University of Modena and Reggio Emilia

Ph.D. School of Agri-Food Sciences,
Technologies and Biotechnologies

XXXV Cycle

Exploring the diversity and functionality of yeasts and lactic acid bacteria from sourdoughs in the Unimore Microbial Culture Collection

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Abstract

Novel sourdough-based biotechnology applications have recently been raised to address customer demand for natural and healthful food as well as provide new inputs for the food industry. This PhD project has focused on the beneficial effects of sourdough yeasts and lactic acid bacteria (LAB) and the production of various secondary metabolites, with both technological and functional roles. Additionally, investigations on the ability of sourdough LAB to produce antifungal and antibacterial compounds to be used as bio-preservatives have been made. The recovery of yeasts and LAB involved in sourdough fermentation was the first step of the project to select strains suitable as starters with optimal technological aptitude and capable of producing desired aromas and/or aromatic precursors. To this aim, yeasts and bacteria strains were isolated from two sourdough samples and their derived doughs were used for Panettone production. They were identified and deposited in the Unimore Microbial Culture Collection (UMCC, www.umcc.unimore.it). The main species detected were *Saccharomyces cerevisiae*, *Kazachstania humilis*, *Fructilactobacillus sanfranciscensis Lactiplantibacillus plantarum, Furfurilactobacillus rossiae, Lactobacillus parabuchneri, Leuconostoc citreum,* and *Leuconostoc mesenteroides*. In addition, volatile organic compounds were identified and correlated to the strains. Additionally, the potential of LAB strains to produce exopolysaccharides (EPSs) was assessed. These compounds are gaining popularity due to their useful features and adaptability to a variety of food-related commercial applications such as gluten-free (GF) products. EPS-producing LAB strains help to enhance the rheological and sensory qualities of GF products which are often characterized by an unpleasant flavour, and fat-richness. To detect LAB with the potential ability to produce EPSs, several strains were isolated from gluten-free rice sourdoughs. Among them, LAB strains belonging to the species *L. plantarum* and *P. pediococcus* were able to produce dextran-like EPS. Moreover, the most promising LAB strains were tested against common spoilage agents including bacteria (*Escherichia coli, Campylobacter jejuni, Salmonella typhimurium and Listeria monocytogenes*) and moulds (*Fusarium graminearum, Aspergillus flavus, Penicillium paneum* and *Aspergillus niger*). A strain-specific behavior against the moulds was detected and a wide activity against bacterial spoilage was proved. Subsequently, the best-chosen strains including *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, and *P. pentosaceus*

UMCC 3010, were used to design a novel strategy for the cultivation of selected starters with a bio-protection activity by valorizing bread waste and cheese whey by-products. During the third year of PhD, further investigation on the best performer LAB from the UMCC collection and other LAB from Danmarks Tekniske Universitet collection were done to detect specific bacteriocin and antifungal compounds. Different assays against specific spoilage bakery agents *Bacillus* spp. *Lysinobacillus* spp. and *Paenibacillus* spp. were performed showing bacteriostatic activity and genomic findings revealed the presence of functional operons of IIc/b class bacteriocins. Specific bakery moulds were also challenged and GC-MASS and HPLC-DAD were performed along with co-culture and detoxification assays. The obtained results highlighted the specific features of the various strains giving the possibility to build starter culture with specific features.

Sintesi

Negli ultimi anni, la crescente richiesta da parte dei consumatori di cibi definiti “clean label” e salutari ha influenzato le industrie alimentari e le biotecnologie verso lo sviluppo di colture starter in grado di soddisfare la richiesta di nuovi prodotti. In questo contesto, la riscoperta dei prodotti fermentati, ed in particolare del lievito madre, ha portato alla ricerca di lieviti e batteri lattici con spiccate abilità tecnologiche per la creazione di colture starter innovative. In particolare, questo progetto di dottorato si è concentrato sugli effetti benefici del lievito madre, sul suo pool microbico di lieviti e batteri lattici (LAB) e sulla produzione dei vari metaboliti secondari, con ruolo sia tecnologico che funzionale. Nello specifico, sono stati condotti diversi studi sulla capacità di bio-preservazione dei LAB e la loro abilità di produrre composti ad impatto tecnologico come aromi ed esopolisaccaridi. La prima parte del progetto si è focalizzata sull’ottenimento di ceppi di lieviti e LAB caratterizzati da spiccate abilità tecnologiche e aromatiche, isolati da paste acide per la produzione di Panettoni artigianali e dai relativi impasti. I ceppi di diverse specie ottenuti dai vari isolamenti sono attualmente depositate nella Unimore Microbial Culture Collection (UMCC, www.umcc.unimore.it). Sia i lieviti che i batteri lattici ottenuti risultano tipici colonizzatori degli impasti acidi e sono rappresentati da: *Saccharomyces cerevisiae*, *Kazachstania humilis*, *Fructilactobacillus sanfranciscensis Lactiplantibacillus plantarum*, *Furfurilactobacillus rossiae*, *Lactobacillus parabuchneri*, *Leuconostoc citreum*, e *Leuconostoc mesenteroides*. Nello specifico, sono stati condotti studi di correlazione tra il volatiloma e i vari microrganismi, e successivamente la capacità di produzione di esopolisaccaridi da vari LAB è stata investigata. Questi ultimi infatti, rappresentano un potenziale prodotto microbico per il miglioramento tecnologico e nutrizionale dei prodotti da forno senza glutine (GF), normalmente caratterizzati da assenza di gusto e scarse qualità nutrizionali. Diversi ceppi appartenenti alle specie *Lacp. plantarum* e *P. pentosaceus*, isolati da una pasta madre GF ottenuta da farina di riso, hanno mostrato la capacità di produzione di simil-destrani. Inoltre, i LAB ritenuti maggiormente significativi per le per produzione aromatica e/o esopolisaccaridica, sono stati utilizzati in studi per il controllo/eliminazione di egenti deterioranti sia batterici (*Escherichia coli*, *Campylobacter jejuni*, *Salmonella typhimurium* e *Listeria monocytogenes)* che fungini (*Fusarium graminearum*, *Aspergillus flavus*, *Penicillium paneum* e *Aspergillus niger)*. I risultati ottenuti hanno mostrato una capacità di inibizione ceppo-specifica

e, i ceppi *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, e *P. pentosaceus* UMCC 3010, ritenuti migliori sono stati impiegati nella realizzazione di coating naturale ad azione bio-preservante e un terreno ottenuto da scarti di pane e sieri di latte. Il terzo ed ultimo anno di dottorato, ulteriori analisi sui ceppi di LAB più promettenti della collezione UMCC e della collezione della Danmarks Tekniske Universitet sono state condotte, con lo scopo di identificare la presenza di eventuali batteriocine e/o composti antifungini. Pertanto, diversi esperimenti a carico di agenti deterioranti e/o patogeni specifici per i prodotti da forno, sono stati condotti. I risultati ottenuti hanno evidenziato la presenza di batteriocine di classe IIb/c e la possibile azione detossificante nei confronti dei metaboliti fungini. In conclusione i risultati ottenuti nel corso del dottorato hanno evidenziato la possibilità di una creazione di colture starter con spiccate abilità tecnologiche.

Preface

Nowadays, consumers in industrialised countries require their daily food to be “clean label”. This trend could be practically translated into more natural, organic food free of additives or preservatives. Consumers are developing a more conscious approach to sustainability and health issues. They become cautious about food labels and food processing, avoiding ultra-processed products and labels with E- numbers supposedly associated with negative health effects [1]. Meanwhile, lactic acid fermentation is a straightforward and natural process that might be used to address new consumers' requirements. Lactic acid bacteria (LAB) are responsible for lactic acid fermentation and are frequently found in plenty of fermented foods and drinks. Through their metabolism, LAB contributes to the safety, shelf life, nutritional value, and sensory quality of fermented foods. Homofermentative LAB produce lactic acid as the main end-product of carbohydrate fermentation, while heterofermentative LAB in addition produce other compounds such as acetic acid, CO_2, ethanol, acetoin, and diacetyl [2-3]. Due to the production of lactic acid, other by-product and their synergistic actions (e.g. pH lowering, bacteriocins, organic acids) LAB can serve as an alternative to chemical food additives like nitrite and sulphite as antibacterial agents [4-5]. Moreover, the nutritional and sensory quality of the food improves due to the increasing amount of vitamins, minerals, dietary fiber (DF), antioxidants, probiotic effects, and the production of exopolysaccharides, aroma compounds, and vital enzymes [6]. LAB has a long history in human use and they were probably applied unintentionally in the beginning as homemade fermentation occurred spontaneously with no control or reproducibility [7]. The food industry later came to embrace the fermentation of food matrices with the direct addition of selective starter cultures due to the benefits of high levels of control over the fermentation process and the consistency of the final product. Starter cultures are made up of microorganisms (bacteria and/or yeasts) that may easily be added to food materials with the objective of obtaining consistent sensory and quality features in the

product [8]. However, the basic selection criteria for the industrial starter culture's strain primarily focusing on dairy starters resulted, in limited biodiversity leading to a loss of the unique quality of the original product and opening the research for new promising strains. Food sectors have recently looked for strains with desired features and functionality in natural and wild-type LAB from vegetable matrix, traditional fermented food, and microbial culture collection due to their variety of metabolic activities [2]. Culture collections are the heart of microbial biodiversity with the principal aim of preserving and providing pure cultures for industrial and research purposes, playing a significant role in the development of innovative products [9]. Consequently, the research of new lactic acid bacteria in traditional fermented food and microbial culture collection (mCC) has raised the demand for promising starter cultures to reach a "clean label" attempt without artificial additives in food products. In this context, this Ph.D. thesis exploited the metabolic potential of different species of LAB isolated from various matrices and preserved in mCCs. Specifically, five different chapters introduced the main research topics and the consequent publications. **Chapter I** introduces the main protagonist of fermentation, yeast and lactic acid bacteria, and highlights the fundamental aspects of fermented cereal and their different positive outcomes both on consumers and product quality. Subsequently, the following **Chapter II,** focused on sourdough, its microbial population, and its aromatic production. The exploitation of the different metabolic abilities of lactic acid bacteria is then continued by **Chapter III**, in which the exopolysaccharide's ability and the use of these microorganisms in bio-preservation and circular economy are introduced. Conclusively, **Chapter IV** and **Chapter V** specifically analysed the antibacterial and antifungal capacity of LAB against bakery spoilage agents such as ropy agents and cereal moulds.

1. Chapter I

This chapter is an introduction to the below review **"Functional and healthy features of Conventional and Non-conventional sourdoughs"** published in *Applied science* on 20 April 2021.

1.1 Yeasts: An Introduction

Yeasts are eukaryotic microorganisms part of a group of organisms defined as "fungi," alongside moulds and mushrooms. [10]. The research conducted over a 30-year period by Emil Christian Hansen helped to distinguish between different yeast species and shed light on their biological characteristics [11]. Yeasts are represented by a polyphyletic group of ascomycetous and basidiomycetous fungi described by a unicellular growth phase and sexual stages that are not confined to fruiting bodies. They are considered ubiquitous and due to their metabolic diversity, yeasts are generally employed in various industrial and food processes such as the production of ethanol, vitamins, organic acids, carotenoids, enzymes, and food fermentations. In fact, yeasts can normally be isolated from different food matrices and are widely used as starter cultures in wine, beer, and fermented cereals products such as sourdough bakery goods [10, 12]. During the decades, more than 1200 species of yeasts from over 186 genera have been identified. However, according to the most recent data, such species represent less than 1% of all the species present in nature [13-14]. Historically, phenotypic features and physiological traits were the major foundations utilised in yeast taxonomy, and comparison between the morphological characteristics, biochemical profiles, and physiological profiles of novel species with those previously identified were necessary to characterise and identify them [15]. Generally, the identification and official description of novel species were based on the fermentation and/or absorption of various sugars, organic acids, alcohols, sugar alcohols, starch, and various nitrogen sources, as well as growth differences. Gradually, novel

biochemical approaches such as the assessments of the cell wall and capsule's polysaccharide composition, mycocin susceptibility, and electrophoretic comparisons of enzymes are employed [16]. However, these methods involve significant quantities of tests, and technical equipment, and are mostly performed only by yeast taxonomist specialists highlighting the global importance of molecular techniques for yeast identification. Beginning with the analysis of DNA base composition, typically expressed as CG content, a ratio of 28 to 50% GC was estimated for yeasts, varying from 50 to 70% GC for basidiomycete yeasts [17]. *Saccharomyces cerevisiae*'s genome was the first fungal genome sequenced, followed by *Candida albicans*, and became a fundamental biologic model establishing the ground for new genomic findings [18-19]. As aforementioned, due to their remarkable colonization ability and metabolic diversity, the various yeasts species are widely used and exploited by the food and beverage industries (Figure 1) such as *S. cerevisiae*, the industry's gold pillar for the wine, beer, bread, and biofuel sectors, *Kazachstania humilis*, fundamental in the sourdough environment, *Cyberlindnera jadinii* (food additives), *K. pastoris* (heterologous protein production), and *Eremothecium gossypii* (riboflavin) [20]. Fermented foods, in particular, cereal fermentation and sourdough, represent a fundamental section of the global food industries. Yeasts could be naturally present in raw materials such as flour, fruit, and vegetables, and consequently in sourdough or externally added, and their contribution is fundamental for the development of the dough and the generation of the various volatile compounds. *Kazachstania humilis* (formerly *Candida humilis*), *Debaryomyces hansenii*, *S. cerevisiae*, *S. uvarum*, *S. servazii*, , *Dekkera bruxellensis,* and *Epicoccum nigrum* are only some of the main species involved in cereals fermentation [21].

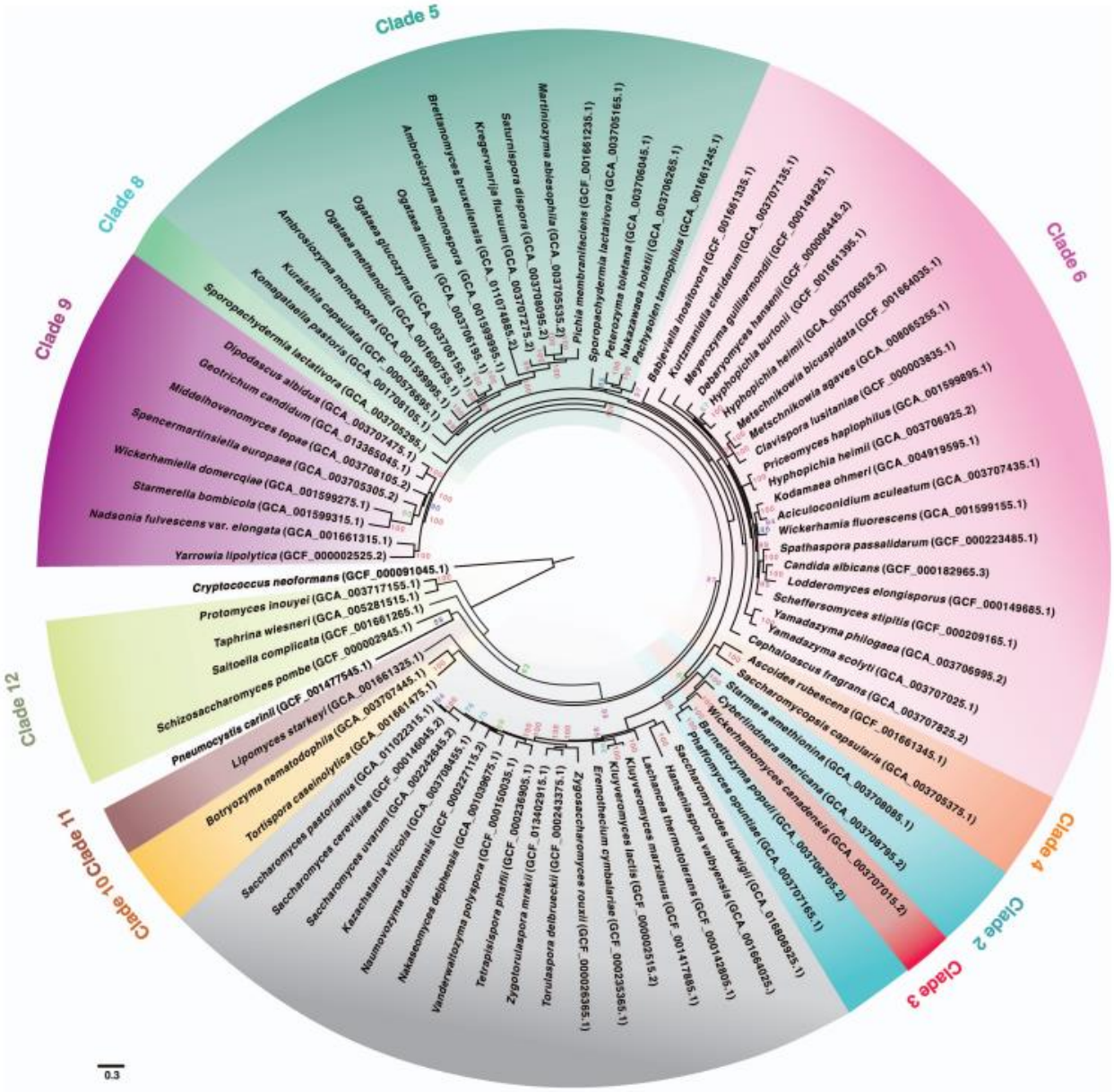


Figure 1. Relationships among ascomycete yeast of industrial importance. Reference taxa were determined from maximum likelihood analysis of the complete genome and *Filobasidiella (Cryptococcus) neoformans* was chosen as outgroup species. Bootstrap values (1000 replicates) >50% are given at branch nodes. Image from [20].

1.2 Lactic Acid Bacteria: An introduction

Lactic acid bacteria (LAB) are a significant group of aerobic or facultative anaerobes with low (≤55 mol%) GC content, Gram-positive cocci or rods, catalase-negative, non-spore-forming microorganisms, low-pH resistant, widely distributed in nature [22]. The morphology, mode of glucose fermentation, growth at specific temperatures, and range of sugar utilization were

previously used to classify the LAB genera [8]. Although the taxonomy has been revised, characters proposed in Orla Jensen's monograph, continue to play a significant role in the current classification of LAB representing a group of bacteria with morphological, metabolic, and physiological similarities. They consist of various phylogenetically diverse genera counting *Lactobacillus, Bacillus, Pediococcus, Leuconostoc, Lactococcus, Enterococcus, Carnobacterium, Oenococcus, Alkalibacterium, Staphylococcus, Weissella, Vagococcus, Streptococcus,* and *Tetragenococcus*, where the first one is the main group comprising at least 261 species [23]. These bacteria can be described as omnipresent in various ecological niches rich in nutrients, ranging from food product habitats such as milk, meat, and vegetables to mouth, intestine, and vagina [24]. They are able to produce a variety of organic acids as a by-product of carbohydrates fermentation such as lactic acid, acetic acid, propionic acid, citric acid, etc. LAB have a long history of use in the food industry and are generally regarded as safe (GRAS). Various LAB species are becoming renowned for their inventive applications in various fermentation-related biotechnologies due to their useful features including rapid growth, acid tolerance, antimicrobial activity, safety for human and animal consumption, and industrial-scale fermentation and are currently being extended to produce chemicals, vitamins, and biochemical peptides and regarded as bio-preservatives.

1.2.1 Phylogeny of LAB and general genomic features

LAB belong to the phylum *Firmicutes,* class *Bacilli,* order *Lactobacillales,* and include various families. According to Vandamme et al., 2014 [25] LAB taxonomy assessment, the order *Lactobacillales* consists of 6 families, 38 genera, and about 400 species. Recently, the species of the *Lactobacillaceae* family have been classified into 25 new genera of *Lactobacillus, Paralactobacillus,* and 23 novel genera, according to a recent publication in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) [23]

(https://www.microbiometimes.com/the-lactobacillus-taxonomy-change-has-arrived-what-do-you-need-to-know/).

The primordial LAB were likely *Bacillus*-like soil organisms, subsequently adapted to rich nutritional ecological niches resulting in the loss of several genes and the related physiological function [26]. Molecular biological criteria, such as 16s rRNA sequencing were used to phylogenetically cluster LAB (Figure 2), and with the abundance of data currently available from whole-genome sequencing, comparable and/or more intricate phylogenetic trees have been built using different gene sets [27]. Though, the general picture of the phylogenetic position of LAB remains unchanged.

Lactococcus latics spp. *lactis* IL1403 in 2001 represents the first reported LAB genome sequence [28]. Since 2001, more than 75 genomes of industrially important LAB have been sequenced, and the recent advance in sequencing techniques in the last decades has led to a rapid increase of complete genome sequence with hundreds of genomes of different LAB genera available in the National Center for Biotechnology Information (NCBI) genome database. Genomes of LAB are characterized by low GC contents and range in size from 1.23 (*Fructilactobacillus sanfranciscensis*) to 4,91 Mpb (*Lentilactobacillus parakefiri*) [29]. The genetic events involving genome reduction, horizontal gene transfer (HGT), and gene duplication have been deemed to contribute to the present genome shape and structure of LAB [30]. This abundance of data not only provides a new comprehension of the taxonomy and evolutionary history of LAB, but also reveals the genetic underpinnings of a number of phenotypic features important for the industrial usage of these bacteria [26].

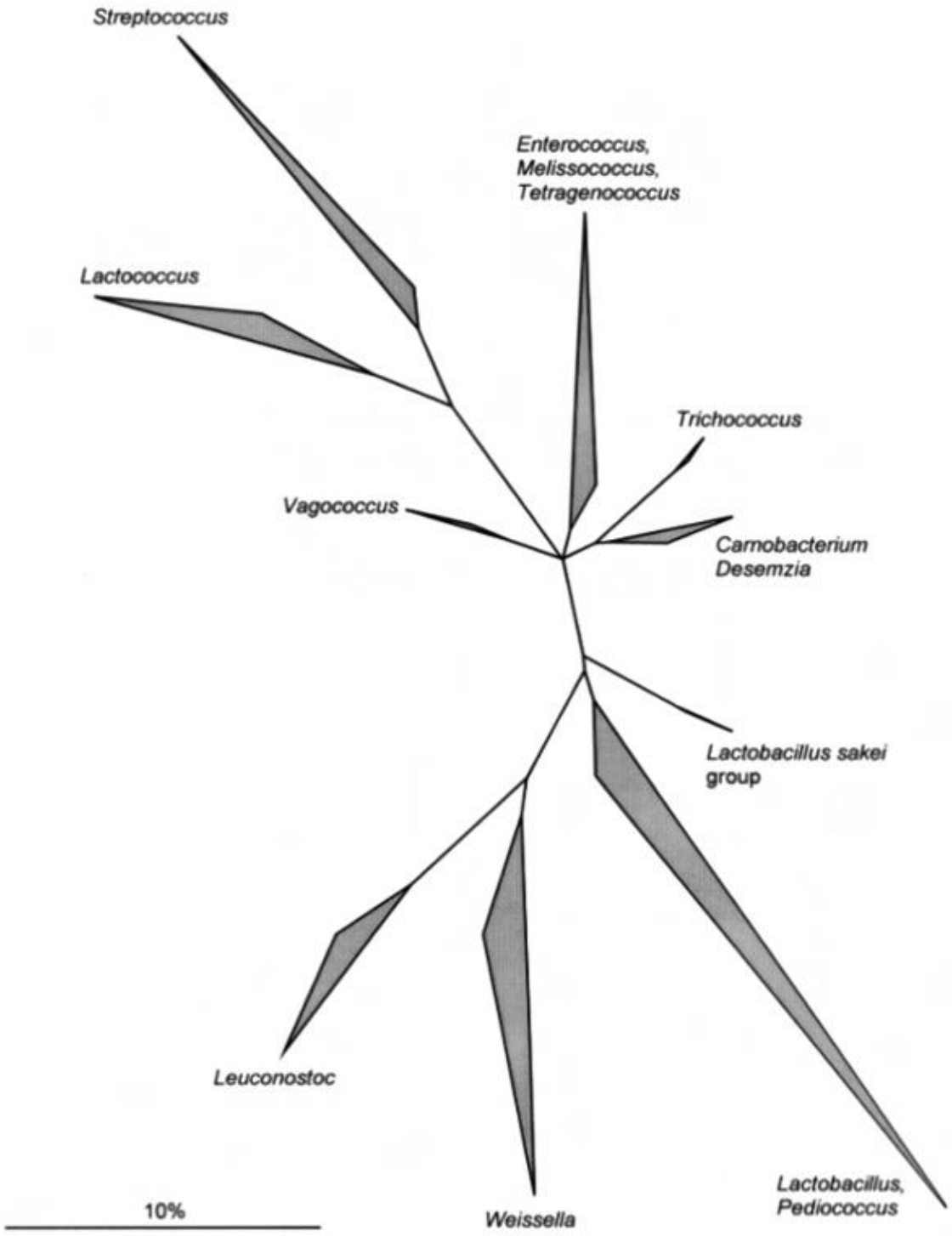


Figure 2. Phylogenetic tree illustrating the position of LAB genera in relation to each other. The consensustree is built on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of gram-positive bacteria. The family *Lactobacillaceae* also includes the genera *Oenococcus* and *Fructobacillus*. The bar signifies 10% estimated sequence divergence [26] with permission.

1.2.2 General Lactic Acid Bacteria Metabolism

LAB hold a relatively simple central metabolism and are identified as chemotrophic microbes as they produce energy (ATP) by substrate-level phosphorylation [31]. Although these bacteria possess a fermentative metabolism and mainly rely on sugar when growing under anaerobic conditions, they can also grow in the presence of oxygen. Several LAB species can convert from fermentation to aerobic respiration metabolism when heme or heme plus menaquinone groups are present, despite having a well-established fermentative metabolism. The first documented electron transport chains and cytochrome evidence in heme-grown cultures came from lactococci, leuconostocs, and certain enterococci [31].

Sugar metabolism

LAB species are generally classified into homofermentative and heterofermentative (facultative or obligate) types depending on the way they fermented carbohydrates. Homofermentative LAB (HoLAB), represented by *Enterococcaceae*, *Streptococcaceae* , and several *Lactobacillaceae* used the Embden-Meyerhoff-Parnas (EMP) pathway (Figure2A) to ferment carbohydrates into pyruvate (terminal electron acceptor), which is then transformed into lactic acid by lactate dehydrogenase (LDH) (theoretically 1 mole of glucose produces 2 moles of lactic acid). L-LDH and D-LDH are stereospecific nicotinamide adenine dinucleotide (NAD)-dependent enzymes that can synthesize the two different forms of lactate isomers, L and D. The homolactic metabolism can also lead to the production of ethanol and acetate through the EMP pathway when switch to a mixed-acid metabolism under particular growth conditions, such as carbon constraint, and formate, acetate, ethanol, and/or CO_2 are produced in addition to lactate [32] (Figure 3A). This is possible because NADH oxidase (NOX) is active in the presence of oxygen and has a high affinity for NADH, it outcompetes LDH for the available NADH, switching the fermentation from homolactic to mixed acid under aerobic circumstances, with the end products depending on the culture pH. *Lactobacillaceae* and

Leuconostoccaceae species are heterofermentative (HeLAB) and typically use the phosphoketolase pathway (PKP, commonly known as the pentose route, 6-phosphogluconate route or the phosphoketolase shunt) to ferment sugars. Pentoses sugar such as xylose and ribose during fermentation lead to the production of pyruvate and acetyl-P later converted into lactate and acetate, respectively while glucose and other hexoses, can be transformed into lactate, CO_2, and ethanol (Figure 2A). A two-domain aldehyde/alcohol dehydrogenase enzyme for ethanol synthesis is present in all heterofermentative LAB [33]. Some homofermentative species also possess this enzyme, in which case they also include pyruvate-formate lyase (PFL), an enzyme not present in heterofermentative LAB, for the anaerobic conversion of pyruvate into acetyl-CoA and formate [33]. The presence or activity of phosphofructokinase (PFK) and fructose-1,6-biphosphate aldolase (FBA) appears to be a significant variation in central carbon metabolism enzymes between heterofermentative and homofermentative LAB that influences the utilization of PKP or EMP. However, the knowledge regarding gene, enzyme, and the fundamental level of the regulation of the various LAB pathway are very little and incomplete to understand the large strain-dependent variations [33]. In addition, LAB are capable of naturally producing a wide range of different end products when grown on carbon sources other than glucose or under aerated conditions, such as aromatic compounds (e.g. diacetyl, acetoin), exopolysaccharides (EPS), B vitamins mainly riboflavin, folate and cobalamin, low-calorie polyols and enzymes (Figure3B).

