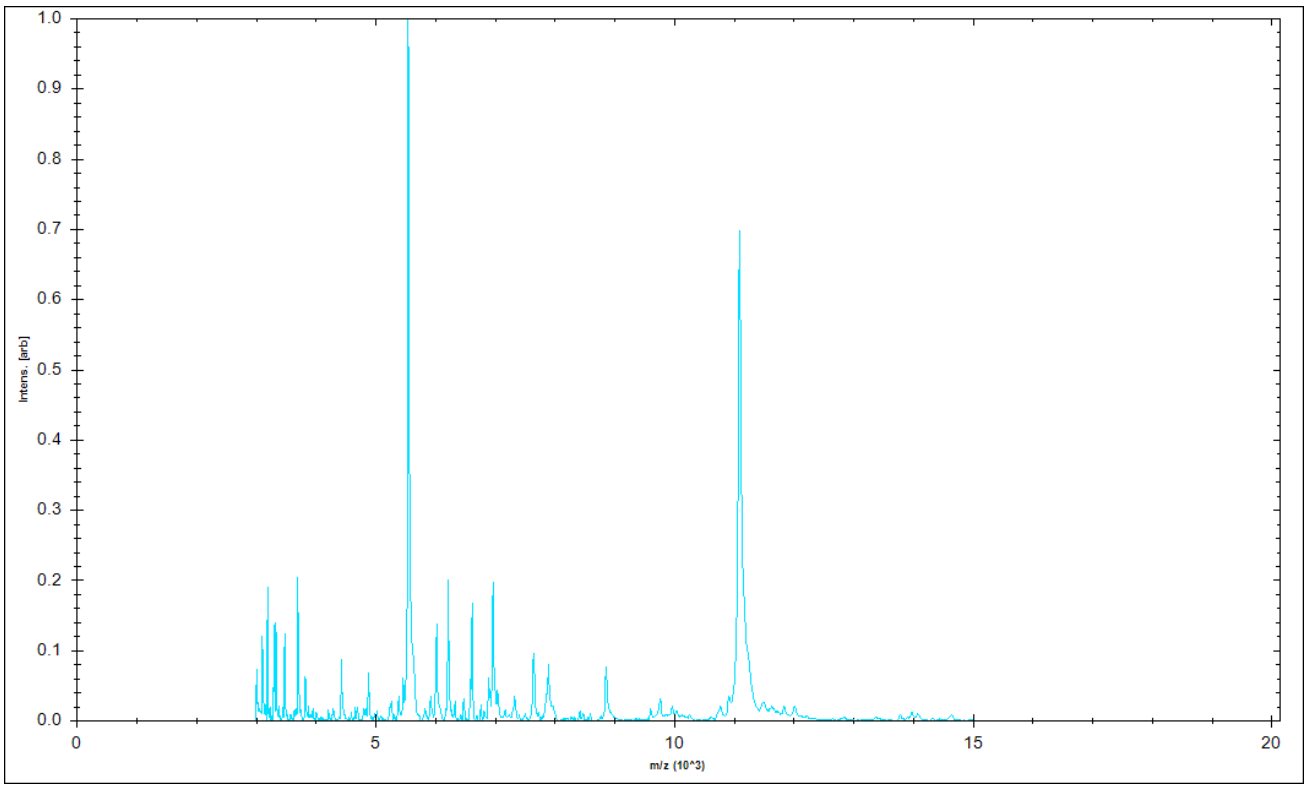
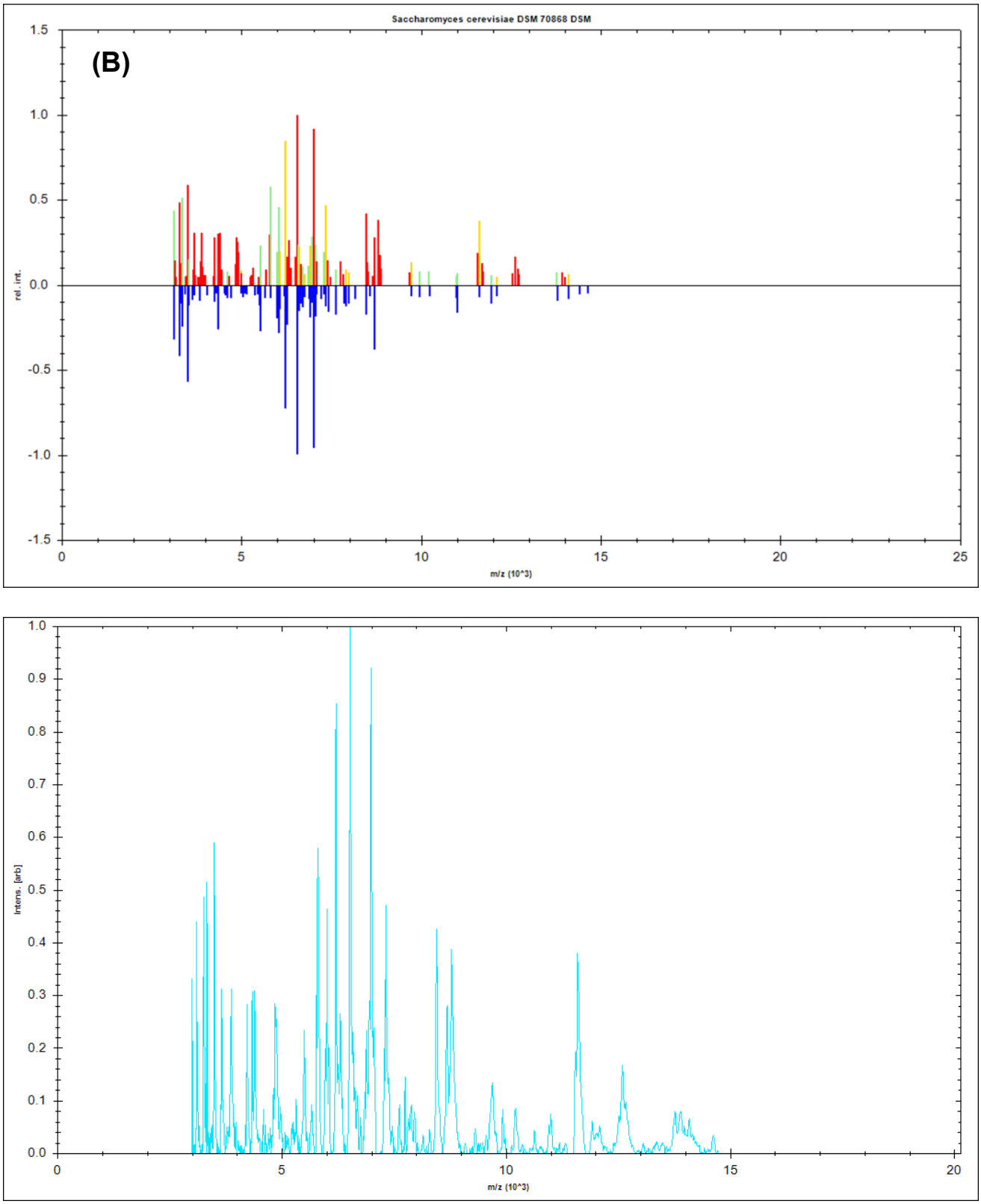