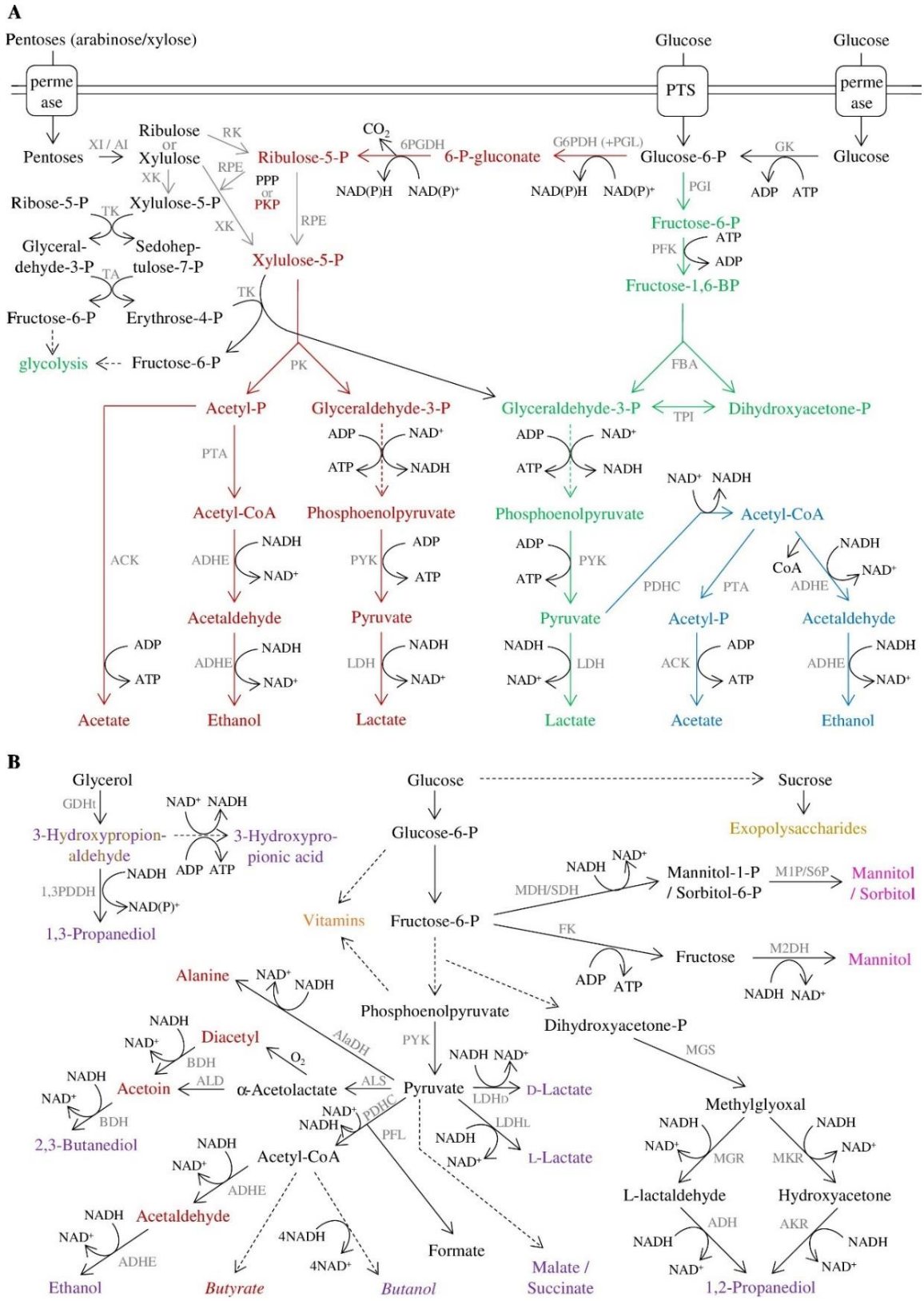


Figure 3. Representations of the common metabolic pathway of Lactic Acid Bacteria (A)
Red: representation of Hetero-lactic fermentation via PKP pathway; Green: representation of homo-lactic fermentation via EMP pathway; Blue: mixed acid fermentation in homolactic species via EMP pathway; Black: pentose phosphate pathway and relative connection to PKP and EMP. Different options for one molecule are reported with grey arrows while dashed one denote multiple enzymatic steps. Enzyme abbreviations: PTS phosphotransferase system; GK glucokinase; G6PDH (+ PGL) glucose-6-P dehydrogenase (+ phosphoglucolactonase); 6PGDH 6-P-gluconate dehydrogenase; RPE ribulose epimerase; RK ribulokinase; XI/AI xylose isomerase/arabinose isomerase; XK xylulokinase; TK transketolase; TA transaldolase; PK phosphoketolase; FBA fructose bisphosphate aldolase; TPI triose-phosphate isomerase; PYK pyruvate kinase; LDH lactate

dehydrogenase; PDHC pyruvate dehydrogenase complex; PTA phosphotransacetylase; ACK acetate kinase; ADHE bifunctional aldehyde and alcohol dehydrogenase. Summary of possible natural end products of metabolism of LAB under specific conditions. Pink: flavoring compounds; Yellow: texturing and medical ingredients; Orange: beneficial products; Purple: chemicals and fuels. The compounds in italic indicates that a metabolic engineering is required to obtained them due to a lack of enzymes. Enzyme abbreviations: GDHt glycerol dehydratase; 1,3PDDH 1,3-propanediol dehydrogenase; MDH/SDH mannitol dehydrogenase/sorbitol dehydrogenase; M1P/S6P mannitol-6-P dehydrogenase/sorbitol-6-P dehydrogenase; FK fructokinase; M2DH mannitol-2-dehydrogenase; MGS methylglyoxal synthase; MGR methylglyoxal reductase; ADH (lact)aldehyde dehydrogenase; PYK pyruvate kinase; PFL pyruvate-formate lyase; PDHC pyruvate dehydrogenase complex; LDH lactate dehydrogenase; ALS acetolactate synthase; AlaDH alanine dehydrogenase; ALD acetolactate decarboxylase; BDH butanediol dehydrogenase; ADHE bifunctional aldehyde and alcohol dehydrogenase. Image from [32] , with permission.

Protein metabolism

Lactic acid bacteria have a very limited ability to synthetized amino acids (aa) from inorganic nitrogen sources relying on their presence in the environment or medium growth [34]. LAB with the ability to hydrolyse proteins into peptides and amino acids include *L. plantarum, L. casei, L. curvatus* and *Streptococcus*. LAB proteolytic activity can be summarize in four steps: protein degradation, peptide transport, peptide degradation and amino acid catabolism [35]. Cell envelope proteinase (CEP), which turns proteins into oligopeptides, marks the start of proteolysis [36]. Following this, oligopeptide (Opp), dipeptide (DtpP), and tripeptide (DtpT) transport systems, respectively, deliver dipeptides, tripeptides, and oligopeptides into cells [37]. Numerous peptidases, such as endopeptidases, aminopeptidases, dipeptidases, tri-peptidases, and proline-specific peptidases, break down peptides in cells into amino acids [36]. These enzymes can be present both at the extracellular or intracellular level [38-39]. Due to their proteolytic activities, lactic acid bacteria have drawn a lot of interest. These activities are particularly significant for faster maturation and enzyme modification of many food products, such as cheese. The flavour of fermented foods is mostly derived from peptides and amino acids, umami taste is develop by glutamic acid whereas alanine is responsible for the sweet taste [40- 41]. Bitterness is frequently attributed to big hydrophobic peptides such as valine. However the "bitter" aa might be tasted as other flavours in co-presence with other amino acids [41-42]. Methionine and leucine, for example, are sometimes linked to the flavours of salt and sweetness, respectively. Free amino acids can also serve as building blocks for later taste

synthesis. According to Ardö [43], two mechanisms can convert free amino acids into volatile compounds: pathways started by amino acid aminotransferases and elimination reactions catalysed by amino acid lyase. Aminotransferases catalyse the start of the transamination pathway by converting amino acids into the corresponding α-keto acid taste precursors and α-ketoglutarate into glutamate. The enzymatic processes that generated α-keto acids can go through reductions catalysed by hydroxy acid dehydrogenase producing α-hydroxy acids and decarboxylation that result in aldehydes. Aldehydes can either be oxidatively decarboxylated to produce acyl-CoA and carboxylic acids, or they can be further reduced to alcohols by alcohol dehydrogenase and oxidised to organic acids by aldehyde dehydrogenase. Esterase and acyltransferases can use the corresponding alcohols or organic acids as substrates to generate esters. Methionine and threonine have their respective elimination pathways defined. Carbon-sulphur lyases' action causes the release of thiols, which can then be transformed into sulphur compounds to produce aromas like that of boiled potatoes, garlic, and cabbage. Proteolysis is considered the key pathway to influence flavour, quality, safety, nutrition and allergies reduction in food.

Lipid metabolism

The breakdown of lipids into fatty acids and glycerol by lipases is known as lipid metabolism. Different strains of lactic acid bacteria have either external or intracellular lipases [44] and species of *Lactococcus* and *Lactobacillus* are known to posses them in highly amount. However, not all LAB strains have the ability to process lipids as reported by Meyers and other in 1996 [45]. In general, lipolysis and fatty acid oxidation in fermented foods are recognised as two significant metabolic processes closely linked to the flavour quality of the final products [46]. Free fatty acids, which are significant aroma components in the majority of fermented foods, are released during lipolysis, which is regulated by particular enzymes, especially lipases and phospholipases. A distinctive flavour is provided by short- and medium-chain free fatty

acids (4–12 carbon atoms) because of their significantly lower sensory thresholds, on the contrary long-chain free fatty acids with high perception (>12 carbon atoms) threshold marginally affect flavour. Through lipid oxidation, free fatty acids produce a number of volatile chemicals, including aldehydes, alcohols, ketones, and esters [47]. Due to their low odour thresholds, aldehydes frequently contribute to the distinctive smells of fermented food. Saturated fatty acids decarboxylate β-keto acids by β-oxidation to get the odd-carbon methyl ketones. Reductases can convert methyl ketones into the equivalent secondary alcohols. Unsaturated fatty acids are oxidised by two distinct mechanisms. One of the processes entails the β-oxidation of unsaturated fatty acids to produce hydroperoxides when free radicals are present. The synthesis of 4-/5-hydroxy acids, which are then transformed into γ/δ lactones that have potent fruity tastes, might be anticipated as a result of another pathway of unsaturated fatty acid metabolism [48]. In order to determine the flavour and quality of food, free amino acid synthesis and subsequent events, including transamination, decarboxylation, deamination, and desulfurization, are crucial. Additionally, specific fatty acid transformation events carried out by lactic acid bacteria strains include isomerization, hydration, dehydration, saturation and can be applied both in food and probiotics industries. Lipase activity of lactic acid bacteria are also employed in the creation of dietetic formulation and have exhibited several health advantages for new-borns, geriatrics, and convalescents patients [49].

1.2.3 Lactic Acid Bacteria in Food

Lactic acid bacteria (LAB) or simply "Lactics" play an important role in food and agricultural application. Nowadays, the presence of LAB in food production is far from accidental. Food-related LAB are among the few microbes that humans have cultivated. Domestication of LAB began millennia ago and they have been connected with fermentation and preservation of many foods and have been use for more than 4,000 years [50]. The employment of LAB as starting

cultures for the production of a variety of products is one of their best employment, and they are used both in the food and feed sector to produce silage, beer, wine, sauerkraut, pickles, sourdough bread, and other fermented foods, presenting various species perfectly adapted to the different substrates [2,51]. By decreasing the pH of the food matrix during lactic acid fermentation, LAB starter cultures provide a bacteriostatic or even bactericidal environment for spoilage and harmful microorganisms. LAB also play a significant part in the development of the product's organoleptic qualities, creating an essential aroma and taste chemicals through their metabolic activities such as lipolysis and proteolysis, and they can also contribute to texture by the production of exopolysaccharides (EPS).

These bacteria produce also a variety of functional oligosaccharides applied as prebiotics, nutraceuticals, sweeteners, humectants, drugs against colon cancer, and as immune stimulators [52]. Additionally later, efforts have been made to utilise LAB as microbial cell factories generating beneficial biochemicals such as vitamins, enzymes, exopolysaccharides (EPS), and antimicrobial compounds [5, 49].

1.2.3.1 Starter Culture

Starter cultures can be defined as standardized microorganisms inoculum that are directly injected into food components to obtained predictable and desired changes in the final products [40]. Production of organic acids, polyphenols, EPS, bacteriocins, and other flavourings are common characteristics that support the use of LAB as functional and effective starter cultures. Enhanced preservation, higher nutritional content, altered sensory characteristics, and increased economic value are just a few examples of these alterations [53].

Although many fermented foods can be created without a culture, large-scale industries require quality, consistency, safety, and foreseeable production and the addition of concentrated microorganisms in form of a starter culture. Starter cultures offers a base for guaranteeing that

goods are manufactured on a regular timetable with consistent product attributes. Commercial starter cultures became a never-ending business in 1878 with Christian Hansen and have been daily employed in the fermented food business since the 1930s and 1940s [53]. The first starter culture was grown in heat-sterilized milk with the addition of calcium carbonate as a buffer, they were characterized by short life and loss of fermentative capacity. Thenceforth, considerable improvements have been made in starter culture companies providing different types of technologies to maintain high cell vitality and fermentative capacity. Modern starter cultures vary from communities of numerous strains and species to single microorganisms or limited specific microbial strains. Different criteria, ranging from the rate of metabolism to specificity for the matrix, are used to select the best starter strains, candidate. Nowadays, LAB are clearly the most important group of bacteria used as starter organisms and they are normally commercially available in lyophilized or freeze-dried form. Both preservative methods are linked with excellent stability and long vitality (years), although rehydration and lag phase might be challenging [34]. Through the formation of organic acids, primarily lactic acid, LAB quickly acidify the food source. In addition, they create a number of enzymes, acetic acid, ethanol, aromatic compounds, bacteriocins, and exopolysaccharides (EPS) [54].

Table 1 presents the LAB mostly employed as starter cultures in food fermentations. Although starter cultures are currently used in the production of a wide range of cereal products as well as fermented meat, fish, pickled vegetables, and olive products, the majority of them fall under the category of dairy goods, specifically cheese, yogurt, and fermented milk even now, using LAB as starter cultures for food fermentation remains their most important industrial application.

Fermented Food	Lactic Acid Bacteria
Dairy products Yogurt, cheese, fermented milks	*Lactobacillus delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus, Lacticaseibacillus casei, Lactobacillus acidophilus, Lacticaseibacillus rhamnosus, Lactobacillus helveticus, Leuconostoc mesenteroides* ssp. *cremoris* [55-56]
Fermented meats and fish Sausages, fish, salami	*Latilactobacillus sakei, Latilactobacillus curvatus, Pediococcus acidilactici, Pediococcus pentosaceus, Lactiplantibacillus plantarum* [57-59]
Fermented vegetables Sauerkrat, pickles, fermented olives	*Leuconostoc mesenteroides, Lactiplantibacillus plantarum, Pediococcus acidilactici, Levilactobacillus brevis, Limosilactobacillus fermentum* [60-62]
Fermented cereals Sourdough	*Fructilactobacillus sanfransiscensis, Companilactobacillus farciminis, Limosilactobacillus fermentum, Lactiplantibacillus plantarum, Lactobacillus amylovorus, Limosilactobacillus panis, Companilactobacillus alimentarius, Leuconostoc mesenteroides* [63-64]

Table 1. Lactic acid bacteria commonly used in a different type of fermented foods.

However, some "weaknesses" must be taken into account. In general, phage resistance and quick acidification were used to select commercial starter cultures and since the initial beginning cultures were microbial brews of many unidentified species, daily replication likely caused ecosystem alterations that caused some strains to vanish. On addition, plasmid-encoded crucial metabolic characteristics in lactic acid bacteria run the danger of being lost during reproduction. Furthermore, it is also possible that genetic material was lost as a result of adaptation to the food matrix. As a result, commercial starters' biodiversity has shrunk [38, 65]. Contrarily, pure cultures obtained from the intricate ecosystems of traditionally fermented foods have a variety of metabolic activities that dramatically vary from those of related strains employed as bulk starters in the industry. For this reason, the food research is focused in wild-type type strains from spontaneous fermented foods to be utilised as innovative starter cultures for food fermentation in small and large industries. Moreover, in the last years, the connection between food and health became a hot topic for the customer resulting in an impressive expansion in the food microorganisms not only for fermentation *per se* but also for the production of natural useful secondary metabolites (bacteriocins, vitamins, EPS, etc.) and health-promoting features [66].

1.3 Microbial Culture Collection

During the last decades, as abovementioned, biodiversity preservation and the exploitation of new microbial characteristics for the creation of innovate starter cultures have earned growing interest. In this perspective, culture collections (CCs) and microbial biological resource centres (mBRCs) are essential for the *ex situ* preservation of microbial biodiversity, facilitating public access to microorganisms, reference strains, and microbial resources for the development of biotechnological strategies focusing on promoting sustainable food production [67-69]. This condition, in fact, is essential for future research, new findings, and the exploitation of microbial resources [70]. The broad notion of CC or BRC, according to Smith et al., 2014 [71] entails "the provision of services and repositories of the living cells, genomes of organisms, and knowledge relevant to heredity and the operations of biological systems." The Organization for Economic Cooperation and Development (OECD), which supports international policies aimed at enhancing social welfare and economic wellbeing, has also acknowledged the significance of microbial CC [71]. At present, majority of European CC are affiliates of the European Culture Collections Organisation (ECCO, https://www.eccosite.org/), founded in 1981, and are part of the World Data Centre for Microorganisms (WDCM, http://www.wfcc.info/ccinfo/), whose collections include more than 3,500,000 million strains and has approximately 250 European CCs listed [68,72].

When it comes to the preservation of biological resources, Europe has been a trailblazer [67]. In 1890, Professor Frantisek Král founded the first CC to offer a public service in Prague [67]. Later, other culture collections were formed, such the Mycothèque de l'Université Catholique de Louvain (MUCL), founded in Belgium in 1894, and the Collection of the Centraalbureau voor Schimmelcultures (CBS), established in Holland in 1906. Then, in 1925, the American Type Culture Collection (ATCC), which keeps various kinds of microorganisms, was founded in the United States. In a short period of time, multiple other significant collections emerged in

a number of European nations, which now contain the largest and most diverse mBRCs and support a significant number of CCs [67]. However, according to historical data, most researchers do not care to place reference strains in public collections. A loss of such crucial microorganisms is also caused by a number of other circumstances, such as employee retirement, project cancellation, diminished financing, researchers' shifting interests, and students moving out after finishing their academic degrees. In order to ensure the cultures' future usability for reference, research, and application, researchers should deposit them in public collections of two different countries in addition to keeping them in their own laboratories [73]. Many nations are currently making efforts to create effective CCs, mBRCs, or Molecular Resource Centers (MRCs) with well-equipped infrastructures. Up to 1920, the primary role of CC was primarily for their value in running both taxonomic and epidemiological researches. Currently, CC, due to the progression of biochemical and physiological findings, have implemented new technologies to characterize and add benefit to the services they provide improving also the preservation of microorganisms [74-75]. Simplify the services provide by CC can be summarize in 4 categories: microbial culture characterization and validation, which serves the purposes of strain identification and preservation, represents the first category [9, 67]. This ought to guarantee clean cultures, a survival of at least 70% of cells, and secure preservation of the microorganisms. The creation of protocols, the second category, aims on standardising the protocols for the management of CC, from the preservation of various types of microorganisms to the maintenance of specialised equipment. Third and fourth categories can be condensed in diffusion, concerning accessibility of the produced material (documents and catalogues), and guidelines and standards, which pursue agreement with national and international laws, rules, and policies regarding safety, shipping and exchange of CC materials, respectively [9,67,72,75]. Thus also include qualified staff in pioneering technology to preserve, identify and validate the different microorganisms. In

addition, various rules and procedures have been established to guarantee an appropriate transfer and exploitation of the material preserved in the CC, such as the Budapest Treaty and the Nagoya protocol [67]. The Budapest Treaty controls the procedure of patent deposits in an international context, while the Nagoya protocols seeks for the equal and fair distribution of the advantages originates by the exploitation of genetic resources. Nowadays, MIRRI (Microbial Resource Research Infrastructure, http://www.mirri.org), a biomedical research infrastructure under the European Strategy Forum on Research Infrastructures (ESFRI, https://www.esfri.eu) initiative, founded in 2010, is the most recent and significant activity of collections and research institutions in Europe. By establishing a pan-European, distributed infrastructure of CCs, mBRCs, and stakeholders, MIRRI aims to increase access to high quality microbiological resources, associated services, and meta data. From 2017, Unimore Microbial Culture Collection (UMCC) (https://umcc.bio-aware.com/) is part of MIRRI network, and secures approximately 2700 strains between yeasts, acetic acid bacteria and LAB. All cultures are preserved in the collection using methods that guarantee long-term genetic stability and preservation. Furthermore, to ensure that the microorganisms match the original cultures deposited, quality controls such as viability, purity, authenticity, and important characteristics are frequently examined.

1.4 Cereals and Lactic Acid Bacteria: from the past to Modern era

Food processing by LAB fermentation has a long history [76]. Neolithic settlement and agriculture began to replace hunting and gathering, thanks to the development of cereal grasses like emmer, einkorn, and barley and the first records of LAB fermentation using cereal can be found in Syria, China, Persia, ancient Egypt, and pre-Columbian America [77]. Various cereal-based meals, including European sourdough rye and wheat bread, Asian flatbreads, numerous varieties of fermented sour porridges, dumplings, and non- or low-alcoholic beers found in

Africa, South America, and elsewhere, are currently fermented by LAB. Cereal can support the growth of microorganisms, including lactic acid bacteria, providing water and hydrolytic enzymes [26]. The native microbiota found in cereal grains typically includes moulds, plant-associated enterobacteria, bacilli, and LAB, which are all in competition with one another for nutrients. Typically, *Enterobacteriaceae* and bacilli are responsible for the start of spontaneous cereal fermentations, later on, acid-tolerant lactic acid bacteria will take over. Traditional production techniques, which are still used in homemade and small-medium size manufacturing facilities, included back-slopping or using a tiny amount of the finished desired product to inoculate a new batch. Unconsciously, this procedure "selects" the best-adapted strains reducing fermentation failure, and in some products such as sourdough is still used. LAB fermentation is the oldest and most popular technique for enhancing the organoleptic and nutritional value of cereal affecting and proving flavour, shelf-life, the safety of the final product, and nutritional advantages. Nowadays, cereals continue to be a basic food in the diet of billions of people worldwide, with high accessibility in terms of cost and availability, and may be the key to millions of people's nutritional problems. Cereals and cereal products, in fact, contain a variety of micronutrients, including vitamin E, several B vitamins, magnesium, and zinc, and are an essential source of energy, carbohydrate, protein, and fiber. According to the United Nations Food and Agriculture Organization's (FAO) global data, the number of undernourished people increased significantly from 777 million in 2015 to over 828 million in 2021, with the majority living in developing nations (https://www.fao.org/newsroom/detail/un-report-global-hunger-SOFI-2022-FAO/en). Besides, according to the World Food Program, 41 million children are suffering from obesity and 795 million individuals have eating disorders with 1% of the population affected by celiac disease [78]. Today a problem-solving to these difficulties can be searched in grain fermentation by LAB due to their numerous beneficial features. LAB fermentation can extend the shelf life of the products and enhance their

nutritional content of necessary amino acids, vitamins, minerals, and fibers improving the diet of underprivileged populations. Additionally, LAB fermentation of cereals and pseudocereals opens up a wider variety of tasty and nutritional balance food products for the increasing population affected by nutritional health issues such as eating disorders and food allergies in particular celiac disease. Partially digested gluten or gluten-free products characterized by poor nutritional values and taste have run the market for the past ten years leading to a rediscovery of ancient technologies (LAB fermentation) and varieties of grains. 21st century consumers screaming for old-new products and to satisfy them old grains variety, plant-based products, and food biotechnology must be merged.

1.4.1 Nutritional value of Cereals

Cereals are the Gramineae grass family's edible seeds or grains and are the most important agricultural plants considered a staple meal from the time of the earliest seed gatherers to the present.

Contemporary high-yielding cultivars progressively replaced the cultivation of ancient and minor cereals, and worldwide, 90% of total cultivated cereals are rice, wheat, and corn used for both feed and food [79]. Wheat (*Triticum aestivum*), einkorn (*Triticum monococcum*), emmer (*Triticum turgidum* subsp. *Dicoccum),* spelt (*Triticum spelta)* Khorasan (*Triticum turanicum*), maize (*Zea mays*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), proso millet (*Panicum miliaceum*), sorghum (*Sorghum bicolor*), oat (*Avena sativa*), rye (*Secale cereale*), and teff *(Eragrostis tef)* are currently the most popular used cereals in the world, while pseudocereals are represented by buckwheat (*Fagopyrum esculentum),* amaranth (*Amaranthus* spp.) and quinoa (*Chenopodium quinoa)* [80]. Nowadays, necessities such as high adaptability crops, biodiversity enhancement and demand for healthy and natural products, have arise renewed attention to minor and ancient cereals. Western countries consider them healthier than

modern grains and the economy marked them as essential in many developing countries. Ancient and minor cereal blends, in fact, are well-matched for the production of extremely nourishing, contemporary, and pioneering baked goods that meet functional and sensory standards in terms of technological and trade aspects. Regardless of the different cultivated varieties, the nutritional values of cereals are generally based on their content of carbohydrates, B vitamins, proteins, zinc, and iron. In Table 2, the nutritional values of the most last decade employed modern and ancient cereals in the world are reported.

Cereals	Carbohydrates	Protein	Fiber	Fat
Barley	77.7	9.9	15.6	9.9
Einkorn	65.5	15.8	8.7	4.2
Emmer	65	14	2.7	1.8
Maize	74	9.4	7.3	4.7
Oat	66.3	16.9	11.6	0.7
Wheat	71.2	12.6	12.2	1.5
Rye	60.7	8.8	13.2	1.7
Rice	80	7.1	1.3	0.7
Sorghum	75	11.3	6.3	3.3
Spelt	53.9	14.6	10.7	2.4
Khorasan	52.4	14.7	9.1	2.2
Proso Millet	72.8	11	8.5	4.2
Teff	19.9	3.9	2.8	0.7

Table 2. Nutritional values of the most used cereals and pseudocereals. All the concentrations are based on 100 grams.

The first cereal grass produced for sustenance in prehistoric times was einkorn. Its cultivation has begun between 8800 and 8400 BCE in Southeast Turkey, although it can grow effectively under low-input conditions, and is still produced as a relic crop in several nations of South and Southeast Europe, Asia, and North Africa. Einkorn, in fact, is economically significant in Ethiopia and is also cultivated in Italy in mountain areas. This ancient crop has been shown to express very few T-cell stimulatory gluten peptides and have a greater protein and bioactive chemical content. Spelt is another ancient wheat-related species that was widely grown until the advent of industrialisation select the crop with highest yield. It is a husked hexaploidy grain with a genome that is comparable to soft wheat and has a relatively high nutritional value among cereals [81].

Successively, barley was one among the earliest crops to be widely planted, although it was eventually supplanted by wheat and rye.