Figure 1-A (Part I). MALDI-TOF mass spectrum analysis of (A) Candida glabrata, (B) Saccharomyces cerevisiae, (C) Pediococcus pentosaceus, (D) Companilactobacillus paralimentarius, (E) Latilactobacillus curvatus, and (F) Hanseniaspora opuntiae.



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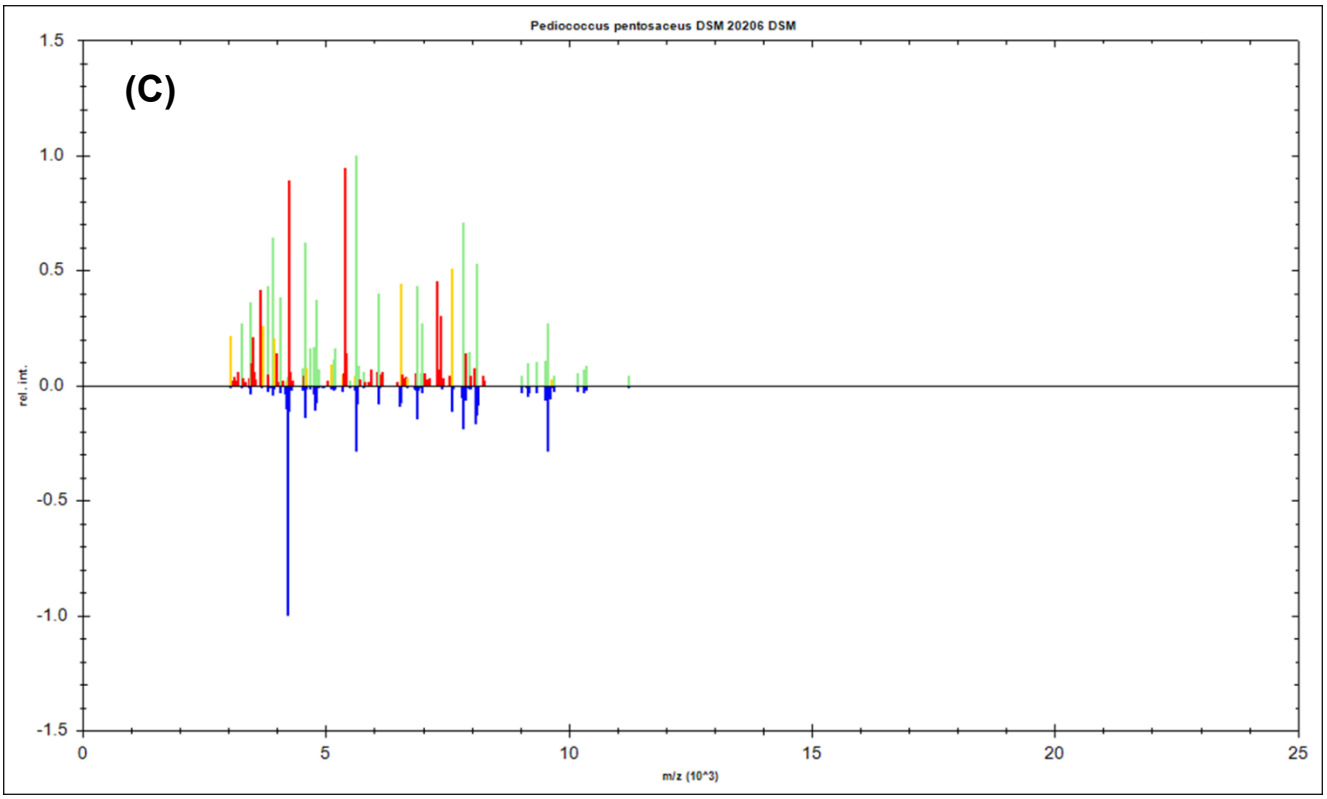