T. eastivum and *T. durum* are the most common type of wheat used for the production of bread and pasta, respectively. It is estimated that the world production of these cereals in 2020 was 766.5 million tons, with 131.168 thousand tonnes (Soft wheat) and 7.824 (Durum wheat) only in Europe in 2021 (https://agriculture.ec.europa.eu/data-and-analysis/markets/overviews/market-observatories/crops/cereals-statistics_it#production). This cereal has a significant content of gluten, results in the combination of starch and protein and contains DF's fructan in the endosperm. Wheat also contains vitamin E, iron, zinc, magnesium, phosphorus, and selenium, as well as vitamins from the B-group like thiamine, riboflavin, niacin, vitamin B6 (pyridoxine), folate, and pantothenic acid [82]. An excellent substitute for wheat for the production of bread and pasta is Kamut. This cereal is rereferred as a high-energy grain owning an excess of fat, protein, and selenium respect the other cereals [83].

Another significant crop, with a world production in 2020 of 510.6 million tons, is represented by rice. Rice is a staple food for a large portion of the world's population, particularly in Asia, is the least allergic grain and is gluten-free. The majority of rice is cultivated for human use, including in breakfast cereals and saké in Japan. There are around 40.000 different rice varieties, but only a select few are produced extensively (Basmati, Jasmine, Arborio, Brown rice). The rice grain is made up of the edible rice caryopsis and an exterior protective layer known as the hull or husk, has a low-fat content, and is mostly composed of carbs, proteins, and insoluble DF. Before rice, millet was regarded as the primary grain in Asia. In fact, this grain is able to thrive in poor soils area such as Russia, Africa, India, and China where is frequently used as a whole grain. Millets are small-seeded cereal grains that include various species are gluten-free and rich in fiber as it's related to sorghum, a plant well-known for DF and B-group vitamins [79].

In 2022, with a domestic consumption of 7560 thousand metric tons (https://www.indexmundi.com/agriculture/?commodity=oats&graph=domestic-consumption), oat represents a great natural source to enhance blood glucose management and lower cholesterol due to its high soluble fiber and β-glucan [84].

Rye, which worldwide consumption was reported to be 13.59 million metric tons (https://knoema.com/data/agriculture-indicators-consumption+rye), appeared in agriculture after wheat, barley, and oats and became popular in cold and severe regions such as Russia and Northern Europe. Rye has a unique and distinct flavours, has a high degree of fiber, high protein level, and lysine, various arabinoxylans and β-glucans, and exceptionally high bioactive substances including alkyl-resorcinol and phenolic acids [85]. A mixture between wheat and rye is represented by triticale, a superior yielding hybrid, with lower quantities of the protein complex responsible for gluten formation.

One of the sweetest gluten-free grains is maize. It is rich in carbohydrates (mostly starch) and dietary fiber. It's characterized by 4%–5% of unsaturated fat, higher concentration of plant sterols and β-carotene than other grains, but generally less protein (about 9%) and lower levels of vitamins and minerals.

Extremely high concentration of β-carotene and vitamins (riboflavin, retinol) are also present in emmer [77]. However, its cultivation was progressively phased out in favour of durum wheat, which is simpler to hull. This cereal has a very peculiar biochemical profile with high grain protein content (12%-23%), lysine (more than 3.65%) and, relatively low levels of fat (1.02%-3.80%). Emmer, also known as farro, was one of the earliest cereals to be cultivated and it served as the Roman troops' basic daily diet. Although teff possess a comparable chemical composition to other cereals, it can also be considered a medicinal grain. According to nutritional research, in fact, teff is primarily responsible for the Ethiopian population's lack of anaemia, osteoporosis, diabetes, and celiac disease [86-88]. This wheat-free cereal's ability

might be related to its high fibre content, iron, calcium and balanced amino acid profile.

1.4.2 Nutritional, healthy and functional aspects of LAB

Fermentation derives from the Latin verb "*fevere*" which can translate in current English as "to boil." Fermentation is still considered one of the oldest and spontaneous food processing technologies and its products represent the 20-40% of the global food supply [89]. Its unique capacity to improve the sensory attributes of raw materials, while also preserving the created product has been recognised throughout human history. Thus, has lead various fermented products to be a crucial part of the culinary and cultural legacy of many countries throughout the world [90]. The industrialization has impacted on food preparation practices and cultivated crops resulting in an elimination of traditional meals and lack of biodiversity. However, the new growing trend for traditional food and the need to preserve genetic variety have all reinvigorated interest in ancient grain species and fermentation. Fermentations system can be divided in liquid and solid. Liquid fermentation is the growth of microorganisms in a nutrient-rich liquid media. The growth and product generation of microbes on solid substances in the absence (or near absence) of free water is referred to as solid-state fermentation [91].

Fermented cereal products can be identified ad plant-based matrices and are idyllic for consumers affected by lactose milk allergies, or following a low lipid or vegan diet [92]. Considerations have also been made regarding their potential as functional food and probiotic delivery functionality [91]. Many of the microbial species colonizing fermented foods have been identified as having probiotic or health-promoting properties, and in some countries such Canada and Italy list of potential prebiotic strains are include in the guidelines whereas in India fermented foods with probiotic cultures are incorporated in dietary guidelines [91,93]. As a result, products containing sufficient numbers of living cells from such strains may provide comparable health benefits [93]. However, in vivo studies involving clinical trials are required

to confirm such assumptions.

Fermentation improves grain palatability through texture and taste changes, minimising the need for flavourings or other chemicals additions such as stabilizer and preservatives. Fermenting microorganisms, employed to create novel products with enhanced sensory and nutritional properties, normally produce other compounds that reduce the growth of spoilage and/or harmful bacteria. Organic acids such as lactic acid, propionic acid, and acetic acid are only some examples of metabolites that are lowering the original pH value, generating an acidic environment and thereby increasing the shelf-life of the fermented product [94]. Furthermore, some LAB such as *P. pentosaceus*, *Lacp. plantarum*, and yeast species create ethanol and hydrogen peroxide, both of which are recognized as potent inhibitors of microbial growth, as well as other secondary metabolites that can serve as antimicrobial agents such as small peptides and fatty acid [95]. *Lactobacillus, Bacillus, Pediococcus, Micrococcus,* and *Streptococcus* are the most prevalent fermenting genera of bacteria [96]. *Lactobacillus* and some *Bacillus* species in particular, play significant roles in grains fermentation through polyphenols hydrolysis and generation of physiologically active molecules [96-97]. In the last years, also yeast fermentation, generally applied for the preparation of alcoholic beverage, its been evaluated as a potential tool to improve cereals flavour and their antioxidant power [98]. Fermentation can be classified based on the state of the fermenting matrix in liquid and solid state. In any fermentation-state, the use of combined cultured strains is always strategic to obtained the maximum benefits from microbial metabolisms. Additional metabolites produced by fermenting microbes may have beneficial impacts on a variety of health-related diseases. The expression of the genes responsible for these metabolites varies according to the microbial species, strain, and matrix in which the microorganism grows. The synergic effect of a mixed culture can maximize the beneficial effect of the fermentation. An exemplar case is represented by the addition of Bifidobacteria in sourdough causing boost phytate hydrolysis, promote

organic acid levels, modifying starch digestibility and contributing to a lower glycaemic index. Thus, cannot be reached by a single-strain fermentation that will lead to an incomplete exploitation of the fermented cereals. Besides the established expanded shelf-life, suggesting that isolated microbial strains from indigenous goods can be employed as starting cultures or as sources to extract metabolites and use them as pure antimicrobial agents, fermented cereals have the potential to improve and increase their health advantages, according to current research [99].

Sourdough fermentation can boost cereal biological activity and modify the nutritional content at the expense of the anti-nutritional component ratio, supporting the development of novel functional meals [96,100-101]. The main cereals components, carbohydrates and protein, play a key role in human health issue through their digestibility and are strongly changed by LAB's metabolic system.

Sugars and protein digestibility

Starch absorption is directly correlated to glycaemic index (GI) which controlled blood sugar level and insulin body response, fundamental in diabetic and polycystic ovarian patients [100-102]. Production of lactic acid limit starch availability and lower the GI of products facilitating starch-gluten interactions. Gluten's presence, strongly linked to celiac disease, can be reduced trough sourdough fermentation or completely avoid producing innovative and full aromatic bakery products from pseudocereals [90,103].

Complete or partial degradation of FODMAP, who stands for "Fermentable Oligo-, Di-, and Mono-saccharides and Polyols", by LAB and yeasts during fermentation, is advantageous for persons with irritable bowel syndrome (IBS) symptoms since their detrimental effects are proportional to the amount of FODMAPs consumed and could help very patient's diet. In fact, patients with IBS are normally advised to avoid cereals and eat a diet low in quickly fermentable carbohydrates. Fructans, galactans, lactose, fructose, sorbitol, and mannitol are not

absorbed in the small intestine and are rapidly fermented by gut bacteria in the large intestine, causing IBS symptoms (meteorism, abdominal discomfort and so on) [104].

Generally, protein digestibility is determined by the protein structure and the existence of antinutrient factors such as protease inhibitors, phytases, as well as other elements such as temperature, ionic strength and pH, which are all directly connected to proteolytic activity [96,105]. Fermentation may modify these characteristics and properties, resulting in improved digestibility of plant proteins. Proteins must be broken down into amino acids or tiny peptides to enter the human circulation after being absorbed by the enterocytes of the small intestine. The obstruction of this process develop in the creation of amines and short-chain fatty acids cause a variety of biological responses via diverse receptors and processes involving signal transduction and modulation of inflammatory responses [106].

Antinutrients and Phenolic compounds

Cereals include a significant number of anti-nutritional components in addition to important nutrient molecules. Tannins, polyphenols, and phytic acid/phytate (myoinositol-1,2,3,4,5,6-hexakis dihydrogen phosphate are among these components [96,107]. In particular, the presence of phytic acid has a significant impact on the nutritional value of the food in which it is found since it may inhibit trypsin and beta-galactosidase activity and form chelates with metal ions, such as iron, magnesium, calcium, and zinc, limiting their bioavailability. The content of this secondary metabolites in cereals ranges from 0.18 g to nearly 6.5 g per 100 g product and it’s mostly found in the aleurone layer and pericarp of wheat, rice or endosperm of maize where it serves as a phosphorus store. Similarly, tannins and polyphenols have a severe detrimental impact on protein digestion because their hydroxyl groups form complexes with protein carbonyl groups. Wheat, barley, corn and millet contain abundant tannin. As a result, proteins precipitate, proteases are blocked, and amino acid deficiency occurs when a diet is based on polyphenol-rich grain products, as is the situation in most underdeveloped

nations or for people following a vegan dietary pattern [105]. Prolonged periods of dietary deficit may also result in osteoporosis, iron deficiency anaemia, and physical development limitations. According to several researchers, sourdough fermentation with starter culture containing various *Lactobacillus* sp. is regarded as the most effective tools to remove anti-nutritional factors [108-109]. In fact, the pH value found in fermented food is believed to be ideal for enzymes to work against and phytic acid in grains. Phytic acid can be destroyed during lactic acid fermentation by endogenous or microbial phytases, increasing the bioavailability of minerals (iron, calcium, and zinc) and tannins undergone an hydrolyzation. After 72 hours of wheat LAB fermentation, the degradation rate of phytic acid and tannins and anti-nutritional components reached its maximum, with tannins decreasing from 0.58 mg/100 g to 0.47 mg/100 g and phytic acid decreasing from 464.1 mg/100 g to 371.28 mg/100 g [101]. Another secondary metabolites with a significant levels in grains are phenolic components [107]. Their production through shikimate and phenylpropanoid pathways involves various biomolecules including acetyl CoA, malonyl CoA, pyruvate, acetate, phenylalanine and tyrosine. Their antidiabetic, anticancer, anti-inflammatory, antimicrobial, antioxidant, as well as neuro, cardio, and hepatoprotective function in human health, are attributed to their ideal chemical structure, which promotes electron transfer or hydrogen donation from their aromatic ring and thus exhibits free radical scavenging activities and metal-chelating potential [107,110]. Nevertheless, to enter the human blood circulation system and exert their antioxidant capabilities, phenolic components must be soluble. Phenolics in cereals can be present free and soluble, conjugated and soluble associated with sugars and sterols, or non-soluble typically connected to polymers such as arabinoxylans and lignin. Increases in cereal phenolic content can be accomplished by addition of hydrolytic enzymes, and fermentation who has also been shown to increase the antioxidant activity of the phenolic fraction itself as reported by various studies on different type of cereals [92]. Temperature, pH value, fermentation duration, type of

microorganisms as well as the kind of cereal or pseudocereal used, all have a critical influence in the release of bound phenolics [78,111].

Reduction of Allergens and immunomodulation activity

Food allergies are a worldwide problem since they appear to affect about 5% of people and 8% of children under the age of 18 [90]. Generally, all dietary proteins can induce allergy responses. However, gluten proteins from wheat and related grains barley, rye, and potentially oat, are supposed to be among the most prevalent allergen sources. Low and high molecular glutenins, α, β, γ, ω gliadins and albumin/globulin fractions are particularly engaged in the IgE-dependent process that causes wheat allergenicity. Celiac disease (CD), is a dietary intolerance that affects approximately one out of every 100-300 people, globally. Celiac disease is defined as an inflammatory illness of the upper small intestine in genetically susceptible individuals caused by gluten ingestion [112]. Hydrolysis of proteins during fermentation by microbial proteases and peptidases might result in reduced allergy responses due to the destruction of the relevant antigenic epitopes. Gluten's disulphide bonds appear to be decreased during sourdough fermentation by *Lactobacillus* sp., which increases the enzymatic activity of cereal proteases and hence improves gluten digestibility [113]. Furthermore, fermented grains have stronger anti-inflammatory properties and can boost gastrointestinal immunity contributing to a better body's immune system function [114].

Dietary fibres and Vitamins

Cereals contains dietary fibres (DF), plant carbohydrates that are resistant to breakdown by human enzymes but can be digested by microbes in the large intestine. They are divided into soluble and insoluble, differs in chemistry and composition according on the kind of cereal and grain tissue in which they are present, and their proportion in food items influences both health consequences and physical-technological qualities [115]. The generation of lactic and acetic acid during fermentation, decrease pH and result in the activation of numerous bacterial

enzymes able to break biopolymer thus results in grain softening and an improvement in the sensory and physiological properties of the fermented product [116].

Vitamins are also classified in two groups based on their solubility [117]: represented by water soluble Vitamin C, B vitamin , and fat soluble Vitamins A, D, E, and K , respectively.

These micronutrients are essential for healthy metabolic function and must be consumed on a regular basis since they cannot be produced in enough amounts (or at all) in the human body. Fermentation with LAB or yeasts might improve cereal's vitamin content and higher vitamin producing strains can isolated from raw matrix and cereal-fermented foods [78,118]. LAB's capacity to create vitamins is strain related, and these bacteria might be utilised as starter or additional cultures to fortify naturally fermented foods for targeted nutritional and quality enhancement. This sort of fortification is critical for various demographic groups who follow unusual dietary regimens, either by choice such as vegans or owing to cultural practises, religious convictions, and a lack of other available food sources (developing countries) [117].

Cereals fermented foods and their prebiotic potential

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defined them as "a substrate that is selectively utilized by host micro-organisms conferring a health benefit" rather than non-digestible oligosaccharides of dietary origin [119]. The location of this beneficial bacteria in the host organism is not only restricted to the human lower gastrointestinal tract (GIT) but it includes the entirety of it, the mouth, the urogenital tract and also encompasses skin. Sources of prebiotic compounds have been found in plants, specifically whole grains, fruit, and vegetables, such as wheat, barley, Jerusalem artichoke, leek, asparagus, garlic, onion, chicory root, and bananas. However, it is thought necessary to investigate a larger range of raw materials and traditional items as potential new prebiotic "pools". Fermented cereal-based products could be investigated as promising sources of prebiotic compounds due to possible presence of β-glucans and arabinoxylans and their oligomeric products, galacto-

oligosaccharides (GOSs), fructo-oligosaccharides (FOSs), resistant starch, phenolics, peptides. [120]. Although current research demonstrated that grains have a significant role in the flora of the human gut, few findings on the prebiotic impact of fermented cereal products are available.

Human Gut microbiota and fermented cereals

Gut microbiome consist of bacteria, viruses, archaea and eukaryotes. Their presence exceed 10^{14} and, genetic, lifestyle, antibiotic and food deeply influenced its composition [121]. Dysbiosis of the gut environment has been linked to intestine-related disorders such as IBS, skin problems, obesity, mental health demonstrating how a gut microbiomes with a favourable and well-balanced composition are crucially related to human health. Dietary choices have a significant impact on microbial gut composition, and may display an inhibitory or stimulating influence on intestinal autochthonous organisms as stated by various research [122-123].

The worldwide consumption of fermented foods varies from 5% to 40% and so does the ingested intake of viable microorganisms in the consumer's gut microbiome that might interact with it [124]. In the last decades, cereal-fermented food and in particular sourdough's effect on the gut microbiome have been investigated. Major nutrients (sugars and proteins), micronutrients, as well as live bacteria and bacterial components from fermentation cultures are able to reach the gastrointestinal system and interact with the host's microbiome.

Due to the remarkable benefits of fermentation, its outcomes and in particular sourdough are regaining popularity**.** The little amount of requirements (equipment, procedure) make it a flexible alternative to boost local economies, raw resources and clean label products. Nevertheless, to maintain basic food requirement such as safety and reproducibility, selected starter cultures must be employed, following optimization strategies for all parameters impacting the fermentation process's outcome.

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Review

Functional and Healthy Features of Conventional and Non-Conventional Sourdoughs

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Abstract: Sourdough is a composite ecosystem largely characterized by yeasts and lactic acid bacteria which are the main players in the fermentation process. The specific strains involved are influenced by several factors including the chemical and enzyme composition of the flour and the sourdough production technology. For many decades the scientific community has explored the microbiological, biochemical, technological and nutritional potential of sourdoughs. Traditionally, sourdoughs have been used to improve the organoleptic properties, texture, digestibility, palatability, and safety of bread and other kinds of baked products. Recently, novel sourdough-based biotechnological applications have been proposed to meet the demand of consumers for healthier and more natural food and offer new inputs for the food industry. Many researchers have focused on the beneficial effects of specific enzymatic activities or compounds, such as exopolysaccharides, with both technological and functional roles. Additionally, many studies have explored the ability of sourdough lactic acid bacteria to produce antifungal compounds for use as bio-preservatives. This review provides an overview of the fundamental features of sourdoughs and their exploitation to develop high value-added products with beneficial microorganisms and/or their metabolites, which can positively impact human health.

Keywords: sourdough; yeasts; lactic acid bacteria; bioactive compounds; exopolysaccharides; antifungal activity

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Citation: De Vero, L.; Iosca, G.; Gullo, M.; Pulvirenti, A. Functional and Healthy Features of Conventional and Non-Conventional Sourdoughs. *Appl. Sci.* **2021**, *11*, 3694. https://doi.org/10.3390/app11083694

Academic Editor: Antonio Valero

Received: 17 March 2021
Accepted: 18 April 2021
Published: 20 April 2021

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1. Introduction

Sourdoughs, in all their different types produced worldwide, represent an awesome ecosystem which can offer several opportunities for conventional and non-conventional microbial exploitation to sustain the ecological and nutritional needs of new consumers [1].

Sourdough is a mixture of water and flour that is fermented by cultures of indigenous yeasts and lactic acid bacteria (LAB) [2–4]. In addition to these microorganisms, *Proteobacteria* may also be present, specially at the beginning of fermentation [5,6]. Among them, acetic acid bacteria (AAB), such as those belonging to *Gluconobacter* sp., *Acetobacter* sp., and *Komagataeibacter* sp., allow faster acidification of the dough and influence the volatile attributes of the final product [7–10]. The indigenous microflora of sourdough is the result of the microbial interaction among microorganisms coming from the flour, the bakery environment, and the vegetable matrices, such as fruits, must, or vinegar, which can be added to the original mixture to accelerate the start-up of fermentation [11].

For many decades the scientific community has explored the microbiological, biochemical, technological, and nutritional potential of sourdoughs and the overall literature, produced in the last 30 years, has been recently reviewed by Arora et al. [12]. What stands out are the novel sourdough-based biotechnological applications proposed, in the last decade, to meet the demand of consumers for healthier and more natural food [13,14].

This review highlights the fundamental features of sourdoughs and their exploitation to develop high value-added products and offer new inputs for the food industry.

In addition to the sourdough technology, this review deals with meaningful studies on the enzymatic activities that have positive effects on human health and the production of compounds with functional properties. Moreover, it reports the potential exploitation of sourdough lactic acid bacteria as bio-preservatives against fungal growth.

2. Sourdough Technology

Based on the technology applied for their production, three main types of sourdough can be distinguished: type I, which is the artisan bakery firm sourdough; type II, referring to industrial liquid sourdoughs; and type III, which indicates industrial dried sourdoughs [15,16] (Figure 1).

Usually, mature type I sourdough contains a mixture of typical yeast and mesophilic LAB strains that characterize sourdough-based products [6]. The stability of the dough depends on the type of flour, the quality and nutritional value of the cereals, the temperature and humidity during processes, and the microbial composition of the inoculum [17,18].

The common yeast species are mainly *Saccharomyces cerevisiae*, *Kazachstania humilis* (formerly *Candida humilis*), *Kazachstania exigua*, *Pichia kudriavzevii*, and *Torulaspora delbrueckii* [19,20]. Moreover, almost 95% of the traditional sourdough population is dominated by heterofermentative LAB alone or in association with homofermentative lactobacilli [15,21]. *Lactobacillus sanfranciscensis* (currently *Fructilactobacillus sanfranciscensis*) was most frequently detected in association with yeasts belonging to *Kazachstania* species, predominantly *K. humilis* [22–24]. Other frequently representative LAB include *Lactobacillus plantarum* (currently *Lactiplantibacillus plantarum*), *Lactobacillus brevis* (currently *Levilactobacillus brevis*), *Leuconostoc* spp., and *Weissella* spp. [1,5,25,26].

To avoid any confusion for the *Lactobacillus* strains reported in previous studies with the former classification of the genus, the old name of the species will be maintained in the present review; the following link is suggested for the appropriate name conversion: http://lactobacillus.ualberta.ca, accessed on 17 March 2021.

The type I sourdough is prepared according to the traditional method, conducted by daily refreshments, also called "back-slopping", which keeps it metabolically active. Accordingly, a selection of sourdough microbiota occurs, due to the back-slopping, which is repeated five to ten times [8,27]. These sourdoughs are typical of various traditional Italian sweet baked products, including those that are commercialized and consumed during holiday seasons, such as *Panettone* and *Colomba* [3,18,28].

Type II and III sourdoughs (the latter is made by dehydrating the stabilized form of type II sourdough) were developed for industries with the aim of providing a more standardized process [6]. Both are made with selected cultures added at a ratio of 100:1 (LAB to yeasts) to obtain specific features of the baked products and inhibit the growth of unwanted microbiota [8].

The selection of microbial strains useful as starter cultures is fundamental for sourdough industries and relies on various metabolic traits that have both technological and functional interest [29,30]. In this context, qualified microbial culture collections, constitute a fundamental cornerstone for the investigation of sourdoughs' microbiota to select strains with desired features [31].

Among LAB, the selection of potential starters is generally made within the *Lactobacillaceae* and the most often used are acid-tolerant strains such as *L. amylovorus*, *L. panis*, *L. pontis*, and *L. reuteri* [27,32,33].

Different non-lactobacillus strains have also been tested as suitable starters because of the positive effect of their compounds on the sourdough flavor, which can open new prospects in the sourdough industries. As reported by Montemurro et al. [34], *Pediococcus pentosaceus* OA1 and S3N3 and *Leuconostoc citreum* PRO17 were selected on the basis of optimal acidification and growth performance, as well as the intense proteolytic activity in whole wheat flour doughs.

Recently, AAB have also been considered useful starters for the production of desired metabolites [7]. Accordingly, *Acetobacter pasteurianus* IMDO 386B and *Gluconobacter oxydans*

IMDO A845 strains were tested for type II sourdough production processes and the latter, in particular, had an attractive impact on the production of volatile organic compounds [7].

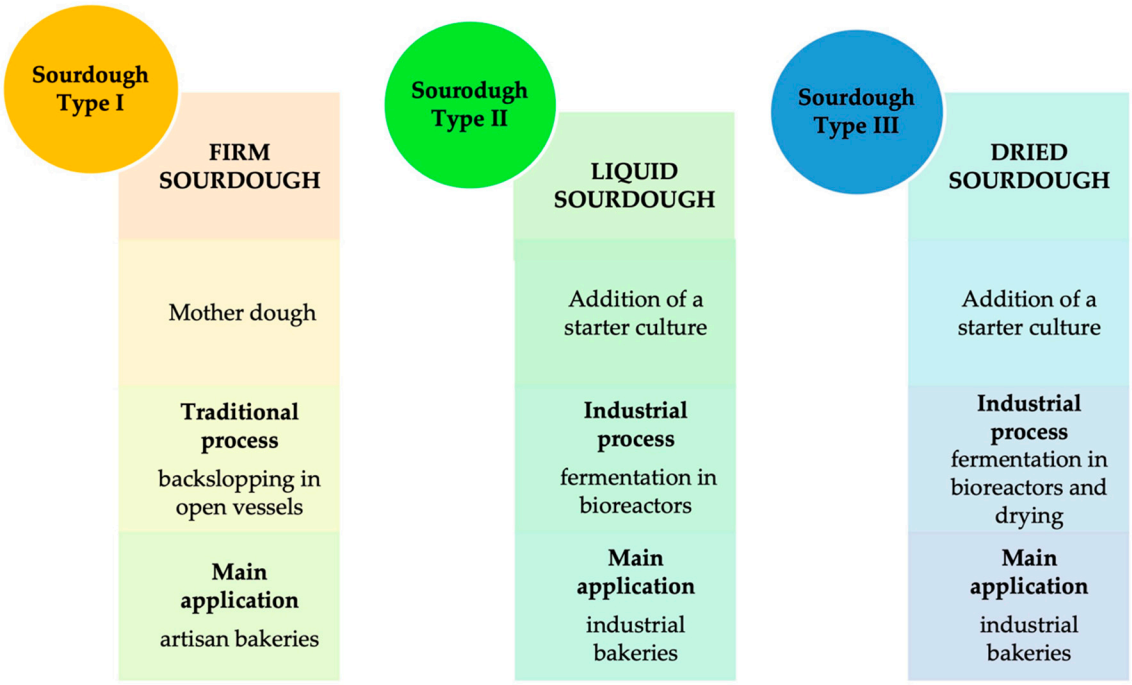


Figure 1. Schematic description of the three main types of sourdough.

3. Nutritional and Functional Features of Sourdough

Sourdough fermentation is certainly the most conventional and efficient tool for guaranteeing rheology, sensory, hygiene and shelf-life features [14]. Additionally, it has the potentiality to enhance the nutritional and functional features of wheat flours [35–38].

The positive effects provided by sourdough technology can be summarized as:

- preservation of food through acetic acid, lactic acid, alcoholic, and alkaline fermentations;
- food enrichment with compounds that originate either from biochemical reactions (e.g., essential amino acids, proteins and essential fatty acids), or biosynthesis (e.g., vitamins);
- development of aromas, flavors and textures in food substrates;
- detoxification during food fermentation processing.

Moreover, it has been reported that sourdough fermentation can lower glycemic index, increase mineral bioavailability, reduce gluten content and reduce starch digestibility, mainly through the organic acids production and other complementary mechanisms [12,39]. The main outcomes of sourdough fermentation are shown in Figure 2.

In recent years the development of high-quality gluten-free (GF) products has become an important socio-economic issue and a new approach in this framework is represented by the application of sourdough fermentation [40].

Common products for celiacs available on the market are often characterized by poor palatability and lack of minerals and fibers [41]. Moreover, they usually have a low amount of health promoting nutrients, such as B vitamins groups, which are essential in the human diet; for instance, folate (B11) is involved in fundamental metabolic reaction, biosynthesis of nucleotides, building blocks of DNA and RNA and prevent neural tube defects in newborns [40]. Consequently, fermentation with LAB and yeasts can be considered an effective methodology in food production suitable for the development of new kinds of GF products with nutraceutical and health-promoting features [42]. Applying microbial selected cultures to suitable raw materials, the fermentation process can be modulated, also

enhancing the content of bioactive metabolites and other compounds, such as glutathione, which exert an antioxidant activity [22,43–45].

Moreover, the sourdough fermentation can be useful in preserving some good sensory characteristics, such as products' structure and/or softness maintenance during storage, which are usually compromised by the lack of gluten [46,47]. Therefore, the selection of specific sourdough cultures has been considered as a new tool for GF food processing [14,48,49].

Various ecological studies have provided useful information on the presence of competitive LAB and yeasts strains, which can be used as candidates for starter development [50]. In rice, maize, teff, and amaranth sourdough, for instance, is frequently used to isolate microorganisms such as: *L. fermentum*, *L. plantarum*, and *L. paralimentarius* [51]. Above them, strains of *L. helveticus*, *L. pontis*, and *S. cerevisiae* are most competitive in different kind of cereals, pseudo-cereals, and cassava sourdoughs [10,52]. Positive cooking performance, obtained with the use of sourdough in GF products, in terms of definite volume, flavor, texture and mouthfeel encourages further studies and the development of an industrial production [40].

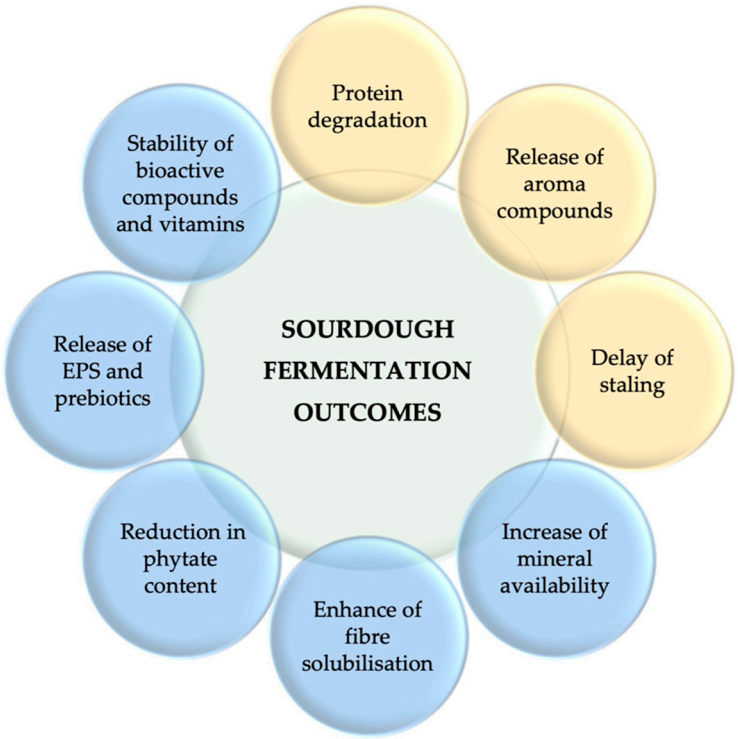


Figure 2. Main technological effects of sourdough on rheology, flavor and shelf life (in yellow), and main health-promoting features (in light blue).

In particular, the flavor profile of the product can be greatly affected by the use of sourdough, depending on type of starter cultures, fermentation conditions, baking conditions and raw material. Recently, researchers have proposed the use of sourdough-fermented ingredients to reach new sensorial profile and enhance nutritional value in pasta [13]. The use of sourdough for pasta fortification has been explored under several aspects and has also been applied with success to mixed flour composed by pseudo-cereals and legumes, allowing an increasing of γ-aminobutyric acid (GABA) levels [53]. GABA is a non-protein amino acid with several physiological functions, such as induction of hypotension and prevention of diabetes as well as diuretic and tranquilizer effects [54,55]. A systematic review on its production from LAB has been recently reported by Cui et al. [56]. The use of non-conventional flours to obtain food products characterized by peculiar flavor, abundance of proteins with high nutritional value, dietary fibers, polyphenols,

and minerals, represent an attractive feature to be explored by food industries for new applications of sourdough microbiota [26,42].

3.1. Enzymatic Activities with Beneficial Effects on Human Health

It is known that cereal grains are significant sources of minerals such as magnesium, potassium, iron and zinc. However, they also contain phytic acid or myo-inositol hexakiphosphate (IP6) (1–4% of dry weight) [57,58]. This compound is an anti-nutritional factor for humans and animals, in fact, the central hexaphosphate ring is highly charged due to six anionic groups and acts as a chelator of dietary minerals reducing their bioavailability [59,60]. Sourdough fermentation, owing to the pH reduction, provides suitable conditions for cereal endogenous phytase activities [36,61]. The phytases that take part in the process are also exogenous and they are produced by a large number of microorganisms among which are sourdough yeasts and LAB. Their activity reduces to less than a half the phytate content of whole wheat bread and allows to increase bioavailability of minerals, free amino acids, and proteins. A recent screening on 152 LAB, isolated from cereal-based substrates, revealed a widespread capacity of the isolates (95%) for degrading phytic acid. Among the isolates, strains *L. brevis* LD65 and *L. plantarum* PB241, showed the highest phytase activity; on the contrary, *Weissella confusa* strains showed low or no phytase activity [62].

Allergies, intolerances, and sensitive individuals are also positively influenced by the LAB present in the sourdough, as they can show specific enzymatic activities toward gluten proteins. The proteolytic activity carried out by endogenous and exogenous proteases during the fermentation process with sourdough seems to lead to a complete hydrolysis of gluten, meeting the needs of individuals affected by celiac disease [32].

3.2. Effects of Organic Acids

Most of the valuable properties attributed to sourdough are due to the acidification activity, due to LAB and AAB. In fact, during fermentation, several organic acids, e.g., lactic, acetic, citric, pyruvic and succinic acid are produced. Among them, lactic and acetic acid are the most important as they can greatly affect the aroma profile and rheological properties of sourdoughs [58]. Their production depends upon several factor including flour type, starter used, metabolic activity, technological performance, and acidification properties of the wheat sourdoughs [63]. Organic acids produced during sourdough fermentation can have preservative and antimicrobial effects, improving storability and safety [64], as well as have positive health effects. For instance, acetic acid, propionic and lactic acid have the capacity of lowering the insulin response [65]. The mechanisms of these acids seem to vary; lactic acid acts to lower the rate of starch digestion in bread while propionic and acetic acids seem to extend the gastric emptying rate [66].

As suggested by the work of Östman et al. [67], lactic acid, in particular, is designed to lower blood sugar and promote an interaction between starch and gluten favoring the reduction of starch bioavailability.

4. Exopolysaccharides

Exopolysaccharides (EPS) are biopolymers of high molecular weight produced by several microorganisms, such as LAB, AAB and microalgae [68–70].

Based on the chemical composition and biosynthesis mechanisms, microbial EPS are classified into two distinct groups: (1) homopolysaccharides (HoPS), such as glucans and fructans, (2) heteropolysaccharides (HePS), e.g., gellan and xanthan [71]. Among the HoPS, dextran, levan, and cellulose are mostl important in the food industry for their significant features [71,72]. In particular, EPS-producing LAB are attractive for application in bakery products thanks to their ability as viscosifiers, texturizers, emulsifiers, and syneresis-lowering agents [62,70]. In addition to polysaccharides naturally occurring in cereal grains flour and dough, microbial EPS from sucrose can be produced in sourdough through the activity of glycosyltransferases [73]. It has been reported that fructan from *L.*

sanfransiscensis can positively affect the dough rheology and bread texture more than the external addition of the same polysaccharides [74]. Furthermore, it has been shown that dextran produced in situ can improve texture and cover unpleasant flavors of wholegrain bread suggesting microbial EPS as a possible substitute of sweeteners [75].

Sourdough technology using EPS-producing LAB strains seem to be a valid solution to improve GF baked products [70]. For instance, microbial EPS can replace hydrocolloids, which are fundamental components in GF products to get acceptable quality levels in terms of texture, volume, and shelf life [76].

Other evidence has been recently reported by Franco et al. [77], who described EPS, produced by *Pediococcus*, *Leuconostoc*, and *Weisella* strains in quinoa sourdough, able to improve the organoleptic and rheological attributes in GF-free doughs. Consequently, the development of EPS-producing starter cultures for different types of flour sourdoughs has been receiving a growing-interest in the last years, also due to the potential health benefits associated with EPS themselves as prebiotics, which can be exploited to make added-value functional products [78]

5. Bioactive Compounds

Cereals contain different phytochemicals, such as phenolic acids, phytosterols, alkylresorcinols, tocols, lignans, and folate [79]. Among other processing conditions, e.g., milling and malting, the sourdough fermentation is the one that most affects the levels and bioavailability of phytochemicals and increases the level of extractable phenolic compounds [14,80]. Furthermore, bioactive compounds are synthesized during fermentation, while other components involved in grain-related digestion problems or pathologies, such as gluten sensitivity or gastrointestinal syndromes, are reduced [81].

As reported by Katina et al. [82], folate and other free phenolic acids increased up to seven and ten times in germinated rye during sourdough fermentation. Moreover, comparing the capability of different yeasts and LAB to affect the folate content in a rye sourdough, it was demonstrated that the synthesis of folate by bacteria was minimal, while the yeasts were able to increase its content over three-fold in the best case [83].

Regarding the vitamin E, tocopherol and tocotrienol, a reduction during the sourdough preparation and dough making has been reported, probably due to the sensitivity of the compounds to the air [14].

Several authors have stated LAB as the microbes most suitable in bioactive peptides enrichment [55,84]. According to Rizzello et al. [49,55], selected sourdough lactobacilli with specific proteinase and peptidase activities toward cereal proteins, were effectively used for releasing Angiotensin I-Converting Enzyme (ACE)-inhibitory peptides during a long-time sourdough fermentation. These peptides are of great interest for functional foods as they may be used for avoiding hypertension and for other therapeutic purposes [55]. Another study showed that sourdough LAB can increase the concentration of lunasin, a cancer-preventing peptide, during fermentation of various flours, including those of wheat, barley, amaranth, soybean, or rye [85]. Specifically, *L. curvatus* SAL33 and *L. brevis* AM7 strains used as sourdough starters were able to synthesize this compound, increasing its concentration up to 2–4 times during fermentation.

6. Antifungal Compounds

One of the big issues in the bakery product industries are moulds – the primary cause of spoilage, off-flavors and potentially producing harmful secondary metabolites commonly called mycotoxins. Aflatoxins are the most common hazardous mycotoxins, causing both chronic and acute toxicity to humans and cattle. Usually, chemical preservatives are successfully used against fungal growth, however, the exploitation of sourdough LAB, as bio-preservatives, has gained great interest among researchers and industries driven by the growing demand for clean label products in which chemically derived ingredients are replaced by natural alternatives [86].

Generally, LAB can be considered protective microorganisms not only for their production of lactate and acetate, which act as effective preservatives, but also for releasing other active compounds during fermentation. Indeed, a synergistic effect between pH and antifungal metabolites seems to be responsible of the LAB protective activity [87,88]. The majority of antifungal substances produced from LAB include organic acids, hydrogen peroxide, reuterin, proteinaceous and phenolic compounds, hydroxyl fatty acids, and other low-molecular-weight compounds [89,90].

Several studies have investigated the ability of sourdough LAB to prevent moulding events on leavened goods; some examples referred to specific strains are reported in Table 1. A comprehensive review on the ability of LAB to serve as antifungal and anti-mycotoxigenic agents have been recently provided by Sadiq et al. [86].

Table 1. Some lactic acid bacteria strains from sourdoughs tested for their antifungal activity.

LAB Strains	Compounds with Antifungal Activity	Fungal Target Tested	Reference
L. sanfrancisencis CB1	Acetic, caproic, formic, propionic, butyric and n-valeric acids	*Fusarium graminearum* 623	[91]
L. plantarum 21B	Phenyllactic acid and 4-hydroxyphenyllactic acid	*Aspergillus niger* FTDC3227	[92]
L. plantarum CRL 778 *L. reuteri* CRL 1100 *L. brevis* CRL 772 *L. brevis* CRL 796	Lactic, acetic, and phenyllactic acids	*Penicillium* sp. *Aspergillus niger* *Fusarium graminearum*	[93]
L. buchneri FUA 3525 *L. diolovorans* DSM 14421	Propionate and acetate	*Aspergillum clavatus* *Cladisporium* spp. *Mortierella* spp. *Penicillium Roquefort*	[94]
L. rossiae LD108, *L. paralimentarius* PB127	Lactic acid, acetic acid, phenyllactic acid and diacetyl	*Aspergillum japonicus*	[95]
L. amylovorus DSM 19280	Lactic acid, acetic acid, 3-phenylpropanoic acid, p-coumaric, (E)-2-methylcinnamic acid, 3-phenyllactic acid and cyclic dipetides	*Fusarium culmorum* FST 4.05 *Aspergillus niger* FST4.21 *Penicillium expansum* FST 4.22 *Penicillium roqueforti* FST 4.11	[96,97]
L. paracasei subsp. *tolerans* L17	Cell-wall binding and enzyme-mediated degradation	*Fusarium proliferatum* M 5991 *Fusarium proliferatum* M 5689 *Fusarium graminearum* R 4053	[98,99]

7. Sourdough Effectiveness on Rheology, Shelf-Life and Safety

The impact of sourdough on properties and rheological behavior of dough has been widely investigated. Generally, changes may be attributed to several intrinsically related factors, including variations in the rate or amount of acid produced [100].

For instance, a low proteolytic degradation of wheat proteins affects the physical properties of gluten and, thus, influences the firmness and staling of the final baked product [101]. The specific proteolytic activity of sourdough microorganisms also has a great impact [102]. As proved by Clarke et al. [103], the use of sourdough, prepared either from a single strain or a mixed strain starter culture, significantly influenced the rheological properties of wheat flour dough; sourdough prepared with starter cultures increased the softening level of the dough.

Regarding gas production in sourdoughs, Hammes and Gänzle [27] proved that the contribution of yeasts and LAB changes according to the type of starter and the dough technology. This aspect is particularly important in the GF products where the lack of the viscoelastic gluten system is responsible for low expansion and gas retention during leavening [104]. The efficiency of the addition of sourdough on GF bread quality was demonstrated in various formulations made of a different kind of flour by Picozzi

et al. [105], which used a Type I GF-sourdough with a stable association between *L. sanfranciscensis* and *C. humilis*. The positive effects of sourdough on rheology and texture, in terms of volume and softness, led to an extended shelf-life of the baked products. This aspect is certainly of great interest for the bakery industry which has recently reevaluated the traditional sourdough fermentation to contrast the short shelf-life of baked products, like bread, mainly caused by spoilage microorganisms.

In fact, sourdough can act as a natural preservative able to replace the use of chemical preservatives. Above the antifungal activity described in the previous section, antibacterial activity has been also scientifically proven [106]. Bread quality and safety can be affected by spore-forming bacteria, such as *Bacillus subtilis*, which mainly occur on the outer parts of grains, and consequently can contaminate also the other ingredients and/or bakery environment [107,108]. The antibacterial effect of sourdough is generally attributed to the synergistic activity of several compounds produced by yeasts and LAB, which includes the synthesis of organic acids, EPS, antimicrobial compounds, bioactive peptides as well as the conversion of phenolic compounds and lipids [106,109,110].

Therefore, sourdough technology combined with the use of high-quality flours represent a tool for improving both organoleptic and healthy features of the baked goods. Optimized sourdoughs obtained with non-conventional flours are the new input for food companies which want to satisfy the needs of consumers affected by allergies and food intolerance.

Legumes and pseudocereals, such as amaranth and quinoa, which have very different chemical composition and technological properties compared to wheat, can be a valid alternative useful for the development of new food products included in different kinds of bread, pasta, or snacks [13]. Regarding the allergies issue, the European Regulation No. 1169/2011 have been adopted with the aim to improve the labeling of foods with the clear indication of ingredients and nutritional values. Accurate food labeling can, in fact, allow consumers suffering from allergies or intolerances of knowing the specific ingredients present in food products and help them to make healthier choices [111].

8. Conclusions

Sourdough fermentation has emerged in human history and since then, it has been empirically used for the improvement of the organoleptic properties, texture, digestibility, palatability, and safety of different food matrices. Nowadays, sourdough fermentation is widely employed to enrich food with beneficial microorganisms and/or their metabolites, which positively impact human health. This result can be achieved either through the exploitation of the wild microbiota naturally associated to raw materials or as the result of the inoculation of selected starters.

The wide and successful use of sourdough, maintained over time for its peculiar and unique features, confirms this ancient biotechnology as an effective answer to the modern world demand for natural, healthy, and eco-friendly food.

Author Contributions: L.D.V., conceptualization, writing—review and editing; G.I., writing partially the original draft and editing; M.G., writing, review and editing; A.P., supervision and review. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the EU Project "Implementation and Sustainability of Microbial Resource Research Infrastructure for the 21st Century" (IS_MIRRI21, Grant Agreement Number 871129).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The JRU MIRRI-IT (http://www.mirri-it.it/) is greatly acknowledged for scientific support.

Conflicts of Interest: The authors declare no conflict of interest.

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2. Chapter II

This chapter is an introduction to the paper **"Yeasts and Lactic Acid Bacteria for Panettone Production: An Assessment of Candidate Strains"** published in *Microorganisms* on 20 May 2021.

2.1 Sourdough through the centuries

Food or lack thereof, which is both essential for living and influenced by the environment, may either create or ruin a community. The strength of old civilizations lay on cereals reserves for six millennia [1]. The population needed to be fed, and grains could be kept in storage for a long time in case of war or siege. It took thousands of years for wild grasses to develop into plants that produced grains. In and around the Fertile Crescent, the region between the Tigris and Euphrates rivers, agriculture originated in the Neolithic Age. In Iraq, an archaeological dig uncovered wild and domesticated wheat that was grown as early as 8,000 BC (Before Christ). Between 10,000 and 7,000 BC, people in Syria and Palestine farmed wheat and barley, while in Anatolia, more modern forms of grains, containing six chromosomes, were produced [1]. Without the ability to initially grow and nurture crops, man would not have been able to maintain civilizations, and from at least 3,000 BC, early varieties of grains were being planted, produced, harvested and used in unintentional fermentations.

Bread-making has approximately 14,400-year of history dating back to Neolithic Asia and ancient civilizations [2]. The oldest known sourdough bread was discovered in Switzerland around 3700 BC, but this fermenting process of cereal is believed to have originated in the Fertile Crescent several thousand years earlier. When flour and water are combined and left in a warm environment for a few hours, sourdough fermentation will begin spontaneously, due to the endogenous microflora of the raw materials.

Early Egypt and the Roman Empire both had extensive, virtually industrial-scale bread production [3]. Egyptian hieroglyphs revealed the use of sourdough fermentation in the making of bread at that period and later, Pliny the Elder's Natural History from the first century explained in details sourdough fermentations, including information on the usage of back-slopped, acidified dough and winemaking yeast [4]. Bread-making, from Egypt, extended north to ancient Greece, where it was a luxury item initially made in the house by women but subsequently at bakeries. The Greeks taught the Romans the craft of producing bread, which became an essential part of the Roman diet (*panem et circenses*).

When it comes to leavening dough in Europe, sourdough fermentation was still the preferred method until the fourteenth century, when the overuse of brewer's yeast became widespread. During the seventeenth century, in France sourdough was raised and fed three times before incorporating it in a dough. The enigma of leavening has also been solved scientifically from antiquity to the most modern-day and Louis Pasteur provided the final explanation of microbial leavening in 1857.

The history of the United States also includes the usage of sourdough. American pioneers who were trekking west in slow-moving wagon groups relied heavily on sourdough bread. Every time settlers or gold seekers left behind the infrastructure that would have provided alternate methods of dough leavening, sourdough was required as a leavening agent. The explorers looking for gold in Alaska and the Yukon in 1898 used sourdough so frequently that it became known as a "sourdough," as depicted in Robert Service's poetry and Jack London's books. Throughout North America, the history and technique of making sourdough bread continued in the century after the California gold rush, in countless small-craft bakeries, and San Francisco, (California), is typically associated with it and with one of the most relevant sourdough lactic acid bacteria *Furfurilactobacillus sanfranciscensis.* In the late nineteenth century, specialised yeast manufacturing for use as a leavening agent began, and sourdough

was no longer used to make wheat bread. The compressed yeast sold throughout Europe was not available until 1867 and dry yeast make its appearance only in 1945 during Second World Word. Bakery yeast was fitting better the demands of contemporary baking processes, due to its ability to leaven quickly and easily, and its adaption to mechanised bakery production. However, sourdough bread remains a major player in the market throughout most of Europe, mainly in nations where rye bread is popular, such as Scandinavia, Germany, eastern Europe, and the former Soviet Union, as well as in some regions of the Middle East [2]. Nowadays, researches on sourdough fermentation as a natural tool for enhancing technological and nutritional features of cereal and pseudocereal products comprise all continents and 47 countries. In the contemporary era, dominated by consumer demand for products with a pronounced flavour, high nutritional value, healthy properties, prolonged shelf-life, and fewer additives, sourdoughs employment and popularity have emerged again, even with the Italian market leading sourdough European production with more than 30 different varieties of salty and sweet baked products.

History of Sourdough in Italy

The majority of the grains grown in early Italy were barley, millet, emmer, and other grains used to make polenta and non-fermented focacce. Emmer was utilised in religious ceremonies as a means of transmission as well as for cooking food. The majority of the grains that the Romans initially ate were roasted or boiled, seasoned with olive oil, and served with vegetables. The Romans acquired knowledge of baking and the method for creating bread ovens after coming into contact with Greek culture. The use of dried grapes as a starter culture, air-dried sourdough after three days of fermentation, and back-slopping of dough as the most popular method to produce dough leavening are all mentioned by Pliny the Elder as alternate methods of dough leavening in the first century A.D. In *De agricoltura* (160 B.C.), Cato the Elder described numerous types of bread that by that time had already reached Rome, including the

erneum, a type of Pandoro, and the *mustaceus*, bread made with grape must. The Middle Ages experienced no unique advancements in baking following the victory of classical baking. Due to the agricultural revolution, hunger, and illnesses that were raging at the time, it was difficult to find flour during these centuries. Later, the custom of hosting banquets at the lords' courts throughout the Renaissance was a success for sourdough products, which were given in a variety of shapes to accompany the various courses. For the Easter holidays, a delicious bread made with sugar, eggs, and butter, was cooked in Milan and "Panettone" make its first appearance. Subsequently, the use of steel rollers, made flour refining more affordable, marking a significant advancement in the skill of baking in the year 1700. Additionally, the industrial revolution's advancements led people to prefer to get their bakery products from bakeries as opposed to baking them at home.

Despite the technological evolution, during the last 30 years, sourdough fermentation has attracted more and more interest both from the scientific and baking communities [5]. Nowadays, sourdough is regarded as a “functional” ingredient and it’s used by a growing number of bakers as a source of taste, nutrients, and health-improving ingredients meeting the request of the new conscious consumers [6-9].

2.2 Sourdough preparation and different types

Various procedures can be followed to prepare sourdough, the French and American systems are two main traditional processes. In any case, the major goal is obtaining a well-leavened dough, with pleasing sensory quality, good texture, and a long shelf-life [10]. Four different forms of sourdough may be distinguished based on technical aspects such as the technique of production and the consistency of the dough.

Sourdough type I

According to Gobbetti and Gänzle [10], traditional sourdough (sourdough type I), is typically

made using a firm dough, with a dough yield (DY) of 150–160, a relatively short fermentation time of 6–24 h, water activity (a_w) of 0.96-0.98, and final pH of 4.0 (Figure 1). The microorganisms are kept continuously metabolically active thanks to regular refreshment, commonly known as backslopping. This type of sourdough is characterised by spontaneous fermentation, which is started by the microbial strains already present in it and occurs at room temperature (20–30 °C). The initial dough can be made only with flour and water or the inclusion of additional raw material, generally high in microorganisms, such as fruits, yoghurt, and manure can be done. The sourdough main microbiota is then "shaped" during daily backsloppling [11]. The number of daily backslopping is variable and dependent on the type of microorganisms and the final preferred sensory features. Due to the pH drop and the presence of organic acid reached through the various refreshment an inhibition of sensitive microorganisms is obtained favouring yeast and LAB. They commonly include *Leuconostoc*, *Weissella*, *Fructilactobacillus sanfranciscensis*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Companilactobacillus* (formerly known as *Lactobacillus*) as LAB species and *Saccharomyces cerevisiae* and *Kazachstania humilis* as yeasts [12-13]. Along with the yeast and LAB, this sourdough occasionally supports acetic acid bacteria such *Acetobacter* species [14] Throughout the years, the preservation of these type of sourdoughs has led to a "collection", the Puratos Sourdough Library (www.puratos.com/en/innovation/Center-for-Bread-Flavor/Sourdough-library/) where some of them are preserved. Traditional sourdoughs, like San Francisco sourdough and different Italian sourdoughs, are typical examples. These breads are frequently legally protected as protected designations of origin (PDO) or protected geographic indications (PGI) such as Pane di Altamura PDO and Pane di Matera PGI [14-15].

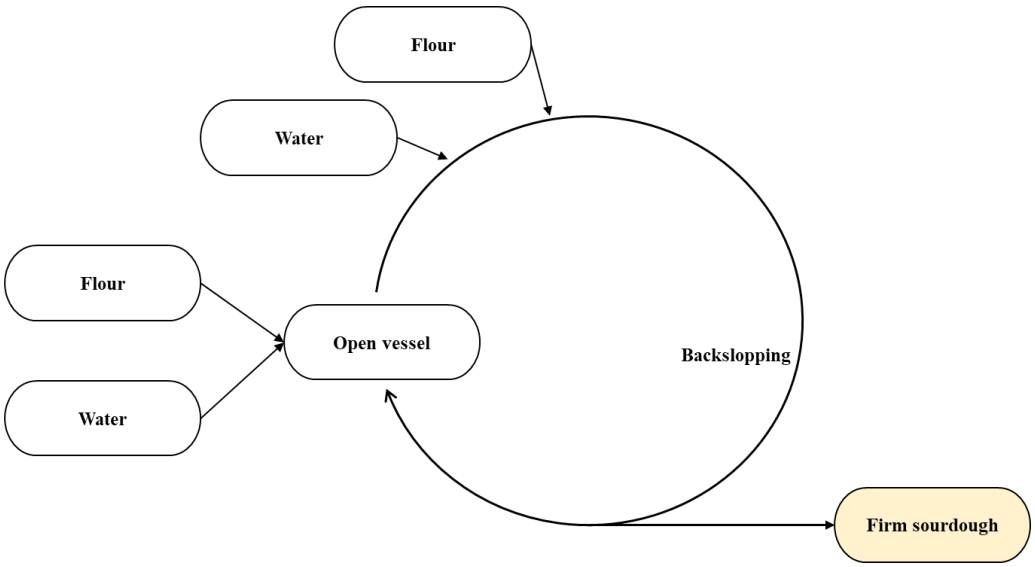


Figure 1. Sourdough type I technology process

Sourdough type II

Sourdough type II (Figure 2) is obtained through an industrial procedure, which involves a single fermentation phase realized at 30-37 °C and a more liquid texture (DY of 200 and a_w of 0.98,), with only lactic bacteria or LAB and yeast [16]. This enables quick and intense acidification that can stop autochthonous yeast from growing and simple handling of the sourdough for mixing, pumping, and dosing processes [9]. The entire process takes one to three days, frequently producing pH values lower than 4.0. The LAB most commonly utilized in this form of dough are acid-tolerant, such as *Limosilactobacillus* species and *Lb. acidophilus,* and after storage the mature sourdough could be employed as an inoculum for the production of bread, generating a final product characterized by higher acidity and traditional sourdough fragrances [12,17]. Sourdough type II has a number of advantages perfectly matching the industrial needs, including requiring one fermentation phase, improved control over temperature, pH and acidity of the fermentation process, and the ability to standardize final products.