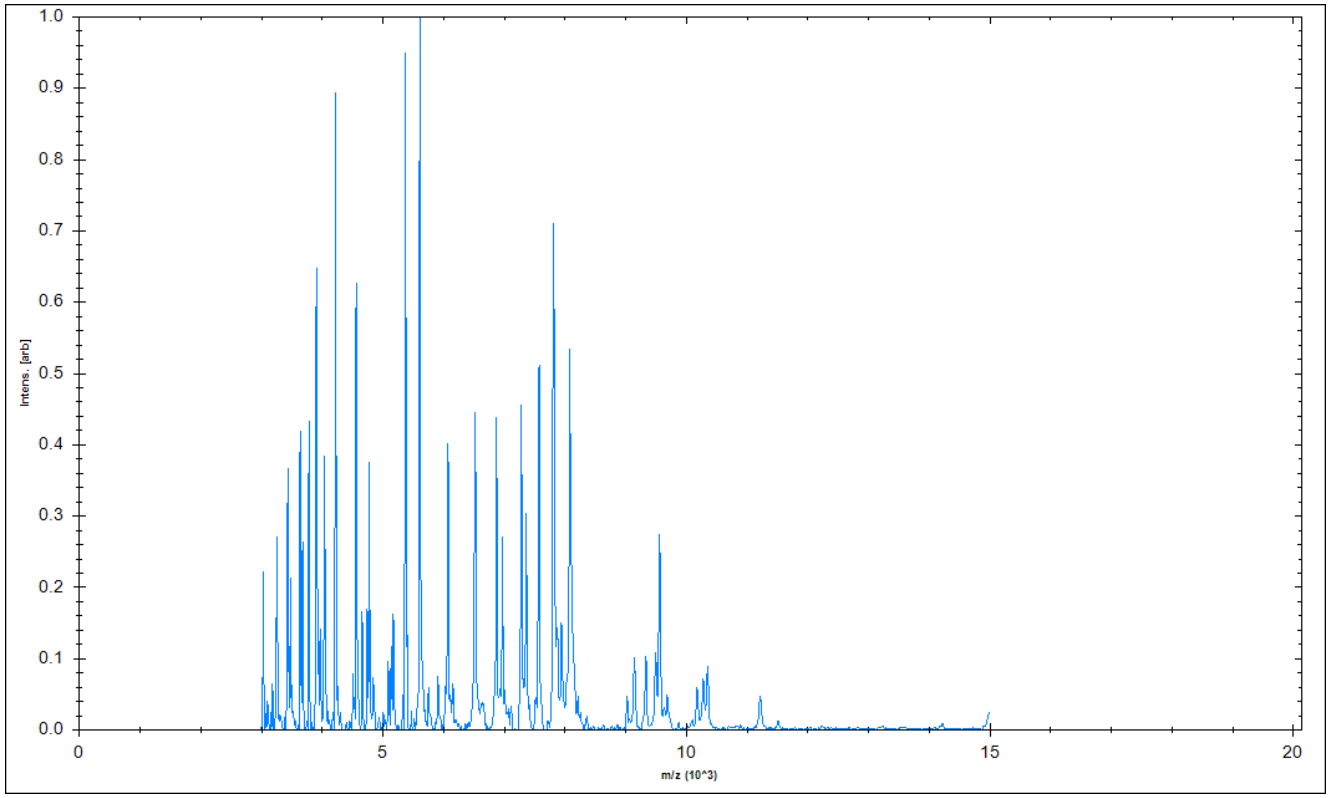

Figure 1-A (Part III). MALDI-TOF mass spectrum analysis of (A) *Candida glabrata,* (B) Saccharomyces cerevisiae, (C) *Pediococcus pentosaceus,* (D) *Companilactobacillus paralimentarius*, (E) *Latilactobacillus curvatus*, and (F) *Hanseniaspora opuntiae.*