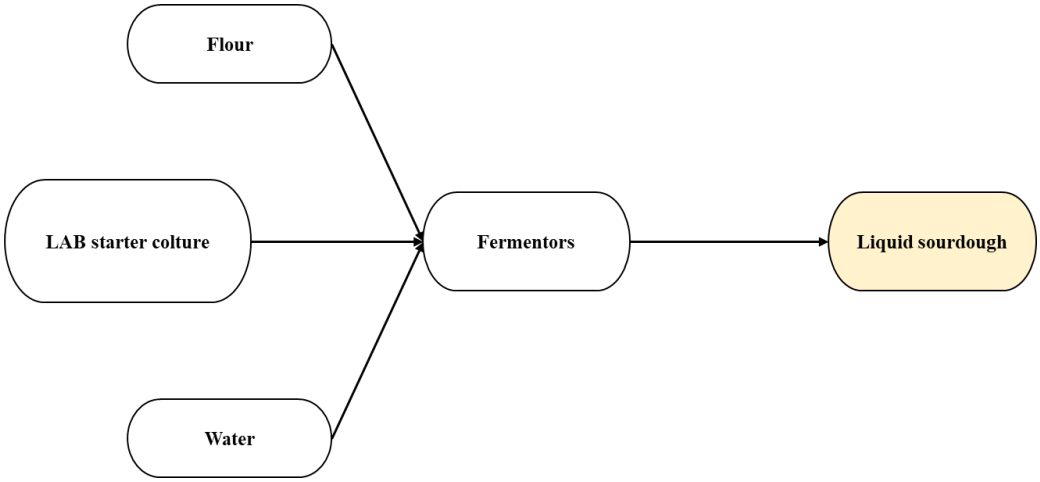


Figure 2. Sourdough type II technological process

Sourdough type III

Sourdough Type III (Figure 3) production techniques result in sourdoughs that are created similarly to Type II but are then dried using a spray or drum drier. Generally, these pre-made sourdoughs are not metabolically active, making this form of product more beneficial for enhancing the flavour and texture of the final products and baker's yeast must be added to enable leavening [9,18]. Drum drying prevents the Maillard process by evaporating water when tiny sourdough droplets are still in the air and away from the equipment's surfaces [19]. In contrast, for the rotary drying, due to the high temperatures needed, compounds with flavour and scent similar to caramelised and roasted malt are created and acetic acid, is reduced [19]. In addition, due to its dehydrated state, the long shelf life, and the easy handling of this sourdough, not only do industrial and artisanal bakeries use it as flavour or acidifying agents in bread doughs but it is also commercialized in supermarkets.

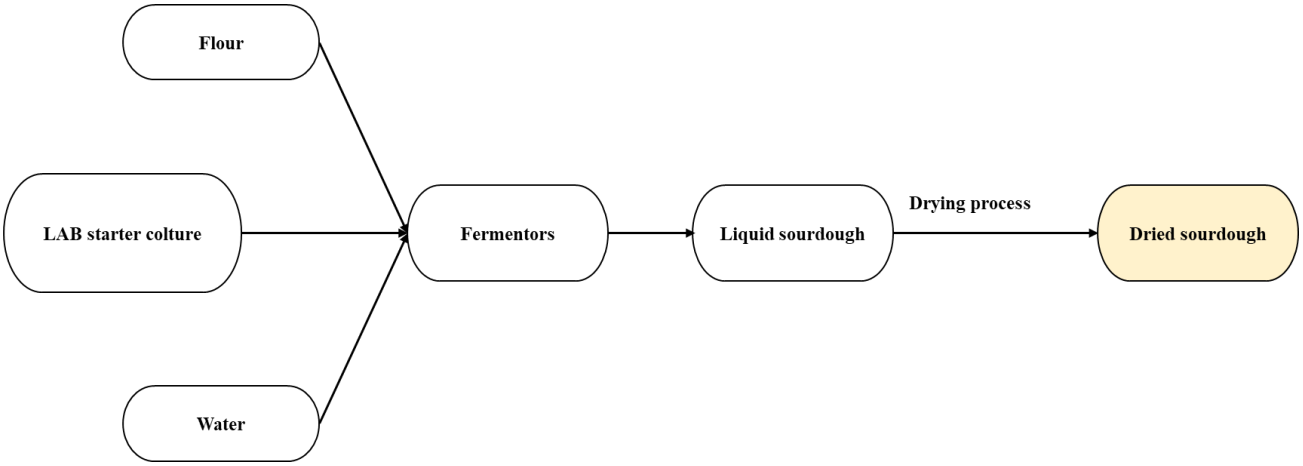


Figure 3. Sourdough type III technological process

Sourdough Type IV

Sourdough type IV (Figure 4) known also as mixed sourdough is more common in research laboratories or select artisanal bakers [18]. The production of it relies on starting cultures like commercial yeast or LAB cultures lately propagated using backslopping technique. In this situation, the dissemination and drift of other microbes may have an impact on the starting culture. The occurrence of more aggressive or well-adapted strains in the dough will result in their dominance in the sourdough microbiota. As a result, there will be natural selection among organisms that can thrive in the sourdough ecology. It's possible that starter cultures are less competitive and, as a result, less able to adjust to the environmental factors that affect sourdough ecosystems [20].

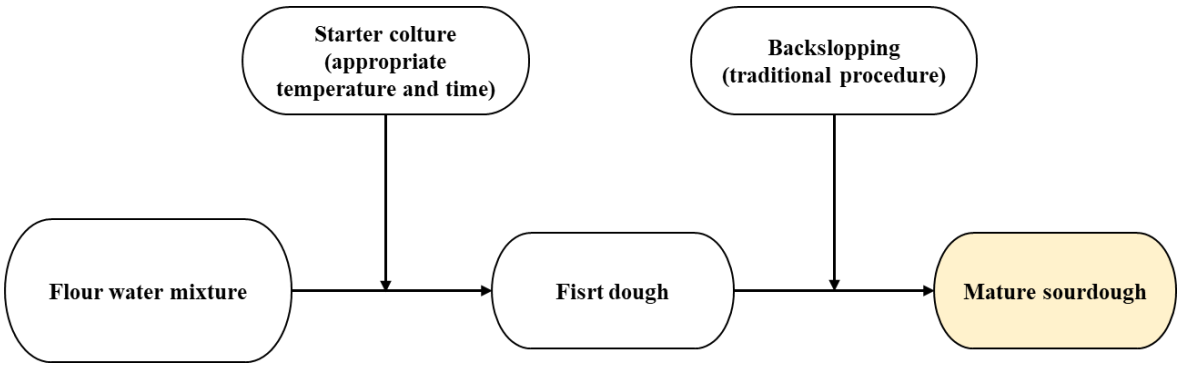


Figure 4. Sourdough type IV technological process

Panettone: a historical Italian use of sourdough type I

The iconic Christmas dessert from Italy known as Panettone is devoured all around the world, and it was created by an accident [10,21].

Legend tells that eminent visitors gathered in Milan at Ludovico il Moro's invitation to participate in one of the most luxurious feasts ever. As lunchtime approached, waiters and chefs flitted around the beautifully set tables bringing wines, meat, and delectable pies flavored with the finest and rarest spices and herbs. The head cook was having a nightmare in the kitchen, though, as the cake he had meticulously made and garnished with cream and flowers was falling apart on a large silver platter. The kitchen assistant, named Toni, didn't give up and rapidly combined the ingredients for candied fruit, flour, yeast, butter, sugar, and spices. He saw a container of raisins in the corner just before putting the dough in the oven and added them to the recipe. While the guests in the dining room continued to enjoy the dinner, the loaves that the kitchen hand had baked in the oven were leavening and developing a lovely golden colour. The head cook's attention was drawn by a sweet, mouthwatering aroma that began to permeate the kitchen, and decided to serve the cake. After a protracted period of stillness, the guests started praising the novel and peculiar cake with great enthusiasm and Ludovico il Moro invited the creator of the cake to join the banquet. The young server was suddenly shoved into the center of the dining room and received a huge, unplanned applause. “What is your name?" Ludovico il Moro enquired. “Toni is my name” said the hesitant and perplexed servant. "Evviva il pan de Toni!" was yelled in the subsequent fervor. As a result, the word "Panettone" was coined and has been used to describe this cake all over the world since then. Historically, both at traditional and industrial level sourdough type I has been used for the production of this sweet [10]. The manufacture of Panettone, due to its high demand, starts months before the actual selling season. Nevertheless, thanks to the employment of traditional sourdough, this sweet results still exceptionally fresh months after the baking [19]. Panettone's distinctive

flavor, which is produced from the dough's components including water, flour, butter, sugar, eggs, salt, and others and processing, gives it a soft structure with regular holes [10]. During the production process, the sourdough might be backslopped up to seven times, adding supplementary sugar to the dough each time. This allows the sourdough to adjust to the high sugar content of the final dough. Moreover, various studies have associated the presence of dextran-producing *Leuconostoc mesenteroides* in sourdough, which employs sucrose as an exopolysaccharide (EPS) precursor, to the longer softness and shelf life of Panettone [19]. Due to its high notoriety, from 2003 the production of traditional Panettone is subjected to a production disciplinary with the aim of protecting this ancient product [22].

2.3 Sourdough process, parameters, and impact

The characteristics and performances of sourdough as well as its microbial community and the overall quality of the final product are influenced by a variety of endogenous and exogenous parameters (Figure 5) [10].

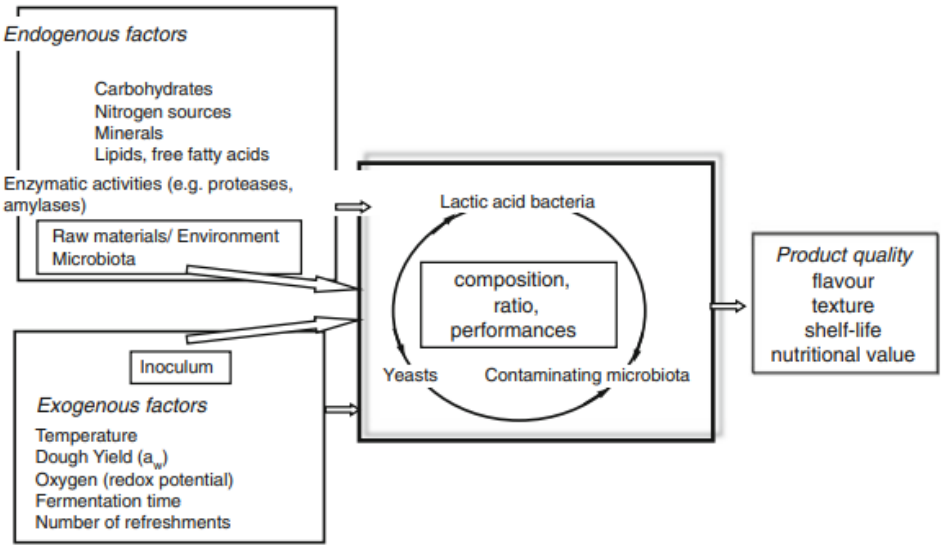


Figure 5. Endogenous and exogenous factors affecting sourdough and final product's quality, image from [10] with permission.

Although the control of endogenous factors is quite limited, the physicochemical

characteristics of the sourdough (exogenous factors) can be assessed influencing the equilibrium between LAB and yeasts population, the microbial ecosystem in terms of species and strains variability, and its final performance to obtain the desired final product. Specifically, dough acidity, pH and Total Titratable Acidity (TTA), dough Yield (DY), fermentation quotient (FQ), a_w, redox potential and number of backslopping are the parameters taken into concern.

Dough Yield

The DY, which designates the consistency of the dough, is the proportion of flour to water in the dough, and its value can be obtained through the following formula:

$$DY = \text{dough weight} \times 100 / \text{flour weight}$$ [9,10]

DY and fermentation temperature also significantly influence sourdough's fragrance, in particular, the molar ratio among lactic and acetic acids (FQ). Based on DY value, three different doughs can be discrete: firm wheat flour sourdough (DY between 150 and 160) generally fermented at 25-30 °C containing a higher level of acetic acid, liquid sourdough (DY near 200), and soft dough indicated by intermediate levels of DY, fermented at 37 °C and characterized by a higher level of lactic acid [10,19]. According to different authors, DY directly influences the water activity (a_w) and acidity of ripe sourdoughs [17, 23]. A low value of DY, in contrast to a higher value, deepens the impact of the flour providing the microbiota with more fermentable carbohydrates, increasing the amount of ash in the dough, affecting the activity of the endogenous flour enzymes and lowering the rate of acidification resulting in a higher pH value [24]. Furthermore, a high DY favors the development of LAB over yeasts, and due to pH changes, it might influence the microbial community equilibrium also in terms of species diversity [17].

pH

Generally regarded as an indicator of a mature sourdough, the final pH, typically drops between

3.5 and 4.3. However, this value, in the final products, is strictly dependent on the amount of employed sour inoculum and may vary between 4.7 and 5.4 when a standard inoculum (20% referred to as the dough weight) is used [10]. Low-pH values generally promote the dominance of acid-tolerant LAB, whereas higher pH positively influences the growth of *Enterococcus, Lactococcus, Leuconostoc, Pediococcus*, and *Weissella* species [15]. Furthermore, the presence of *Fructilactobacillus sanfranciscensis* (optimal growth pH 5.0) is crucially dependent on the sourdough acidification rate while its associate *Kazachstania humilis* is not limited by pH value [25].

Total Titratable Acidity

The total organic acids produced during sourdough fermentation are quantified by the TTA value, stated as 10 g of dough/ ml of NaOH 0.1 N. Although, the ideal TTA for sourdough differs depending on the type of bread, values from dried and liquid sourdough generally vary from 40-220 ml NaOH 0.1 N/10 g to 30 to 150 ml NaOH 0.1 N/10 g, respectively.

Water activity

Majority of LAB and yeast species can develop without being hindered by the a_w of the various type of sourdoughs [17]. Nevertheless, the addition of several ingredients such as salt or sugar may affect the development of the microbial community, shaping the promotion of osmotolerant species and/or strains [26]. Higher osmotic pressure due to the addition of NaCl, alongside low a_w values, deeply influences the LAB community inducing stress response and promoting yeast thriving. Alternatively, low salt concentration (below 0.7 g) favors the LAB community [17].

Fermentation Quotient

FQ expresses the lactic/acetic acid molar ratio during sourdough fermentation, is commonly determined using enzymatic or chromatographic methods, and is equal to:

FQ= (g of lactic acid in 100 g of dough/g of lactic acid's molecular weight) :(g of acetic acid

in 100 g of dough/g of acetic acid's molecular weight) [10]

This parameter is drastically connected to the endogenous and exogenous factors affecting sourdough and is strictly related to the type of metabolic dominant LAB.

Redox potential

Backslopping and kneading are the main responsible processes for the inclusion of oxygen in sourdough where it's utilized by enzymatic and microbiological activity resulting in a semi-anaerobic fermentation [10]. Consequently, the dynamics of the microbial population, the metabolism rate, and the characteristic of the final products are transformed by their presence and quantity [10, 17]. In fact, oxygen presence directly affects the production of aromatic compounds through lipoxygenase activity and exercises a stress response on LAB altering their enzymatic reactions and flavour production based on their metabolism [27].

Backslopping activity

Backslopping is the practice of constant reinoculation of the dominant microorganisms into a fresh batch of flour and water while maintaining the same process parameters [10]. According to De Vuyst and Neysens [28], this practice generally encourages the presence of HeLAB, while the frequency and amount of refreshed material influence the dynamics and stability of the microbial communities. Acid-tolerant LAB species, such as *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* are easily adapted to hostile environments and thrive under prolonged backslopping periods or complete absence of backslopping [23]. Oppositely, short-term backslopping benefits fast acidifiers LAB species and the presence of *Fl. sanfranciscensis* especially at ambient temperature and moderate pH. Whereas yeast population, apart *from K. humilis*, mutualistic associated with *Fl. sanfranciscensis*, might suffer it, and be more competitive in long-term refreshment (≥ 12 hours) as demonstrated by *Saccharomyces cerevisiae* [23]. Nonetheless, this procedure encourages the thriving of the best-adapted LAB and yeast species, strongly selecting the core microbiota and resulting in an ecological

redundancy [29].

2.4 Sourdough microbiota

According to a commonly recognized technical definition, sourdough is a combination of flour and water that is spontaneously fermented by wild lactic acid bacteria (LAB) and yeasts and has the ability to both acidify and leaven food [30]. *Fructilactobacillus sanfranciscensis* (earlier known as *Lactobacillus sanfranciscensis*), a LAB species, and *Kazachstania humilis* (formerly *Candida humilis*), a yeast species, are typical microorganisms found primarily in traditional sourdoughs [16,28, 30-32]. The ecosystem of sourdough is a stressful environment for the colonizing microorganisms, and as a result, it promotes growth through the adaptation of a particular microbiota that expresses the physiological and stress responses required for metabolic activities and survival [26, 32-33]. Yeasts are responsible for extended flavour formation, in particular, higher alcohols such as 3-methyl-1-butanol and esters compounds like ethyl acetate, and leavening via the generation of carbon dioxide. While, LAB are responsible for acidification of the flour-water mixture by the production of lactic acid and/or acetic acid as well as for flavour formation (primarily the acidic flavour) [32-33]. Although other microorganisms, such as acetic acid bacteria (AAB) might be present in small quantities, they do not represent the essential component of sourdoughs' microbiota and might not be fundamental for its positive development [17]. Oppositely, they might negatively affect the dough rising slowing the process. As debated by various authors, their metabolic requirement of oxygen would promote moulds development and flour oxidation [9, 17, 34-36]. Additionally, the oxidation of ethanol and lactic acid would drastically raise the quantities of acetic acid and acetoin affecting flavour and changing the composition of volatile organic compounds of the dough. Although sourdoughs starters, as a habitat, may comprise more than 20 species of yeasts (Figure 6a) and 50 species of LAB (Figure 6b), it possesses a very low

level of microbial variety, particularly phylogenetic diversity, as compared to several environmental and biological systems such as soil or intestines [37].

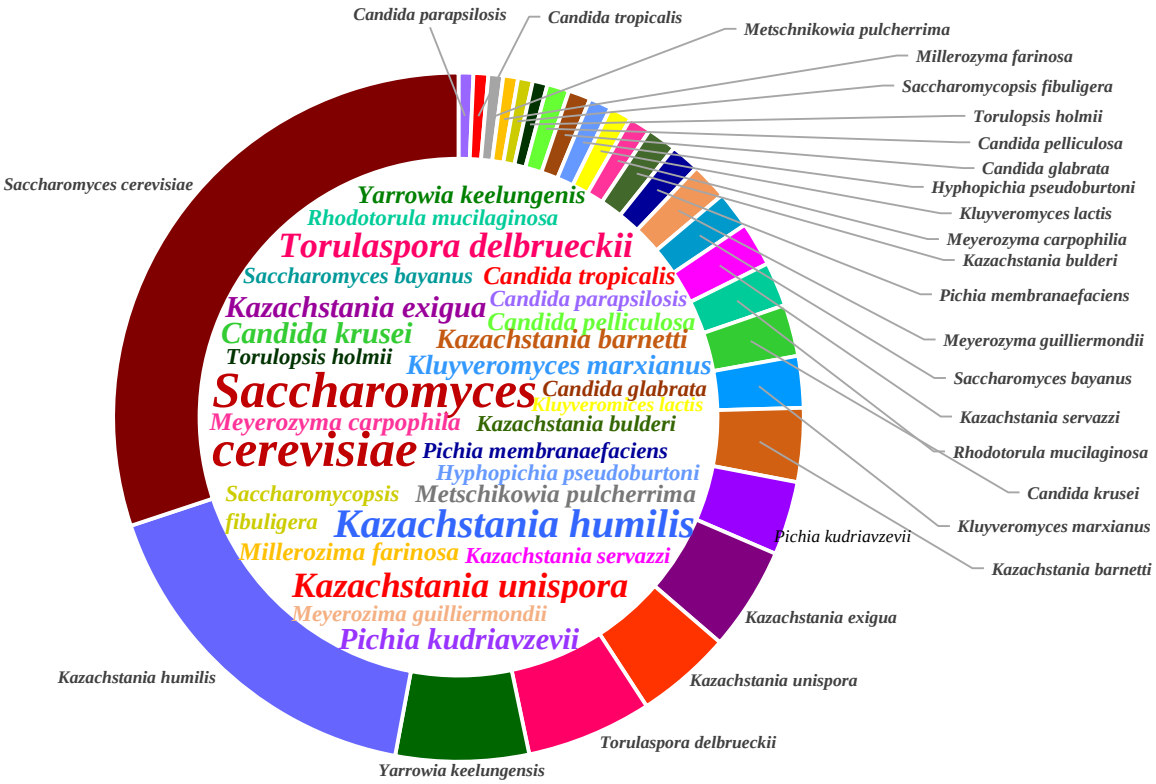


Figure 6a. Pie chart representing the most isolated species of yeasts from sourdough over the last decades. The various size of the pie chart represents the incidence of isolation of each yeast species in sourdough. Image based on [5].

Numerous environmental and technological factors affect the diversity of sourdough's microbiota [26, 32]. They are reliant on one the four processes influencing microbial ecology, the dispersion mechanisms, connected to the species and strains that happen to be the first to colonise [38]. Additionally, the nutritional interactions between lactobacilli and yeasts, and the inherent robustness or weakness of the various microorganisms have an impact on the microbial diversity of sourdough [26]. they are affected by exogenous elements including baking procedures, storage temperature, starter usage during propagation, the number of stages in propagation, and fermentation duration [26]. Yeasts (Ascomycota), LAB (Firmicutes,

mostly *Lactobacillaceae*), and occasionally AAB (α-proteobacteria) are involved.

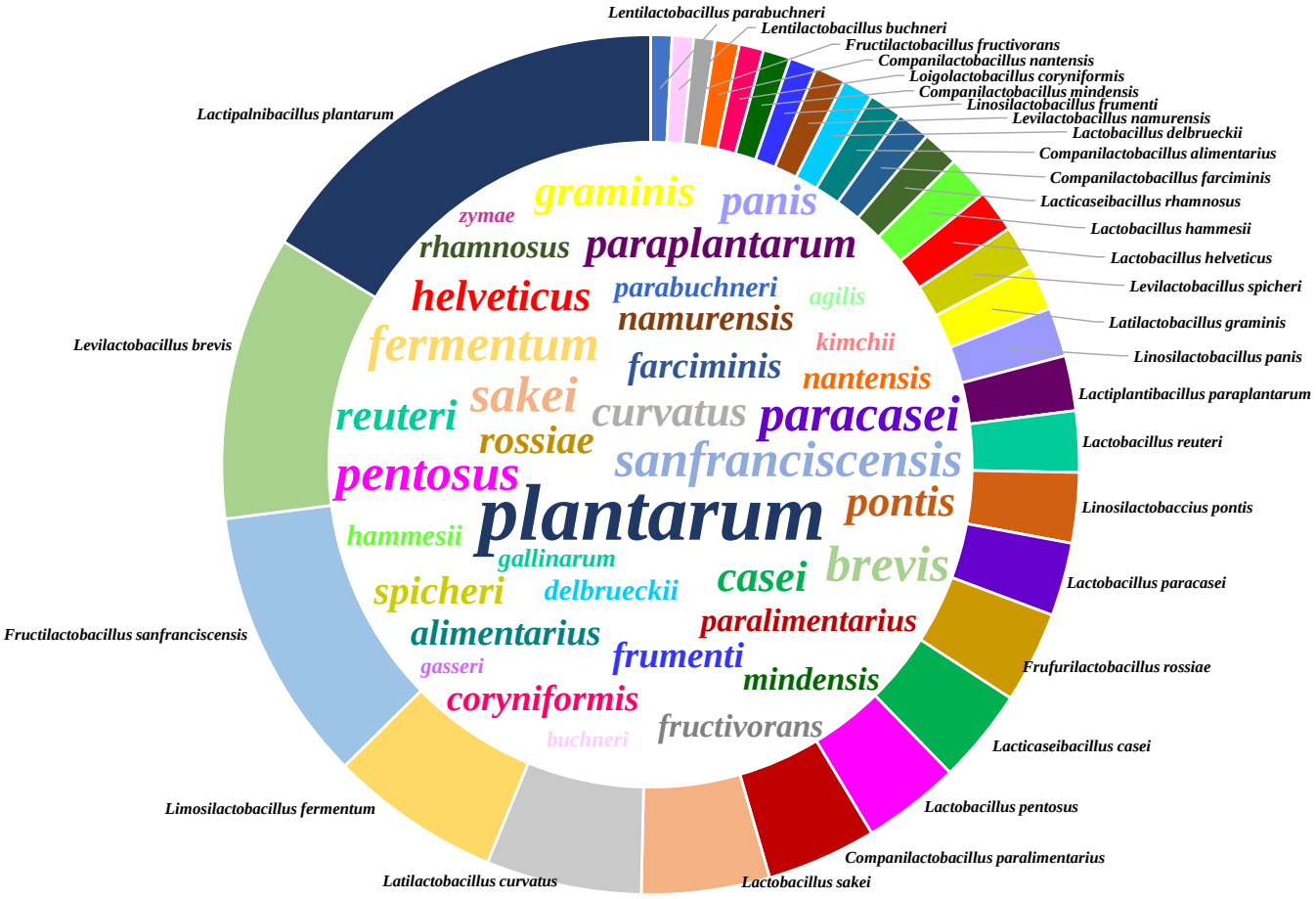


Figure 6b. Pie chart representing the most isolated species of lactic acid bacteria from sourdough over the last decades. The various size of the pie chart represents the incidence of isolation of each LAB species in sourdough. Image based on [5].

2.4.1 Yeasts

Yeasts are single-celled fungi that are capable of clonal reproduction but are unable to create genuine hyphae. They are mostly facultatively aerobic and reproduce either asexually mainly by budding, or sexually by spore production under starving conditions [39]. These microorganisms are crucial in the manufacture of fermented foods (e.g. wine, bread) by causing desirable changes in various raw products. Alternately, in some cases, they are considered food spoilage and are responsible for the product's deterioration.

Ascomycota yeasts, which are fermentative, acid-tolerant, and suited to the sourdough ecology,

are the majority of the yeasts found in sourdoughs [16]. The genesis of yeast in the sourdough ecosystem is assumed to be tied to a number of elements, such as the flour or the immediate environment in which a starter is stored (home kitchen vs a professional bakery). Nevertheless, the time-appearing of these microorganisms in sourdough might vary. Yeasts can predominate over bacteria during the early stage of propagation or not be present until the fifth, or seventh day of multiplication. Although the ratio of LAB to yeast can range from 1:1 to 1000:1, a typical sourdough starter includes 100 times as many bacterial cells as yeast cells (100:1 ratio) [28]. They play a crucial part in the sourdough ecosystem by creating CO2 for leavening [40]. Yeasts also interact both positively and negatively with LAB, generating a variety of classes of alcohols, esters, and organic acids that have an influence on the activity of the LAB as well as the flavour of the sourdough [16-17, 41-42]. Embden-Meyerhof-Parnas (EMP) route is used by sourdough yeasts to convert the maltose, sucrose, glucose, and fructose present in the flour into pyruvate. By further converting pyruvate into ethanol and carbon dioxide through alcoholic fermentation, ATP and reducing power (NADH + H+), are created replenishing the cofactor NAD+ used in the upper part of the EMP pathway (Figure 7) [17].

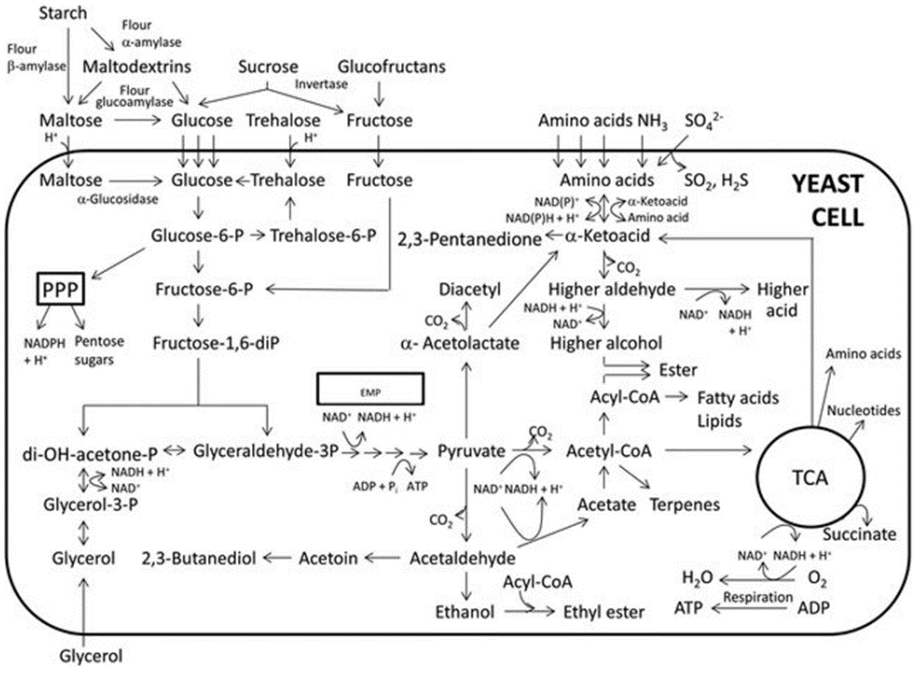


Figure 7. Representation of yeasts pathway EMPP, pentose phosphate pathway (PPP), and Krebs cycle (TCA). Image from [9] with permission, licence: CC BY-NC-ND 4.0).

Most live cells can degrade glucose via the pentose phosphate pathway (PPP), which can function in tandem with glycolysis. Glucose-6-phosphate dehydrogenase is an important enzyme in its unidirectional oxidative portion, which produces NADPH. It also contains a non-oxidative portion that involves the reversible transketolase and transaldolase processes, which exchange PPP metabolites with glycolysis. The non-oxidative branch supplies the precursors for the synthesis of nucleic, fatty, and aromatic amino acids, but the oxidative branch is essential for dealing with oxidative stress. The PPP has been found to have just a minimal part in glucose catabolism in *Saccharomyces cerevisiae*, other yeasts, however, use both PPP and glycolysis to contribute nearly equally to the breakdown of glucose [43].

An additional mechanism for redox balancing (NAD+ regeneration) is the synthesis of succinic acid via the reductive tricarboxylic acid (TCA) cycle and glycerol from dihydroxy acetone phosphate produced by the EMP pathway. Although the generation of ethanol, glycerol, and other substances such as succinic acid help lower the pH and the development of gluten network and positive rheologic characteristics of the bakery products, they are not as crucial as the generation of carbon dioxide during dough fermentation. Yeasts have a significant role in the taste development of bread in addition to their capacity to leaven (carbon dioxide) and affect on the strength of the gluten network (ethanol and succinate). As a result, the yeast's metabolic activity, which depends not only on the strain but also on the LAB species present in the same matrix, determines the volatile of the sourdough. Through the Ehrlich Branched-chain amino acid conversion, yeasts can produce higher alcohols and esters. Leucine-derived 3-methyl-1-butanol plays a key role in the crumb scent of wheat bread, even at low concentrations, and through the catabolism of pyruvate or Ehrlich pathway diacetyl and its buttery aroma can be produced. The flavour of the bread is largely influenced also by esters,

which are condensation products of ethanol and fatty acids (ethyl esters) or higher alcohols and acetate (acetate esters). Due to the yeasts' improved aerobic development, the addition of air during the mixing and kneading phases of dough preparation increases the synthesis of the yeast metabolites. Moreover By elaborating fructose, which heterofermentative LAB species can use as an alternative external electron acceptor (instead of pyruvate) to produce mannitol (for NAD+ regeneration) and acetic acid (instead of ethanol production for NAD+ regeneration, thereby enabling extra ATP formation), the yeasts also indirectly contribute to the formation of acetic acid and flavour, especially in type I sourdough [17, 33]. The order *Saccharomycetales* contains species of yeast from several genera, including sourdough yeasts. According to the literature, in the sourdough environment, there are over 40 distinct species of yeasts that have been documented and initially found and named from sourdough starters [33,37,42]. Despite, in the last year, 70 variants among 500 sourdough starters gathered from 17 different nations, were found, the ordinary starter often only contains a small number of species [13]. A single bakery sourdough frequently contains one, two or sometimes no yeast species, showing a low α-diversity but higher γ and β diversity [9,13]. *Saccharomyces cerevisiae* and *Kazachstania humilis* are the main protagonist yeasts found in sourdough along with *Candida krusei, Kazachstania exigua, Torulaspora delbrueckii, Wickerhamomyces anomalus,* and *Pichia kudriavzevii* [17, 32-33]. These yeast species include several generalists that can survive in a variety of microbial environments. For instance, *S. cerevisiae* and *W. anomalus* fall under this category, while *K. humilis* and *K. exigua* display a perfect adaptation to the sourdough niche. Two dominant yeast species can be isolated in Italian sourdoughs: *S. cerevisiae* and *K. humilis* [44].

Saccharomyces cerevisiae

Saccharomyces cerevisiae (Figure 8), is a unicellular model organism, presenting in a very well-conserved way approximately all biological functions found in eukaryotes [45].

Saccharomyces cerevisiae employs a mechanism known as budding to reproduce, which means that the "mother" cell produces an ellipsoidal daughter cell made up of completely new cell surface material and its life cycle consists of an alternation between haploid and diploid phases.

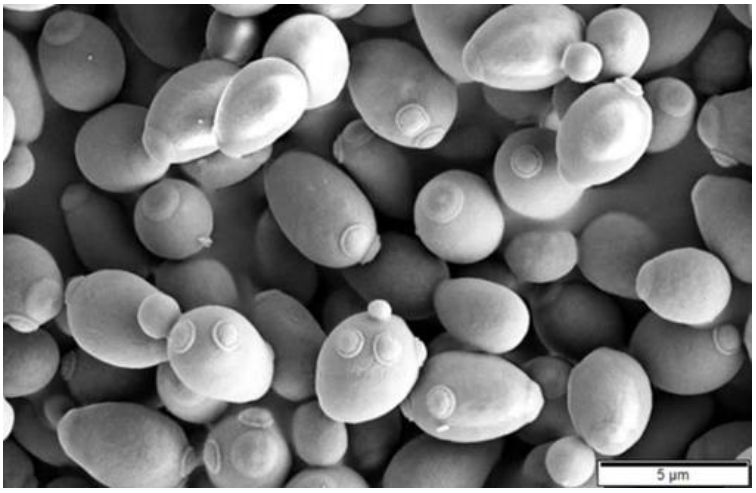


Figure 8. *Saccharomyces cerevisiae* budding by scanning electron microscopy (Picture by Mogana Das Murtey and Patchamuthu Ramasamy)

Its life cycle could be described as haplo-diplobuiontic [46-47]. Haploid cells from different cellular types unite to create diploids, which multiply by mitosis in nutrient-rich environments. Under nutrient-poor conditions, diploids go through meiosis and create tetrads, which are made up of four haploid spores encased in an ascus. Additionally, because *S. cerevisiae* is homothallic, haploid cells can convert to a different mating type while the organism is still in the vegetative phase of development. This enables haploid mother cells to change their mode of reproduction after producing a daughter cell. By haploselfing, mother and daughter cell mating restores the diploid state. The MAT locus, which either encodes the a1 gene (MATa) or the α1 and α2 genes (MATα), defines the mating type of haploid cells [46-47].

S. cerevisiae, in contrast to other model organisms, is also crucial for a number of biotechnological uses. It is generally regarded as safe (GRAS) due to its non-pathogenic behavior and has been historically used in different food industries and bio-pharmaceuticals.

S. cerevisiae S288C strain genome was the first fully sequenced eukaryotic genome, and it was accomplished in 1996 [48]. The sequencing of the *S. cerevisiae* genome, which is regarded as an amazing achievement in eukaryotic biology, also shed light on a variety of elements of genome organisation and evolution. The biological traits that make *S. cerevisiae* particularly helpful for biotechnology include its ability for fermentation, which results in the formation of alcohol and CO2, as well as its resistance to unfavorable osmolarity, low pH conditions, and high concentrations of ethanol and sugar. *S. cerevisiae* is frequently regarded as the "canonical" sourdough yeast because it is present in the majority of starters and is connected to the earliest leavened bread [13], is maltose-positive and moderately acid-tolerant [33]. The ubiquity of this yeast is due to its rapid proliferating ability and may spread on sourdough helped by human compounds (baker's body), containers, and tools. This yeast can be addressed as a generalist due to its sugar utilization ability. Glucose, sucrose and other sugar such as maltose, can be used as a carbohydrate source, creating a competitive environment with specific sourdough lactic acid bacteria *Fl. sanfranciscensis* [49]. However, due to a specific mechanism, the LAB is able to theoretically arrests *S. cerevisiae*'s maltose uptake [49]. The leavening properties of this yeast plays a crucial function in sourdough products by creating carbon dioxide through the alcoholic fermentation of carbohydrates, increasing the volume of the loaf [50]. Additionally, yeast contributes to the construction of gluten and the synthesis of aromatic substances such as alcohols, aldehydes, carbonyls, and esters [50].

Kazachstania humilis

As a maltose-negative budding yeast, acid-tolerant (pH 3.5 and 7.0) and fairly thermotolerant (36 °C) *Kazachstania humilis* frequently coexists in the sourdough environment with the maltose-positive lactic acid bacteria *Fl. sanfranciscensis* [49]. The relationship between *Fl. sanfranciscensis* and *K. humilis* is one of the most prevalent LAB/yeast relationships in sourdough [26]. The San Francisco sourdough from which the species *Fl. sanfranciscensis* was

isolated represents the isolation matrix where this connection was first discovered [51]. These two species' trophic connection is considered positive/positive, is based on a mutualistic exchange of nutrients and is found especially in sourdough type I. In the process of glycolysis, *K. humilis* degrades the glucose that *Fl. sanfranciscensis* releases from maltose. This yeast also destroys the dough's gluco-fructans and consumes glucose while leaving the fructose for *Fl. sanfranciscensis*. The LAB will then reduces fructose to mannitol by using it as an electron acceptor, making possible the production of extra ATP and a metabolic transition from ethanol to acetate synthesis. The nutritional relationship spread also to the provision of amino acids, complementing auxotrophies of *Fl. sanfranciscensis*, and vitamins by *K. humilis* to the lactic acid bacteria. Furthermore, *K. humilis* generates oxidative stress by thiol oxidation, which *Fl. sanfranciscensis* is capable to resist thanks to its redox homeostasis through NADH oxidase activity. Meanwhile, through the production of lactic and acetic acid, *Fl. sanfranciscensis* creates acidic stress, which *K. humilis* can withstand thanks to its acetic acid tolerance. Nevertheless, this reciprocal association for both species appears to be strain-dependent [49,51]. It is possible, nonetheless, for other, more intricate interactions to take place, including both hostile and mutualistic ones [49,51].

2.4.2 Acetic Acid Bacteria

A different group of bacteria that can occasionally be found in sourdough is represented by Acetic acid bacteria (AAB) [17]. According to De Roos and De Vuyst [52] acetic acid bacteria are Gram-negative, catalase-negative, oxidase-positive, non-sporulating, aerobic bacteria, members of the α-proteobacteria, mostly found in ethanol and carbohydrate-rich environments. In the past 10 years, it has been clear that the microbial community in spontaneous sourdoughs frequently includes acetic acid bacteria, and an interest in functional starter culture of AAB able to produce exopolysaccharides from sucrose has risen. [34, 52-56]. Besides, recent studies

on sourdough composition revealed their contribution in aromas and flavours production. A higher percentage of acetic acid bacteria in the dough provides a larger variety of aromas and fruity flavours, affecting the smell and the taste of the final products [13]. Nevertheless, the improved aromatic variation, due to the higher presence of acetic acid bacteria, results in slower yeasts growth and leavening disadvantages [13]. Acetic acid bacteria, use specialised cofactor-dependent dehydrogenases that are embedded in the cytoplasmic membrane at the periplasmic location to partially oxidise gluconic acid and or ethanol from glucose in the cells' periplasm, to create acetic acid. They are also capable of producing fructans, which may contribute to the texture of gluten/gluten-free sourdough bread and dihydroxy acetone, which may also contribute to the colouring of the bread crumb. Though it is still unclear how widespread acetic acid bacteria are in sourdoughs, AAB probably do not predominate in the final sourdoughs due to their aerobic metabolism, and incorporation of oxygen in the dough is unfavourable due to spoilage agents colonization (moulds) and oxidation. The various species of AAB isolates from sourdough reported in different studies include various *Acetobacter* and/or *Gluconobacter* species [34]. In particular, species of the *Acetobacter malorum* and *lovanensis* group were found, along with *Acetobacter cerevisiae*, *Acetobacter fabarum*, *Acetobacter indonesiensis*, *Acetobacter orleanensis*, *Acetobacter oryzifermentans, Acetobacter pasteurianus*, *Acetobacter senegalensis, Acetobacter sicerae*, *Acetobacter tropicalis, Gluconobacter cerinus* [29-31, 39,49]. Additionally, the *Gluconobacter frateurii* group species, *Komagataeibacter sucrofermentans*, and *Komagataeibacter xylinu*s were isolated [13, 55].

2.4.3 Lactic Acid Bacteria

LAB are Firmicutes, which are gram-positive, catalase-negative, anaerobic (aerotolerant), nonsporulating bacteria. In Italian wheat sourdough, *Fl. sanfranciscensis*, *Levl. brevis*, *Liml. fermentum* and *Fl. fructivorans* (obligately heterofermentative) are the most prevalent LAB

followed by *Lacp. plantarum*, *Coml. alimentarius* (facultatively heterofermentative), *L. acidophilus*. *L. delbrueckii* subsp. *delbrueckii*, and *Coml. farciminis* (obligately homofermentative) [17-18, 30]. Generally, according to several studies [9,17,28], sourdoughs mostly include heterofermentative LAB species from the *Lactobacillaceae* family that are especially suited to this stressful environment. A perfect example of this is represented by *Fl. sanfranciscensis*. Due to its limited genome size of 1.3 Mbp and a constrained metabolism, this LAB specie is commonly connected to the sourdough environment. Similar behaviour is ascribable to *Coml. paralimentarius*, only sometimes detected in environments different than sourdough, despite the fact that this LAB species has a genome that is twice as big (2.55 Mbp) as that of *Fl. sanfranciscensis*. However, a genetic explanation for matrix specificity is not always obvious. In various sourdoughs investigated in the last decades, different heterofermentative sourdough LAB species include *Fl. sanfranciscensis, Levl. brevis* , and *Liml. fermentum*, were detected. In addition, homofermentative LAB species such as *Lacp. Plantarum, P. pentosaceus*, and *Coml. paralimentarius* are found, while *Leuconostocs* and *Weissella* species are heterofermentative and typically found in sourdoughs that ferment at low temperatures and relatively high pH levels [9]. Different types of sourdoughs can be distinguished by their presence, such as the adapted *Fl. sanfranciscensis*, typical for Type I sourdoughs and often associated with *Companilactobacillus* species, the *Lactobacillus* and *Limosilactobacillus* species typical colonizers of Type II sourdoughs, and the environmental *Liml. fermentum*, *Lacp plantarum*, and *Levl. brevis*, typical for laboratory sourdoughs, Type 0 sourdoughs, and sourdoughs made from flours other than wheat or rye [12]. Generally, a bakery sourdough hosts less than three distinct species of LAB. The adaptations of species like *Fl. sanfranciscensis*, *Liml. fermentum* and *Levl. brevis* to the sourdough ecosystem includes a variety of physiological characteristics that vary according to the surrounding environment [12]. The preferred fermentation of maltose through an energy-efficient constitutively

expressed maltose phosphorylase supports their mutual relationship with maltose-negative yeasts, in particular *K. humilis*, and avoids glucose repression. LAB strains' competitiveness is upgraded, due to extra ATP formation, and the production of acetic acid can be increased by avoiding ethanol formation and activating the acetate kinase branch of the pyruvate metabolism by using alternative external electron acceptors, such as fructose, constantly elaborated by yeast activity, which is then reduced to mannitol to recover NAD+. This also applies to the use of fructose 6-phosphate which is reduced to erythritol, oxaloacetic acid that is converted to malate, which can then be further reduced to fumarate, which is then reduced to succinate via the reductive TCA cycle, oxygen gas that is reduced to hydrogen peroxide by pyruvate oxidase activity, lipid oxidation-derived aldehydes that are converted to alcohols through alcohol dehydrogenase and the use of glutathione reductase to obtain glutathione from glutathione disulfide. Furthermore, LAB species, such as *Coml. paralimentarius* and *Lacp. Plantarum*, have the capacity to ferment both hexoses and pentoses present in the flour via xylulose 5-phosphate through the phosphogluconate route. It is also desirable to utilize sucrose in the form of an energy-efficient sucrose phosphorylase. Dedicated strains of *Fl. sanfranciscensis* utilise sucrose through extracellular levan-sucrase activity, resulting in the production of free glucose. However, glucose as an energy source and fructose as an alternative external electron acceptor, are primarily supplied through yeast invertase activity. Nonetheless, according to Gobbetti and Gänzle [3], Gänzle [10], and Gänzle and Zheng [12], the whole of these activities depend on the species and/or strain of LAB. *Limosilactobacillus* species and *Leuconostoc*, using fructansucrase or levansucrase activities, are able to convert sucrose into homo-oligosaccharides that contribute to the dough texture. Additionally, acid-tolerant *Limosilactobacillus* species provide protection against acidic stress through amino acid conversion. LAB species posses specific reactions to oxidative and acidic stressors activating glutathione reduction and cystathionine conversion, respectively. Moreover, pH increases, as

a response to the acidic stress, and the formation of additional ATP might be achieved due to the arginine deiminase pathway that releases ammonia and enhances the strains' competitiveness [17]. However, *Fl. sanfranciscensis* lacks the action of the above-mentioned metabolic strategies, making it extremely reliant on regular sourdough back-slopping and sensitive to low pH [49]. Additionally, with their metabolism, LAB are accountable for creating various flavour compounds.

Fructilactobacillus sanfranciscensis

The most common LAB in sourdough is *Fl. sanfranciscensis*, and despite it is a bacterium that is related to insects, few findings of it outside the sourdough environment have been discovered [57]. Fruit flies have been suggested as a potential host for this LAB since a strain of *Fl. sanfranciscensis* was isolated in the stomach of *Drosophila simulans* Stuvervant (a fruit fly species closely related to *Drosophila melanogaster*) [58]. In addition, this assumption is supported by the presence of fruit flies in artisanal bakeries and flour after storage periods and its absence in the cereal plant [51]. This LAB represents the most prevalent and stable species of the local LAB diversity (α-diversity) of sourdoughs, and it also predominates in sourdoughs globally (β-diversity) [14, 35]. According to the findings, at least 33 genome assemblies of this LAB have been described [59]. These genome assemblies revealed a median total length of 1.29554 Mb, a median protein count of 1,251, and a median G + C% of 34.7 [59]. This LAB becomes dominant only following numerous refreshment steps of the sourdough, and cannot be detected in the initial flour [35]. Indeed, when the ideal circumstances occur (propagation temperature, inoculum size, and fermentation period) such as in traditional sourdough, this LAB thrives more quickly than other species [48, 57]. As aforementioned, due to its remarkable adaptation to the sourdough environment, *Fl. sanfranciscensis* possesses the largest density of ribosomal RNA operons per Mbp genome among the wholly sequenced LAB species, which supports this particular adaptation ability [12]. *Fl. sanfranciscensis* is distinguished by a

prolonged latency phase, increased acid production, high acidic tolerance, and a particularly marked oxidative stress resistance [28]. It also possesses heat shock proteins (DnaJ, DnaK, GrpE, and GroES), that according to various research, function as response regulators and give the bacterium tolerance to a variety of stressors, including acid stress [60-61]. Moreover, *Fl. sanfranciscensis* metabolism is fundamental for rheological, aromatic and bio-preservation of the final products.

Lactiplantibacillus plantarum

Lactiplantibacillus plantarum is an aerotolerant LAB able to thrive between 15 and 45 °C, and is considered a typical plant bacterium. This LAB has been isolated from silage, pickled vegetables, sourdough, and other nutritive-rich habitats such as meat, fish, and dairy products. Its capacity to consume a wide variety of carbohydrates, from which both isomers of lactic acid are generated as the main end metabolite, explains *Lacp. plantarum*'s remarkable ecological adaptation to such diverse ecological surroundings [62]. This LAB can express cytochromes and breathe oxygen if the growing media contains heme and menaquinone. In the absence of heme and menaquinone, NADH-peroxidase consumes oxygen, producing water as the final product and hydrogen peroxide as an intermediate. Presumably, the peroxide acts as a weapon to exclude competing bacteria from the food source, and in place of the protective enzyme superoxide dismutase present in almost all other oxygen-tolerant LAB, *Lacp. plantarum* accumulates millimolar quantities of manganese polyphosphate. One of the biggest LAB genomes is represented by *Lacp. plantarum* [63]. The median overall length of the 820 *Lacp. plantarum* genome assemblies is 3.26356 Mb, the median protein count is 2989, and the median G + C% is 44.5 [63]. Due to its consumption of dextrins after the depletion of the fermentable sugars, *Lacp. plantarum* is the dominating microbe in sourdough and many cereal-based fermented products impacting also their final aromatic scent [64]. This LAB strains possess an acidophilic starch hydrolyzing enzyme able to degrade soluble starch, amylopectin, glycogen,

pullan, and various amylolytic strains of this LAB have been isolated in fermented doughs. The degradation of starch due to this bacteria during fermentation results in sugar being able to provide the growth of a secondary flora[64]. In addition, during the last few years, various studies have been focusing on its exopolysaccharides production and antibacterial/antifungal capabilities [65-66].

Leuconostoc citreum* and *Leuconostoc mesenteroides

Leuconostoc species are widespread in the environment and have been found in plant material, human clinical sources, and foods including refrigerated and fermented meats, fermented vegetables, and fermented dairy products. *Leuconostoc* spp. have been linked to a small number of human illnesses; nevertheless, these strains are thought to be opportunistic pathogens of susceptible immunocompromised people, and the genus is usually accepted as harmless [67]. *Leuconostocs* are obligately heterofermentative mesophilic with optimal growth at 25 °C. This LAB are picky eaters and need a source of vitamins, amino acids, and a fermentable carbohydrate for energy and cannot metabolize arginase [67]. According to NCBI database, 147 genome of *Leuc. mesenteroides* have been sequenced, with a median length of 1.97853 Mb, a median protein count of 1903 and a median G+C % content of 37.7 [68] . While regarding *Leuc. citreum* 60 genomes have been assembled with a median length of 1.89974 Mb, median protein count of 1836, and median G+C % content of 38.8402 [69]. *Leuconostoc* species, in particular *Leuc. mesenteroides* display a high proliferation in sourdough with a lot of sucrose, such as Panettone where dried fruit is generally included during the manufacturing [70-71]. For the microbial habitat and culinary qualities of sourdough, the presence of *Leuc. mesenteroides* strains in pre-ferments can be essential and this microorganism help to provide acid-lactic, fruity, or toasted undertones to the final product [70]. Instead, the dextran-producing species *Leuc. citreum* is believed to have probiotic potential due to a putative plasmid-encoded protein that is cell wall-anchored and contains five mucus-binding domains

that are crucial for gastrointestinal colonization [67]. In recent years, the use of EPS for the production of baked goods has been investigated in an effort to improve the quality of various bakery products. Essentially, due to their hydrocolloid activity, EPS have a significant impact on the textural qualities of dough, which affects a number of bakery products characteristics, such as better structure and volume, increased softness, and delayed staling.

Furfurilactobacillus rossiae

Furl. rossiae appears to be widely distributed in sourdough from central and southern Italy [72-73]. In the past years, it was assumed that *Furl. rossiae* was an indigenous species whose only established habitat was sourdough [73]. However, lately, *Furl. rossiae* isolates were discovered in fermented pork, spelt flour, pineapples and pineapple juice, human faeces, pig faeces and brewing environment [74-77]. Since maltose is the most prevalent fermentable carbohydrate in beer and sourdough, *Furl. rossiae*'s colonization of the beer habitat is not unexpected, although in beer this LAB is considered a spoilage agent [78]. According to De Vuyst and Vancanneyt [79], this LAB is strictly related to the *Levl. brevis* and *Lacp. plantarum* groups, indicating that these species may have adopted similar adaptation tactics [80]. *Furl. rossiae* is microaerophilic, it can grow at 15°C but not at 45°C, positive arginine hydrolysis results in the production of DL-lactic acid and possess a peptidoglycan structure type A3α (L-Lys-L-Ser-L-Ala2) [81]. The total amount of assembled genome is 9 with a median length of 2.86229 Mb, a G+C% content of 43.44, and a median protein count of 2638 [82].

Lentilactobacillus parabuchneri

Generally isolated from artisanal cheese, silage, spoiled beer, and whiskey mashes, this species is strictly related to *Lenl. buchneri* [83]. According to NCIB database a total of 33 genomes have been sequenced, revealing a median length of 2.61508 Mp with median protein count of 2414 and a median G+C% content of 43.4 [84]. This heterofermentative LAB is principally accountable for the development of histamine in a variety of cheeses [83]. Although the presence of this LAB might be detected also in fermented cereal such as sourdough, where it

could be accountable for exopolysaccharides production and anti-spoilage activity [66].

2.5 Influence of LAB and yeasts on the formation of taste and aroma compounds

In sourdough, enzymatic and microbiological reactions during fermentation are the main causes of odorants being produced. The most frequently stated chemical classes for these substances normally include aldehydes, acids, alcohols, ketones, esters, and pyrazines [27]. The flour already contains certain compounds, however, those introduced by raw materials are insignificant in comparison to the total volatile chemical concentration. Moreover, lipid oxidation, the Maillard and caramelization reactions, as well as fermentation, are the main causes of other flavour compounds [85]. The key sensory characteristics that best described the distinctiveness of sourdough products are acidic taste and smell, strong aftertaste and fragrance, freshness, fruitiness, and sourness (Figure 9). Two types of volatile substances can be detected in sourdough: non-volatile substances, including the organic acids produced by the LAB, and volatile substances such as alcohols, aldehydes, ketones, esters, and sulphur [86]. The two main producers of volatile compounds in sourdough are LAB and yeast. LAB and yeast fermentations differ from metabolic and kinetic processes resulting in the creation of specific volatile compounds. In particular, monosaccharides are consumed in glycolysis to produce pyruvic acid, which provides the foundation for both yeast and LAB fermentations. Lactic acid, propanoic acid, propanol, ethyl lactate, ethanol, acetic acid, 2,3-butanedione, 2-butanone, and butanol, are produced from pyruvic acid, and the following generated volatile compounds differ based on the sourdough microflora. In addition, according to Hansen and Schieberle [87], competition between yeast and LAB has an effect on the generation of volatile compounds.