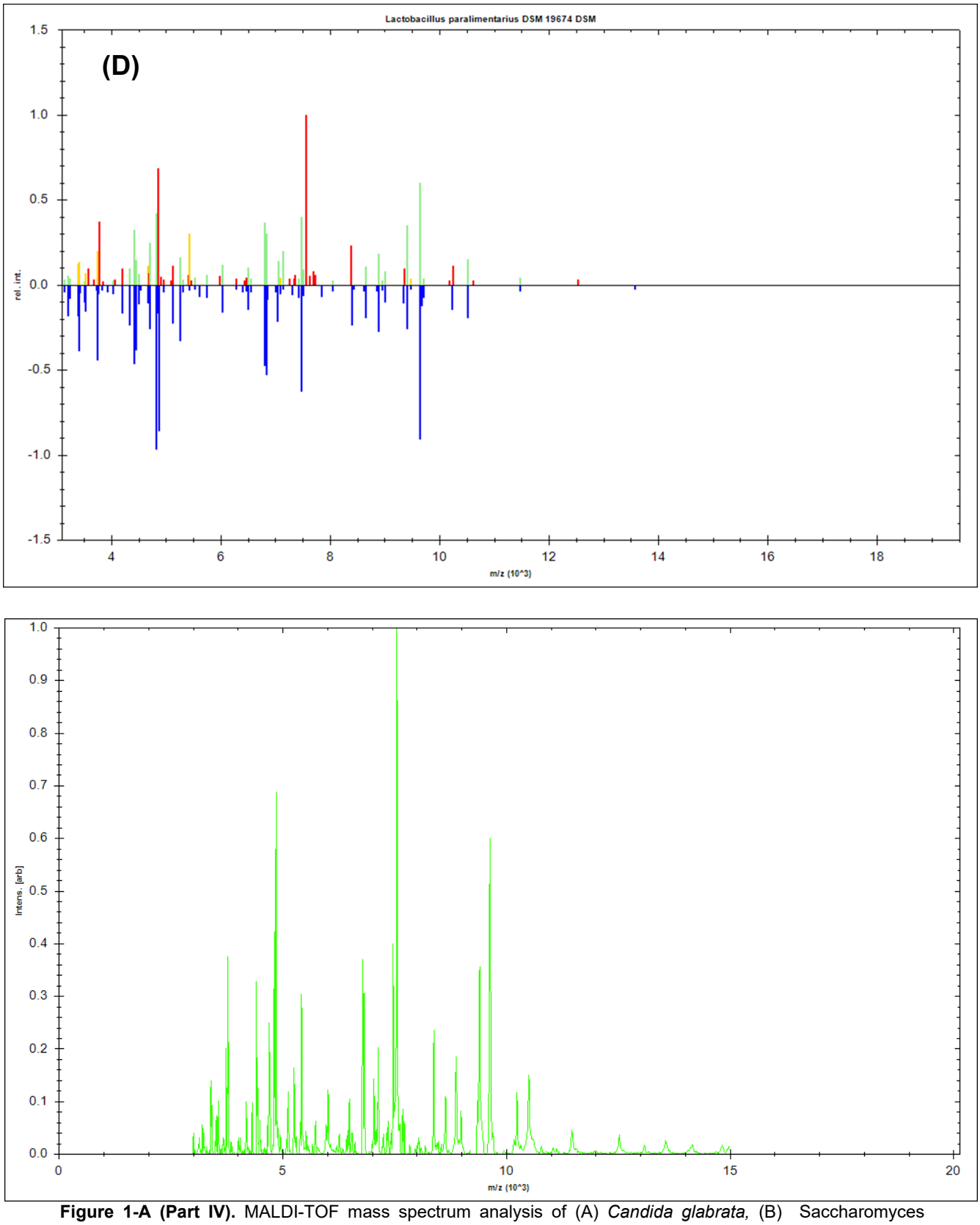

Figure 1-A (Part IV). MALDI-TOF mass spectrum analysis of (A) *Candida glabrata,* (B) Saccharomyces cerevisiae, (C) *Pediococcus pentosaceus,* (D) *Companilactobacillus paralimentarius*, (E) *Latilactobacillus curvatus*, and (F) *Hanseniaspora opuntiae.*

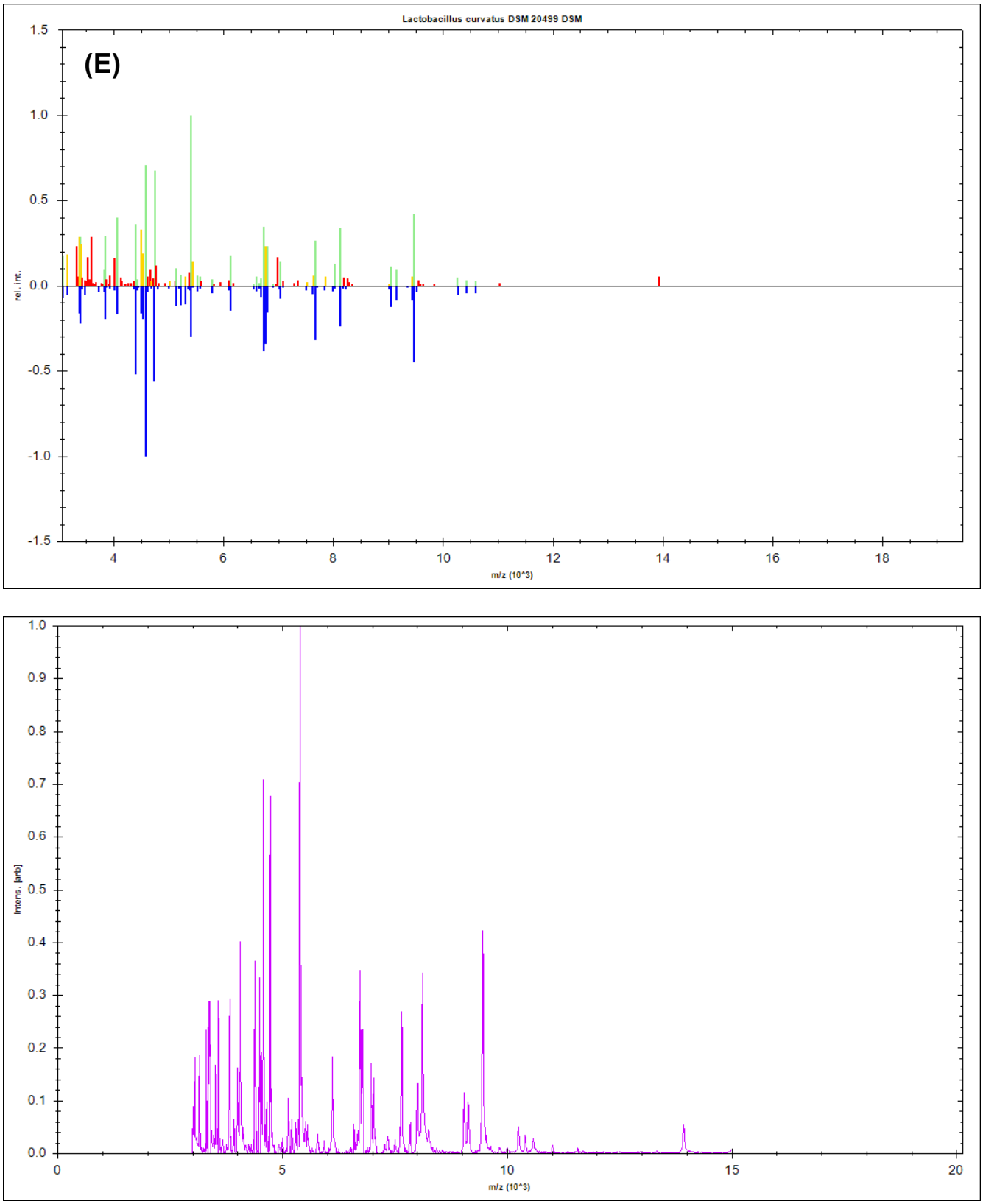

Figure 1-A (Part V). MALDI-TOF mass spectrum analysis of (A) *Candida glabrata,* (B) Saccharomyces cerevisiae, (C) *Pediococcus pentosaceus,* (D) *Companilactobacillus paralimentarius*, (E) *Latilactobacillus curvatus*, and (F) *Hanseniaspora opuntiae.*