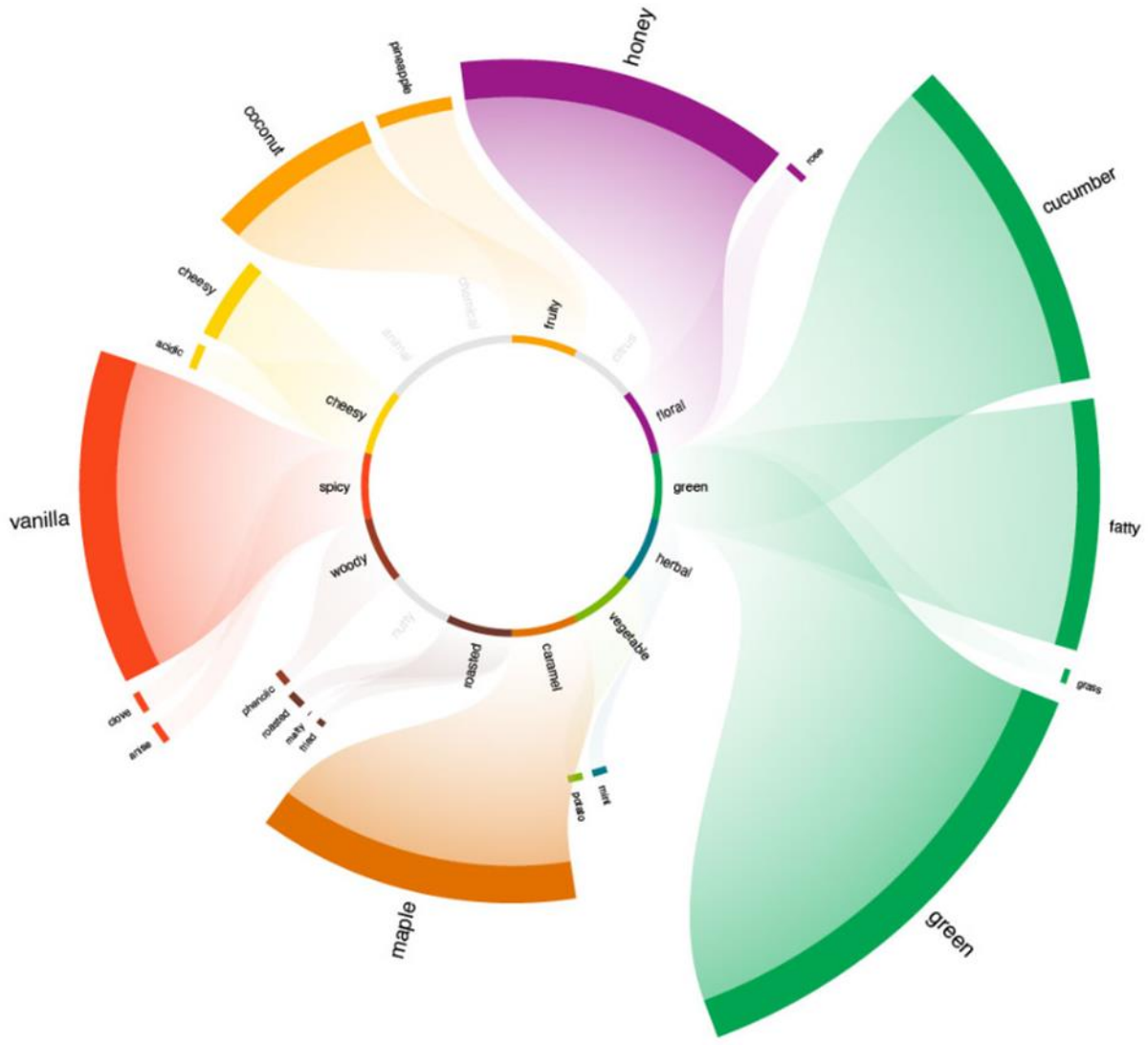


Figure 9. Representation of aromatic scent commonly detectable in wheat sourdough (https://www.foodpairing.com/the-science-behind-the-aroma-of-sourdough/)

LAB, both heterofermentative (HeLAB) and homofermentative (HoLAB), deeply affect the aromatic bouquet of the final product and are mainly responsible for the release of fragrance precursors such as free amino acids, which break down into aldehydes or the equivalent alcohols. Generally, in a few hours of fermentation, yeasts can generate a considerable number of volatiles in particular ethanol and isoalcohols as opposed to LAB that produce hexanoic acid (goat), hexanol (floral), pentanol (sweet), 2- and 3-Methylbutanol (malty), 2-pentylfuran (fruity), and dimethylsulfide (sulphurous) in enough quantity only after 12 hours of fermentation [86,88]. Through the Ehrlich mechanism yeasts break down amino acids during

fermentation resulting in odor-active aldehydes or the equivalent alcohols. Yeasts are the main producers of ethanol, aldehydes, iso-alcohols, and ethyl acetate, as well as 2-methyl-1-propanol (ethereal), 3-methylbutanol (alcohol), 2-methylbutanol (roasted), aldehydes including acetaldehyde, hexanal, octanal, nonanal, 3-hydroxy-2-butanone (sweet), benzyl alcohol (floral) or octanoic acid (fatty) production [82-84]. Moreover, yeasts' amino acids conversion, is accountable for the production of scents such as 2-phenylethanol (rose) from phenylalanine, methional (potato) from methionine, 2-methylpropanal (aldehydic) from valine, 2-methylbutanal (musty) and 2-methylbutanoic acid (acidic) from isoleucine [87-89]. A clear incidence of yeast contribution on the volatilome of sourdough is represented by 3-Methyl-1-butanol, responsible for the ''fermented" flavoring, alongside other alcohols including 1-pentanol, 1-hexanol, 1-heptanol, 1-octen-3-ol, 3-octen-1-ol, 1-octanol, phenylethyl alcohol, and 1-nonanol [89]. In contrast, other detectable volatile compounds are considered specific to various species of LAB including benzaldehyde (almond) commonly associated with *L. helveticus*, and 2-heptenal (sour) assigned to *Lacp. plantarum* [88]. As above mentioned the aromatic LAB contribution is strictly dependent on their metabolism and species. For instance, heterofermentative LAB primarily generate acetic acid and ethanol along with esters and alcohols originating from aldehydes transportation. Contrarily, homofermentative species generate 2,3-butanedione (butter-like), acetaldehyde (aldehydic) and 2- and 3-methylbutanol (fruity) [86,88]. Furthermore, the addition of various ingredients, in particular, fatty substances, and the consequent activation of the exogenous and endogenous lipoxygenases during the mixing process also lead to the formation of flavour volatiles and affects dough rheology [90]. Lipoxygenase oxidase polyunsaturated fatty acids into free radicals, peroxides, and hydroperoxides, which are then transformed into volatile compounds during the baking process [90]. Commonly, lipid oxidation in sourdough leads to the formation of aldehydes, ketones, alcohols, and esters. In addition, the HoLAB present in sourdough could stimulate lipid

oxidation and the production of nonenal and decanieal, contrary to obligatory heterofermentative which accumulate glutathione or low molecular weight thiol compounds and reduce the oxidation-reduction potential of sourdoughs. As well, during development in sourdough, HeLAB convert flavor-active 2-nonenal and 2,4-decadienal to the corresponding alcohols [90]. Flour, added ingredients, fermentation conditions (pH, temperature), microbial community and elements affecting their activity like temperature and water content, will therefore impact the amounts of flavours produced, as well as the baking process [87,91]. In fact, even if the contribution of the Maillard reactions to the aromatic bouquet is ascribable only to some compounds (2-methylbutanal, 3- 474 methylbutanal and 2,3-butanedione) and strictly related to the amount of sugars and amino acids present in the dough, its contribution is still important for the final product flavour [91].

Nevertheless, the outcomes of LAB fermentation are not limited to the development of desired flavour compounds in the final products but comprise different substances such as EPS and antimicrobial compounds that might be used and added as external ingredients in multiple applications in the food industry.

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Yeasts and Lactic Acid Bacteria for *Panettone* Production: An Assessment of Candidate Strains

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Abstract: The recovery of yeasts and lactic acid bacteria (LAB) involved in sourdough fermentation is the first step in the selection of starters with suitable technological aptitude and capable of producing desired aromas and/or aromatic precursors. In this work, two sourdoughs samples (MA and MB) and the derived doughs (samples A and B) were collected from a bakery during artisanal Panettone manufacture. Yeasts and bacteria were isolated at different fermentation steps on selective agar media. A total of 77 isolates were obtained and characterized. Representative strains of yeasts and LAB were identified by sequencing the D1/D2 domain of the 26S rRNA and the 16S rRNA genes, respectively. Moreover, the volatile organic compounds (VOCs) produced in the collected samples were detected and correlated to the species found in the same samples. The results highlighted the occurrence of *Kazachstania humilis* in both samples A and B, while *Saccharomyces cerevisiae* strains were detected only in samples B. Among LAB, *Fructilactobacillus sanfranciscensis* was the main species detected in both sourdoughs. Furthermore, strains belonging to the species *Lactiplantibacillus plantarum*, *Furfurilactobacillus rossiae*, *Lactobacillus parabuchneri*, *Leuconostoc citreum*, and *Leuconostoc mesenteroides* were assessed in the dough samples.

Keywords: sourdoughs; starter culture; sequencing; volatile organic compounds; Panettone

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Citation: De Vero, L.; Iosca, G.; La China, S.; Licciardello, F.; Gullo, M.; Pulvirenti, A. Yeasts and Lactic Acid Bacteria for *Panettone* Production: An Assessment of Candidate Strains. *Microorganisms* **2021**, *9*, 1093. https://doi.org/10.3390/microorganisms9051093

Academic Editor: Salam A. Ibrahim

Received: 3 May 2021
Accepted: 18 May 2021
Published: 19 May 2021

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1. Introduction

Panettone is a traditional Italian baked cake produced from sourdough, also called mother dough (MD). Sourdough can be defined as a matrix of flour and water including yeasts and lactic acid bacteria (LAB) as main functional microorganisms [1,2].

Commonly, the type I sourdough is specifically used for the artisanal production of Panettone and other traditional Italian sweet baked products, such as Pandoro and Colomba [3–5]. This type of sourdough is characterized by daily refreshments, carried out every 4–16 h at 25–35 °C according to a back-slopping procedure, which consists of the addition of flour and water with an aliquot of previously fermented dough [6,7]. This practice maintains the microorganisms in an active state and promotes the selection of a stable and characteristic microflora, which is well adapted to the specific recipe and manufacturing procedure [8,9].

The main species occurring in type I sourdoughs are the LAB Fructilactobacillus sanfranciscensis (formerly Lactobacillus sanfranciscensis), Levilactobacillus brevis (formerly Lactobacillus brevis), and Lactiplantibacillus plantarum (formerly Lactobacillus plantarum) and the yeasts Saccharomyces cerevisiae, Kazachstania exigua, and Kazachstania humilis [7,10,11].

Technological parameters, such as temperature, flour composition, degree of dough hydration, and sodium chloride content significantly contribute to the selection of the

microflora [12]. Moreover, the richness and diversity of yeasts in sourdoughs can differ considerably as they are influenced by many factors including tolerance to the organic acids produced by the LAB and the availability of carbon sources [12–14].

Generally, LAB activity causes the acidification of sourdough, while yeasts are mainly responsible for the dough leavening [4,6]. In addition, the metabolism of LAB and yeasts contributes to the aroma's formation through the production of important flavor compounds such as diacetyl, other carbonyls, ethyl acetate, and isoalcohols [7,15,16].

Although sourdoughs have been widely studied, currently, there is an interest in exploring their microbial composition, due to the numerous scientific studies that highlight their positive influence on sensory, nutritional, and shelf-life characteristics of naturally leavened products [17–19]. Additionally, the continuous search for starter cultures with new attributes, aimed at enhancing the nutritional and nutraceutical traits of sourdoughs, is driving the interest in exploring their microbial diversity [20–22].

In the present work, we investigated the yeasts and LAB population of sourdoughs for Panettone production, as well as the volatile fraction of dough samples, with the aim to select candidate strains for developing single and/or multiple starter cultures.

2. Materials and Methods

2.1. Panettone Production and Sample Collection

Two sourdoughs, defined as MA and MB, were used to produce an artisanal Panettone in a bakery (Panificio Fantuzzi) of the Emilia Romagna region (Italy). They differed in the kind of storage after the periodical refreshments; specifically, MA was kept in a cotton bag, while MB in a bucket with water. Starting from these, the refreshment procedures were made using only wheat flour (type 0) and water. Three back-slopping steps were made for MA and only two for MB. In both cases, about 28% (*w/w*) of the final refreshed sourdough was used for the first dough preparation by adding the following ingredients: flour (4.0 kg), sugar (1.25 kg), yolks (1.0 kg), butter (1.5 kg), and water (2.1 L). After 18 h of incubation at 20–22°C, a second dough was prepared by mixing the first dough with flour (1.5 kg), sugar (1.0 kg), yolks (1,0 kg), butter (2.0 kg), barley malt (0.05 kg), salt (0.065 kg), and water (0.25 L). The final dough was left to rise for 5–6 h before baking. No aromatic ingredients were added.

During the entire process, six samples were taken concurrently in sterile containers for the laboratory analyses. Specifically, the samples were MA and MB; the first doughs were named IMPA and IMPB, collected after the leavening of 18 h, and the final doughs were called FINA and FINB, collected just before the cooking. A schematic representation of the process with the sampling points is reported in Figure 1.

2.2. Determination of Physicochemical Parameters

The following parameters pH, total titratable acidity (TTA), and water activity (a_w) were evaluated on all the samples collected. In detail, pH was detected with a pH meter XS series pH 70 (Bormac srl, Carpi, Italy), and TTA was determined on the homogenized samples and expressed as the amount (mL) of 0.1 N NaOH necessary to achieve pH 8.3 [23]. The a_w was measured with the AquaLab 4TE instrument (Meter Group, Pullman, WA, USA) following the manufacturer's instructions. Each sample was analyzed in triplicate.

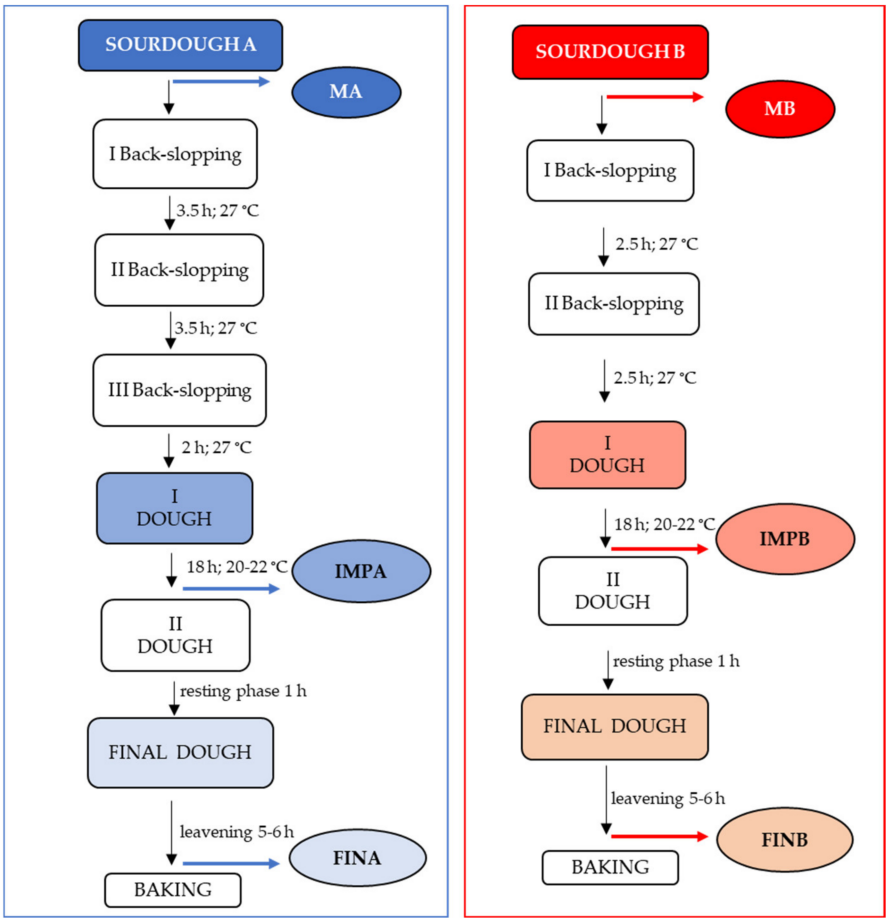


Figure 1. Artisanal Panettone production using two different sourdoughs (MA and MB). The samples highlighted were collected concurrently at the same stages for their investigation.

2.3. Yeasts and LAB Isolation

To obtain microbial isolates, 10 g of each sample was collected and mixed with 90 mL of physiological solution (9 g/L of NaCl) in a Stomacher bag and homogenized for 2 min. From the homogenate samples, serial dilutions from 10^{-1} to 10^{-7} were made and appropriate dilutions were plated in triplicate on different agar media. Specifically, Yeast Peptone Dextrose Agar (YPDA; 10 g/L yeasts extract, 10 g/L peptone, 20 g/L dextrose, and 20 g/L agar) supplemented with 0.1 g/L of chloramphenicol (Sigma-Aldrich, Milan, Italy) was used for yeasts isolation. Plates were incubated at 27 °C for 2 days. LAB isolation was made on de Man Rogosa Sharpe (MRS agar; Oxoid, Milan, Italy) and Sourdough Bacteria Agar (SDB; 6 g/L trypticase, 3 g/L yeast extract, 20 g/L maltose, 3 mL of 10% Tween 80, 1.5 % of fresh yeast extract, 15 g/L agar), both supplemented with 0.1 g/L cycloheximide (Sigma-Aldrich). Plates were incubated in jars with the Anaerogen system (Oxoid) at 30 °C for 3 days. After the colonies were counted on the plates of each medium, between 3 and 10 colonies were randomly selected at the highest dilutions and purified by streaking on the correspondent isolation medium. Basic phenotypic tests, such as Gram staining and catalases, were done for bacteria. Cell morphology of the isolates was observed using a Zeiss Axiolab microscope (Carl Zeiss Ltd., Cambridge, UK). After the molecular characterizations, yeasts and bacteria strains were preserved in the Unimore Microbial Culture Collection (UMCC) in accordance with standard procedures [24]. A seed lot of the strains was stored at −80 °C, in cryovials containing an aliquot of each culture, grown in liquid medium, and 25% (*v*/*v*) glycerol (Carlo Erba, Milan, Italy).

2.4. DNA Extraction and Molecular Characterization

Genomic DNA from yeast and bacterial cultures was extracted following the protocols of Hoffman and Winston [25] and Ausubel et al. [26], respectively. The quality and concentration of the extracted DNA were checked by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

For a preliminary characterization at species level, PCR-RFLP of the internal transcribed spacer (ITS) region and 5.8S gene of rDNA was performed on yeast gDNA following the protocol described by Pulvirenti et al. [14]. ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) oligonucleotide primers (Eurofins MWG Operon, Ebersberg, Germany), and PCR amplification kit, which included TaKaRa Taq DNA Polymerase (Takara Bio, Inc., Otsu, Shiga, Japan), were used. PCR was made on a final volume of 50 µL by using the thermal cycler BioRAD T100^{TM} (Bio-Rad Laboratories, Milan, Italy). Amplicons digestion was performed with *Hae*III restriction endonucleases (Fermentas, Hanover, ND, USA), according to supplier's recommendation. The fragments were separated on 2% (*w/v*) agarose gel electrophoresis in 0.5X Tris-Borate-EDTA (TBE) buffer at 90 volts for 2 h. The gels were stained with SYBR Safe reagent (Thermo Fisher Scientific) and visualized on the Transilluminator Safe View (Cleaver Scientific LTD, Rugby, UK).

A first rapid detection of LAB strains belonging to *Fl. sanfranciscensis* species was made based on PCR amplification of the 16S–23S rDNA intergenic spacer region (ISR). PCR was performed according to the protocol described by Valcheva et al. [27] by using the oligonucleotide primers 16S p2: (5′-CTTGTACACACCGCCCGTC-3′) and 23S p7: (5′-GGTACTTAGATGTTTCAGTTC-3′) (Eurofins MWG Operon). The fragments were separated on 1.5% (*w/v*) agarose gel electrophoresis in 0.5X Tris-Borate-EDTA (TBE) buffer at 90 volts for 1.5 h.

For strain characterization, repetitive element sequence-based (rep)-PCR genotyping [28] using $(GTG)_5$ oligonucleotide primer (Eurofins MWG Operon) was performed on gDNA of all the isolates according to the protocol described by La China et al. [29]. Fingerprinting profiles were assessed after electrophoresis on 1.5% (*w/v*) agarose gel, carried out as previously described. Pattern band lengths were determined by comparison against a 100 bp plus DNA ladder (Thermo Fisher Scientific).

Digitalized images of the electrophoretic profiles were analyzed by using the GelCompare software v3.0 (Applied Maths, Kortrijk, Belgium). The similarity matrix of the bands' patterns was computed by using Pearson's correlation with optimization and curve smoothening values at 0.5%. Dendrograms were constructed by the unweighted-pair group method using arithmetic averages (UPGMA) clustering method.

2.5. Species Assignment and Phylogenetic Clustering

At least one strain of yeasts and bacteria isolated from each sample was subjected to sequencing analysis for the species assignment and the successive phylogenetic clustering. To this aim, targeted regions were amplified using specific primer sets. In particular, the D1/D2 region of 26 rRNA large subunit from the yeast gDNA was amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′), as reported by Kurtzman et al. [30].

Yeast Ab1 files obtained from the sequencing were processed using CodonCode aligner to trim the sequences based on phred score (phred 20). The sequence ends were trimmed according to primer length. High-quality sequences were aligned against YeastIP database [31]. Reference sequences were downloaded from YeastIP database, based on matches, e-value, and identity scores.

Regarding bacteria, amplification of 16S rRNA gene was performed using primers 27f (5′-CTGGGATCCATTTACTCGAGAGTTTGATCCTGGCTCAG-3′) and 1490r (5′-GGTTCCCCTAAGCTTACCTTGTTACGACTTC-3′), as reported by Sato et al. [32].

PCR amplicons were purified and cleaned using ExoSAP PCR cleanup reagent (Thermo Fisher Scientific) and sequenced by Eurofins MWG Biotech Company (Ebersberg, Germany). The strains sequenced were deposited in the UMCC Database (https:

//umcc.bio-aware.com/, accessed on 20 April 2021).

Bacteria Ab1 files were processed as described for the yeast dataset. High-quality sequences were aligned against 16S rRNA sequence database from NCBI using blast algorithm. The top hit with the highest percentage of identities (considering a minimum threshold of 97%) was downloaded and used as reference.

The sequences of selected strains and references were aligned all-vs.-all using Clustal W v2.1 [33]. For constructing the phylogenetic trees, the multiple sequences alignment was imported into MegaX v10.2 and trimmed to match the sequence length. The phylogeny was inferred computing a neighbor-joining phylogenetic tree based on 1000 bootstraps for both yeasts and LAB dataset. The Tamura-Nei DNA evolutionary model was used, applying a discrete Gamma distribution to model evolutionary rate differences among sites [34].

Newick trees of both bacteria and yeasts were visualized using Interactive Tree of Life (ITOL) v4 [35] and rooted at outgroup reference strains. The sequences of D1/D2 region and 16S rRNA gene were submitted to GenBank/EMBL/DDBJ under the accession numbers from MZ170795 to MZ170810 and from MZ170701 to MZ170718, respectively.

2.6. HS-SPME-GC/MS Profiling of Fermented Products

Volatile organic compounds (VOCs) developed during dough fermentation were determined by headspace solid-phase microextraction (HS-SPME) followed by gas-chromatography/mass spectrometry (GC-MS) analysis.

Seven grams of the six samples collected were weighted into 25 mL screw-cap glass vials provided with Mininert© valves.

Vials were conditioned at 60 °C for 30 min in a thermoblock (Falc Instruments, Treviglio, Italy), and then a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber was exposed in the headspace for 30 min at the same temperature for the extraction of volatile compounds. Chromatographic separation of analytes was carried out by an Agilent (Paolo Alto, CA, USA) 6890N GC followed by mass spectrometer Agilent 5973 Network Mass Selective Detector. After extraction, fibers were desorbed for 3 min into the GC injector port set in splitless mode at 240 °C. The GC carrier gas used was helium at 1 mL/min and the detector temperature set at 240 °C. GC oven temperature program was: start at 50 °C for 3 min, 5 °C/min until 160 °C, hold at 160 °C for 2 min, 20 °C/min until 240 °C, hold at 240 °C for 2 min. Peaks identification was carried out by comparison with system libraries (Wiley, Nist). The analyses were performed in triplicate. A correlation analysis of VOCs and species detected in sourdough and dough samples was obtained using the Corrplot v. 0.84 [36] package implemented in R v 4.0.3 [37].

2.7. Statistical Analysis

pH, temperature, TTA, and a_w data were expressed as mean value ± standard deviation (sd). Microbial counts were converted to log colony-forming units (CFU) per g of sample and expressed as mean ± sd. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test to establish significant differences between means ($p < 0.05$; n = 3). For the statistical analysis, the software GraphPad Prism version 8 was used (GraphPad Software, San Diego, CA, USA).

Total VOCs in sourdough and dough samples were expressed as a mean value of the total chromatographic area detected by GC-MS analysis, while the relative abundance of each VOC was reported as % of the total chromatographic area ± sd.

3. Results

3.1. Samples Analysis and Microbial Counts

The physicochemical parameters detected on the samples collected during the Panettone production are reported in Table 1. The pH values of sourdoughs MA and MB pH were 3.90 and 3.81, respectively. A significant increase in the pH was observed in all the other samples because of the dilution effect of the ingredients added during the production

process. Accordingly, the TTA was higher in the two sourdoughs and decreased in doughs IMPA and IMPB, as well as in the final doughs. The same trend was observed also for the water activity (a_w), which reached a final value of 0.9441 and 0.9411 in FINA and FINB, respectively. In these cases, above the dilution effect, the addition of ingredients, such as sugars, also contributed to lowering the a_w values [8].

Table 1. Physicochemical parameters detected on the samples collected during the Panettone production.

Samples	pH	Water Activity (a_w)	Total Titratable Acidity (mL NaOH 0.1N/10 g)
MA	3.90 ± 0.03^{d}	$0.0.9855 \pm 0.002^{d}$	10.85 ± 0.45^{b}
IMPA	5.44 ± 0.05^{b}	$0.0.9636 \pm 0.001^{b}$	3.35 ± 0.25^{a}
FINA	5.35 ± 0.02^{bc}	$0.0.9441 \pm 0.002^{a}$	3.55 ± 0.45^{a}
MB	3.81 ± 0.02^{d}	$0.0.9894 \pm 0.001^{d}$	10.45 ± 0.45^{b}
IMPB	5.30 ± 0.07^{ac}	$0.0.9562 \pm 0.002^{c}$	3.45 ± 0.30^{a}
FINB	5.21 ± 0.03^{a}	$0.0.9411 \pm 0.003^{a}$	4.05 ± 0.05^{b}

Values are means ± standard deviation of three replicates (n = 3). Values within a column with different superscript letters are significantly different ($p < 0.05$).

Regarding the colony counts of the yeasts assessed on YPDA, it ranged from 6.81 to 7.83 log cfu/g for samples A and from 7.56 to 7.89 log cfu/g for B. The viable bacterial population enumerated on MRS and SDB media was generally higher in MA and MB, with values around 6 log cfu/g; in all the other samples, the values were around 5 log cfu/g (Table 2). In total, 57 yeast isolates were collected and further investigated. Concerning the isolates from MRS and SDB media, 20 were selected as presumptive LAB after the basic microbiological screenings.

Table 2. Colony counts on the growth media used for the isolation of yeasts and lactic acid bacteria from dough samples.