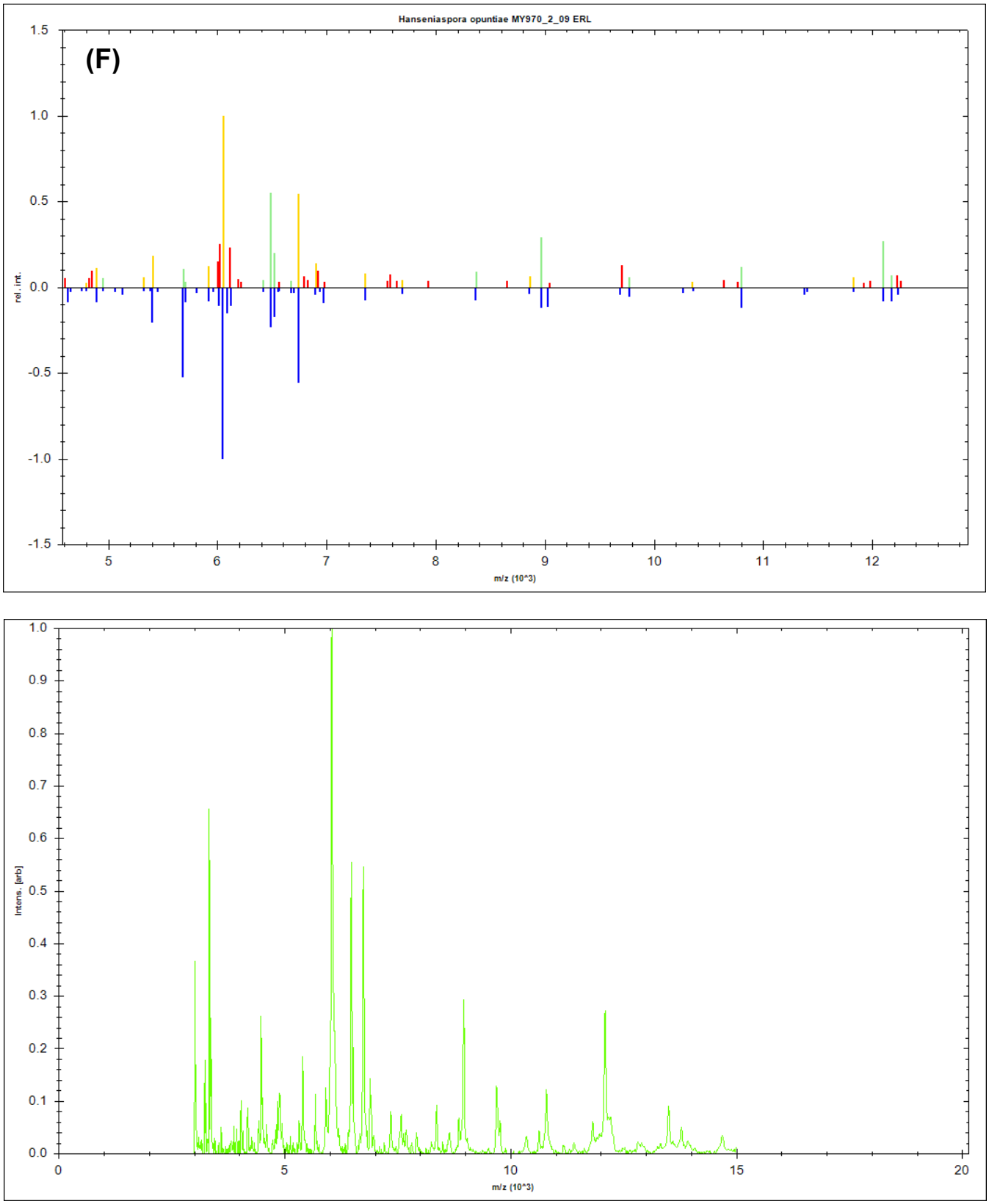

Figure 1-A (Part VI). MALDI-TOF mass spectrum analysis of (A) *Candida glabrata,* (B) Saccharomyces cerevisiae, (C) *Pediococcus pentosaceus,* (D) *Companilactobacillus paralimentarius*, (E) *Latilactobacillus curvatus*, and (F) *Hanseniaspora opuntiae.*

CURRICULUM VITAE

Omar Pérez Alvarado earned the Biotechnology Engineering degree from the Instituto Tecnológico y de Estudios Superiores de Monterrey, Guadalajara Campus in December 2018. He was accepted in the Biotechnology Master program in August 2020, working under the supervision of Dr. Tomás García Cayuela. This thesis represents the termination of her Master studies.

He made a stay in the area of Microbiology at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ) in a project releated to prebiotic evaluation of agave fructans through *in-vitro* digestion. Furthermore, he made another stay in the Sup Biotech Institute of Paris, where he coursed the subject of Food Science and Development of new products. About the laboral experience, Omar Pérez worked 2 years in the Research and Development department at Alpezzi Chocolate.

He participated in two congresses:

- A poster presentation introducing the first part of his investigation thesis project titled “Microbial characterization of sourdough and evaluation of the postbiotic effect in bread” at the 52 Congress of Research and Development of Tecnológico de Monterrey 2021, Monterrey Mexico.
- An oral session presenting “Developing of bread with postbiotic potential using a defined microbial consortium and sourdough” at the 5 Congress of Functional Food and Nutraceuticals, Guadalajara, Mexico.

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