Samples	Means Counts * on the Isolation Media		
	YPDA	MRS	SDB
MA	6.81 ± 0.25^{b}	6.15 ± 0.14^{ab}	6.17 ± 0.21^{b}
IMPA	7.81 ± 0.05^{a}	5.39 ± 0.12^{a}	5.65 ± 0.21^{ab}
FINA	7.83 ± 0.17^{a}	5.46 ± 0.07^{a}	5.45 ± 0.19^{ab}
MB	7.56 ± 0.03^{a}	6.87 ± 0.42^{b}	6.16 ± 0.15^{b}
IMPB	8.02 ± 0.01^{a}	5.57 ± 0.06^{a}	5.27 ± 0.09^{a}
FINB	7.89 ± 0.17^{a}	5.64 ± 0.04^{a}	5.64 ± 0.02^{ab}

* expressed as log of colony forming unit (cfu)/ g of sample ± standard deviation. Values are means ± standard deviation of three replicates (n = 3). Values within a column with different superscript letters are significantly different ($p < 0.05$). YPDA medium (yeast extract, peptone, dextrose, agar); MRS medium (de Man Rogosa Sharpe); SDB medium (Sourdough Bacteria agar).

3.2. Yeasts' Molecular Characterization and Phylogenetic Analysis

The analysis of the RFLP-PCR of ITS-5.8S rDNA and the yeasts' amplicons digestion with *Hae*III allowed us to detect two different profiles (Table 3). Specifically, all the isolates belonging to samples A showed the same profile characterized by two bands of 230 and 400 base pairs (bp). As reported in the literature, this profile was tentatively attributed to the species *K. humilis* (formerly *Candida milleri*) [13,14]. Regarding the isolates from samples B, 12 showed a profile with two bands, as before, whereas a profile with three bands of 150, 180, 230, 320 bp were detected in the remaining ones. These isolates were preliminarily attributed to *S. cerevisiae* species according to Esteve-Zarzoso et al. [38].

Table 3. RFLP-PCR of ITS-5.8S rDNA.

Sample	Strains *	Size of Amplicon (bp)	Amplicons Digestion with *Hae*III (Size Fragments, bp)
MA	LMA2, **LMA3 (UMCC 2973)**, LMA1, LMA4, **LMA5 (UMCC 2974)**, LMA6, LMA7, LMA8, **LMA9 (UMCC 2975)**, LMA10	630	400–230
IMPA	**LIA1 (UMCC 2976)**, LIA9, LIA10, LIA2, LIA3, LIA4, **LIA5 (UMCC 2977)**, LIA7, **LIA6 (UMCC 2978)**, LIA8	630	400–230
FINA	**LFA1 (UMCC 2979)**, LFA2, **LFA3 (UMCC 2980)**, LFA4, LFA5, **LFA6 (UMCC 2981)**, LFA7, LFA8, LFA9, LFA10	630	400–230
MB	**LMB1 (UMCC 2982)**, LMB5	630	400–230
	LMB2, LMB3, LMB7, **LMB9 (UMCC 2984)**, LMB10	880	320–230–180–150
IMPB	LIB1, LIB4, **LIB7 (UMCC 2986)**, LIB10	880	320–230–180–150
	LIB2, LIB3, **LIB5 (UMCC 2985)**, LIB6, LIB8, LIB9	880	400–230
FINB	**LFB1 (UMCC 2987)**, LFB2, LFB3, LFB4, LFB6, **LFB10 (UMCC 2988)**	880	320–230–180–150
	LFB5, LFB7, LFB8, LFB9	630	400–230

* The strains sequenced are shown in bold and in brackets are indicated the correspondent UMCC code.

For strain clusterization, the $(GTG)_5$ rep-PCR fingerprinting techniques were performed. Figure 2 reports the dendrogram obtained from UPGMA analysis using Pearson's coefficient. The discrimination of biotypes was performed considering a similarity threshold of 89%, which allowed the grouping of yeast isolates into 25 clades. The resulting discrimination power, calculated using the Simpson's index, was 0.92. The clades with the highest number of strains (from 5 to 13) included those preliminarily attributed to *K. humilis*. Considering a similarity threshold of 40%, all the tentative *K. humilis* strains (42 in total) were grouped closely in a major clade clearly distinct from a minor clade that included the remaining 15 strains preliminarily attributed to *S. cerevisiae.*

A total of 43 high-quality sequences were used to infer the phylogeny of yeast isolates. Reference sequences belong to *Saccharomyces* and *Kazachstania* genera, detected after alignment in the YeastIP database. After all-vs.-all sequence alignment, sequences were trimmed all at the same length, resulting in a total length of 599 bp. The neighbor-joining phylogenetic tree was reported in Figure 3. The bootstrap values higher than 50%, used to define the accuracy of branch prediction, were reported.

According to sequence clusterization, the phylogenetic tree resulted in three well-defined clades, distinguishing with high accuracy the detected species, as reported by bootstrap values. The total average of dissimilarities among all phylotypes considered was 7.04%. The two major clades included *Saccharomyces* and *Kazachstania* species, supported by a bootstrap value of 98%. A total of four isolates, coming from sample B (LFB1, LFB10, LIB7, and LMB9), were grouped in *S. cerevisiae* clade, having a percentage of identity of 100% with *S. cerevisiae* CBS 1171^T. The remaining *Saccharomyces* species were grouped in three different clades, of which one includes *S. uvarum* CBS 395^T and the hybrid species from *S. uvarum*, *S. bayanus* CBS 380^T, and *S. pastorianus* CBS 1538^T. Another cluster of *Saccharomyces* species includes *S. kudriavzevii* CBS 8840^T and *S. arboricolus* CBS 10644^T, which share 99.2% of sequence identitt. The remaining clade of *Saccharomyces* includes *S. cariocanus* CBS 8841^T, *S. paradoxus* CBS 432^T, *S. jurei* NCYC $D5088^T$, and *S. mikatae* CBS 8839^T. Species included in this clade were phylogenetically closest, except for *S. mikatae*, sharing a dissimilarity percentage of about 1% with the other clade members.

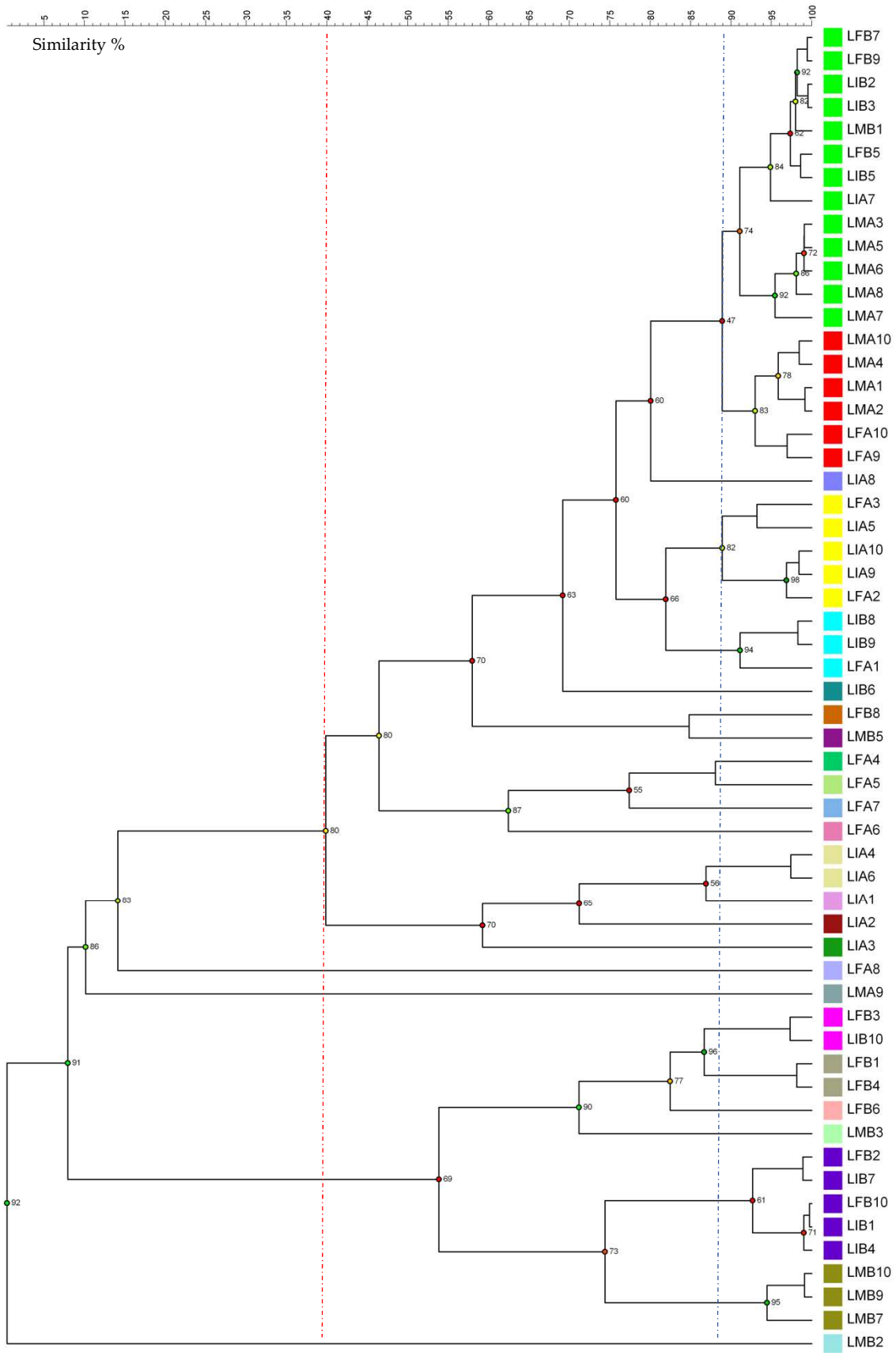


Figure 2. Dendrogram obtained from unweighted pair group method with arithmetic mean (UPGMA) analysis, using Pearson's coefficient, of the $(GTG)_5$ rep-PCR patterns. The similarity threshold for biotypes discrimination was 89% (blue line). The cophenetic coefficient is represented by numbers and dots colored as red, orange, yellow, or green, based on the branch quality. Two major clades are distinct with a threshold of 40% (red line).

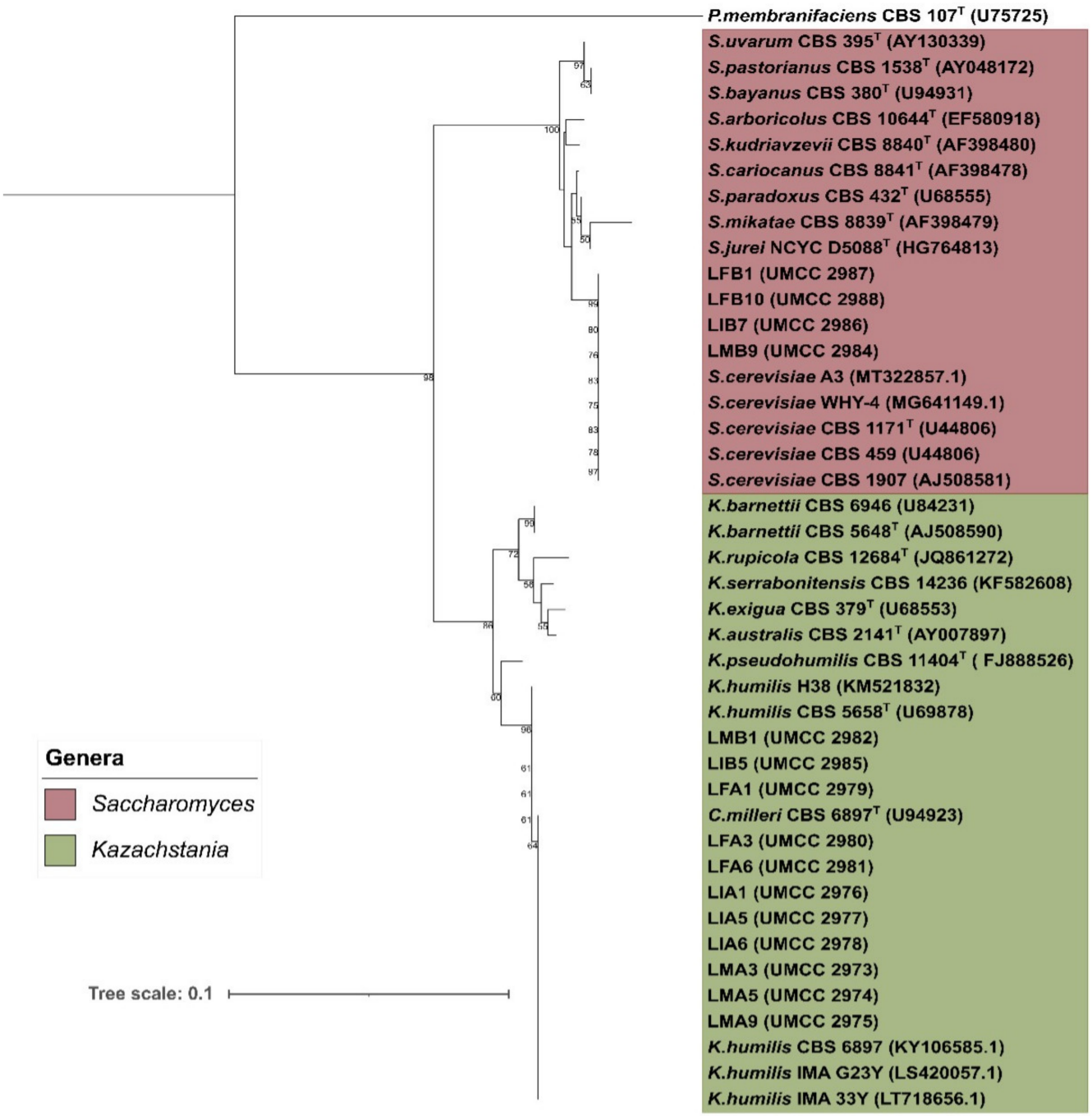


Figure 3. Neighbor-joining phylogenetic tree representing the phylogenetic distances of isolates from sourdough MA and MB and the strain types of identified yeasts species. The tree was made using Tamura-Nei model, and a discrete Gamma distribution was used to model evolutionary rate differences among sites. Node numbers indicate bootstrap values obtained using 1000 replicates.

The *Kazachstania* clade is branched out into three subclades. A clade including isolates coming from sample A and sample B was represented by *K. humilis*. Three isolates, represented by LMB1, LIB5, and LFA1, share 100% identity with *K. humilis* CBS 5658^{T} and *K. humilis* H38. The remaining strains in the *K. humilis* clade, including *C. milleri* CBS 6897 (currently a synonym of *K. humilis*), are phylogenetically quite distant from *K. humilis* CBS 5658^{T}, which shares the 99.8% similarity, indicating a low divergence as also

reported from different studies [39,40]. Given this consideration, *K. humilis* and *C. milleri* are considered conspecific [41]. The most closely related to *K. humilis* strains is *K. pseudohumilis* CBS 11404^T, with divergence supported by a bootstrap of 60%. The remaining *Kazachstania* strains included in the dataset were in a different clade, supported by a bootstrap of 86%. This clade includes *K. barnettii* CBS 5648 and *K. barnettii* CBS 6946^T, forming a single group and the stains *K. rupicola* CBS 12684^T, *K. serrabonitensis* CBS 14236^T, *K. australis* CBS 2141^T, and *K. exigua* CBS 379^T. The represented species are low in divergence since the sequence differs by 0.7% to 1.7%.

3.3. Bacteria Molecular Characterization and Phylogenetic Analysis

The analysis of the 16S-23S rDNA ISR allowed to detect *Fl. sanfranciscensis* species among the bacterial isolates (Table 4). In particular, PCR products from all the MA and MB isolates yielded similar profiles containing three bands of 600, 700, and 800 bp; these isolates were preliminarily attributed to *Fl. sanfranciscensis* [27]. All the remaining isolates showed a profile with only two bands of 600 and 800 bp. This result was consistent with previously reported data for *Lactobacillus* 16S–23S ISR organization [27,42,43].

Table 4. PCR amplification of the 16S-23S rDNA intergenic spacer region (ISR).

Sample	Strain *	16S-23S rDNA ISR Size Fragments (bp)
MA	**BMA2 (UMCC 2989)**, **BMA8 (UMCC 2990)**, **BMA10 (UMCC 2991)**	600–700–800
IMPA	**BIA2 (UMCC 2992)**, BIA3, BIA5, **BIA6 (UMCC 2993)**, **BIA7 (UMCC 2994)**, BIA8, BIA9, **BIA10 (UMCC 2995)**	600–800
FINA	**BFA1 (UMCC 2996)**, **BFA2 (UMCC 2997)**	600–800
MB	**BMB5 (UMCC 2998)**, **BMB7 (UMCC 2999)**	600–700–800
IMPB	**BIB7 (UMCC 3002)**, BIB8	600–800
FINB	BFB3, **BFB6 (UMCC 3004)**, **BFB8 (UMCC 3006)**	600–800

* The strains sequenced are shown in bold and in brackets are indicated the correspondent UMCC code.

The digitized patterns obtained from $(GTG)_5$ rep-PCR, analyzed with the UPGMA method and using Pearson's coefficient, allowed the construction of the dendrogram shown in Figure 4. The biotypes discrimination was performed considering a similarity threshold of 95%, which allowed the grouping of the strains into 19 different clades; only the strains BIA5 and BIB8 were included in the same clade. The calculated Simpson's index was 0.99, indicating a high diversity among the isolated strains including those preliminarily attributed to the species *Fl. sanfranciscensis*. This is consistent with the evidence reported by several authors that highlighted the great variability among *F. sanfranciscensis* strains [7,27].

High-quality sequences obtained from the bacteria dataset were analyzed in order to determine the phylotypes of isolates. After all-vs.-all alignment, sequences were trimmed at the same length, resulting in a total length of 904 bp. Reference sequences were downloaded from NCBI 16S rRNA database, resulting in two genera including *Leuconostoc* and *Lactobacillus*. The sequence of *Bacillus albidus* MCCC 1A02146^T was used as an outgroup.

The average phylogenetic distance among all sequences included in the dataset was 13.17%. The resulting phylogenetic tree was represented in Figure 5. A total of two major clades are depicted, represented by the clade of *Leuconostoc* genus and the clade including the genera *Furfurilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, and *Fructilactobacillus*. In particular, *Leuconostoc* clade includes five isolates and is branched in two subclades, *Leuc. mesenteroides* NBRC 100469^T and *Leuc. citreum* ATCC 49370^T.

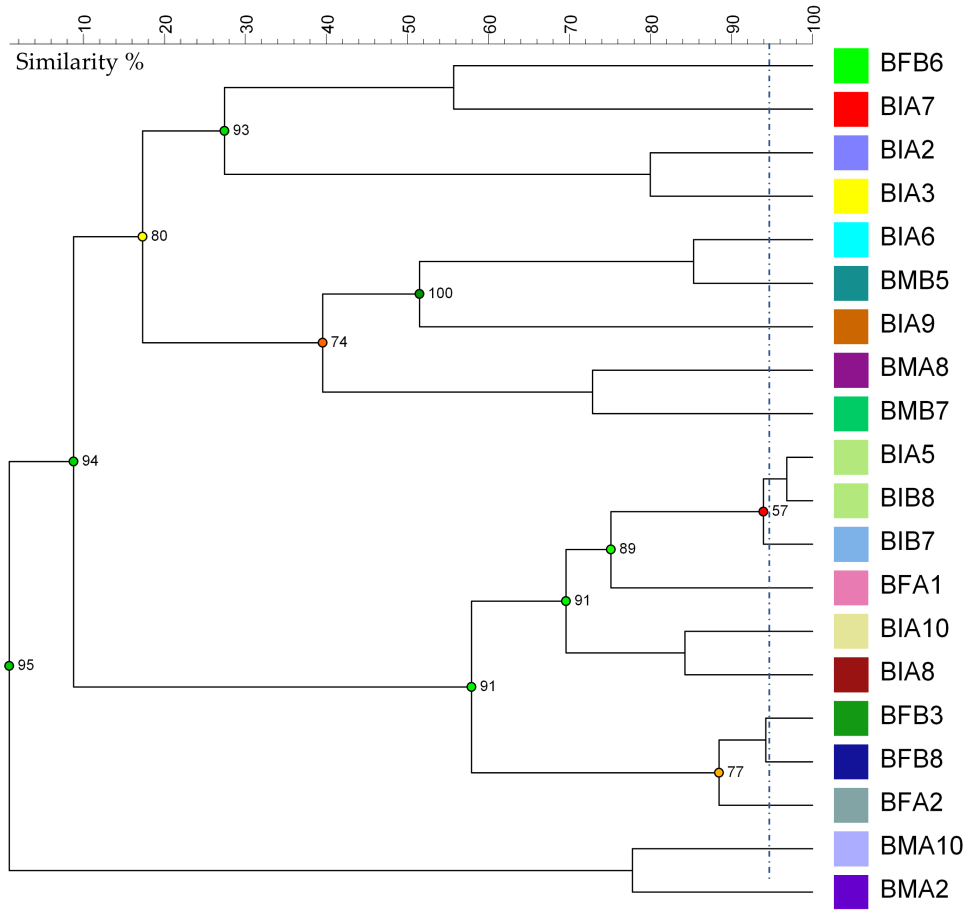


Figure 4. Dendrograms obtained from unweighted pair group method with arithmetic mean (UPGMA) analysis, using Pearson's coefficient, of $(GTG)_5$ rep-PCR. The similarity threshold for biotypes discrimination was 95% (blue line). The cophenetic coefficient is represented by numbers and dots colored as red, orange, yellow, or green, based on the branch quality.

Two strains, BIA7 and BFB6, were grouped with *Leuc. mesenteroides* NBRC 100469^T, sharing 100% similarity.

Three strains, BFA2, BFB8, and BIA6, were clustered with *Leuc. citreum* ATCC 49370^T, sharing 100% of similarity among them and 99.9% of similarity with the reference strain. The phylotype classification was supported by high bootstrap values (ranging between 77% and 100%).

The remaining isolated strains were included in the other major clade, represented by four species: *Furfurilactobacillus rossiae* (formerly *Lactobacillus rossiae*) DSM 15814^T, *Lacp. plantarum* JCM 1149^T, *Lentilactobacillus parabuchneri* (formerly *Lactobacillus parabuchneri*) LMG 11457^T, and *Fl. sanfranciscensis* JCM 5669^T. Branches in this clade, representing species differentiation, were supported by high bootstrap values, ranging between 63% and 100%.

Specifically, BIA10 and BIB7 strains were grouped with *Furl. rossiae* DSM 15814^T. The two strains shared 100% of identity and were phylogenetic divergent with the reference strain of 1.51%. The strain BFA1 was identified as *Lacp. Plantarum,* having 100% sequence similarity to *Lacp. plantarum* JCM 1149^T. The branching was supported by 100% of bootstrap value, meaning an inference with high confidence. Isolate BIA2 clustered with *Lenl. parabuchneri* LMG 11457^T, supported by a 100% bootstrap value and sharing 100% identity. The remaining strains, represented by BMB5, BMA2, BMB7, BMA8, and BMA10, were included in the *Fl. sanfranciscensis* clade (bootstrap value 100%). All references and isolates showed low divergence, since the maximum phylogenetic distances observed was 0.2% in the case of BMB10 with BMA8. Given the high sequence similarities and the high

confidence represented by the bootstrap values, it is reasonable to assume that all of the isolates belonging to *Fl. sanfranciscensis* clade could be assigned to this species. Our results are consistent with previous evidence that reported the remarkable polymorphism of the 16S rDNA within the *Fl. sanfranciscensis* species and placed this microorganism in the *Lacp. plantarum* group based on the 16S rDNA phylogenetic analysis [27].

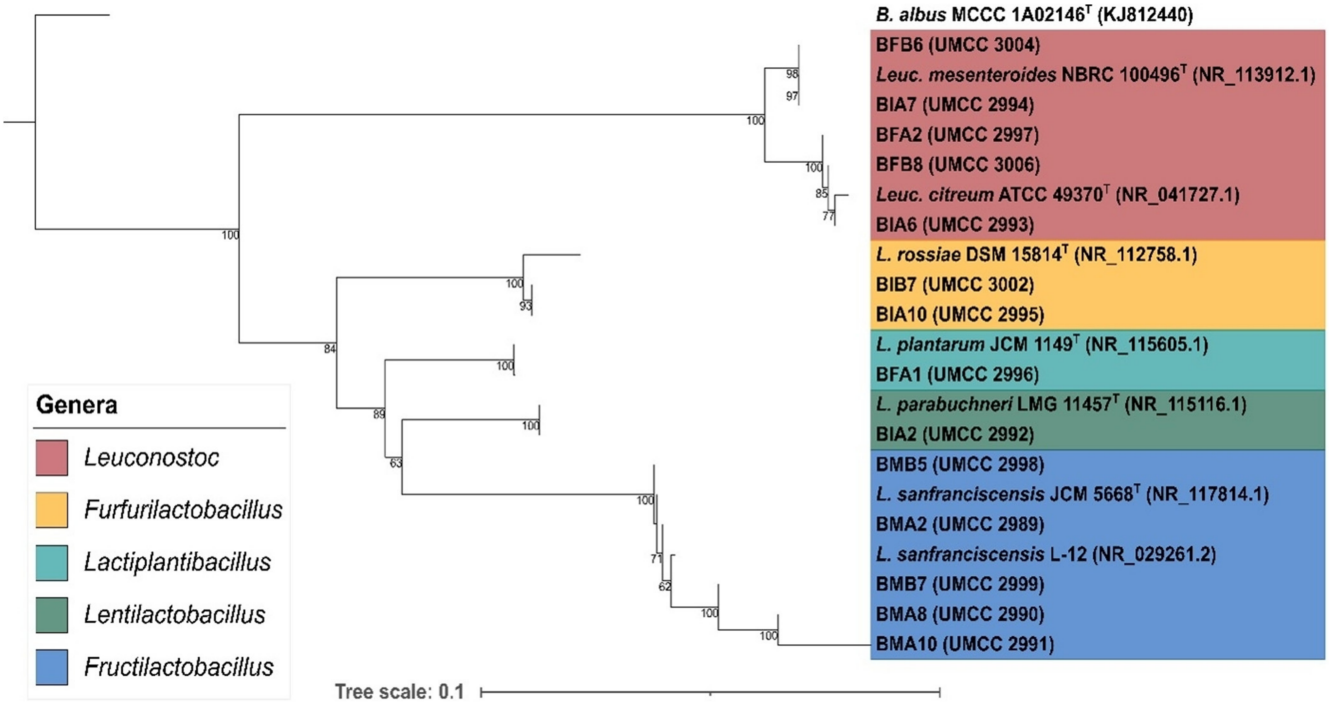


Figure 5. Neighbor-joining phylogenetic tree obtained by 16S multiple alignment. A gamma distribution was applied to model the rate variation among sites. The node numbers indicate the percentage of replicates in which taxa clustered at the same distance (bootstrap test). References species used in the dataset are reported, in the figure, using their former name and the associated NCBI id. Given the recent re-organization of the Lactobacillus genus, the different genera are highlighted with different colors and named using the new taxonomical nomenclature, according to Zheng et al. [11].

3.4. Volatile Organic Compounds Profiling

The VOCs profiling of sourdoughs and following dough samples revealed significant quantitative and qualitative differences, which can be attributed to the different microflora of each of the two systems A and B, given that the ingredients used were the same.

Sourdoughs were characterized by much higher total VOCs, as can be inferred from Figure 6. Indeed, the volatiles produced in the sourdough undergo a dramatic dilution from the addition of ingredients. It is noteworthy that the significantly higher (1.5-fold) VOCs levels, expressed as total chromatographic area, observed in MB (4.76×10^7) compared with MA (3.16×10^7), are followed by significantly higher levels also recovered in IMPB (2.26×10^7) and FINB (1.88×10^7) samples compared with IMPA (1.62×10^7) and FINA (1.56×10^7), respectively, even if the difference is slightly reduced to 1.2-fold.

The relative abundance of chemical classes of VOCs in the volatile fraction of samples is shown in Figure 7. As much as >80% of the volatile fraction of sourdoughs was made up of alcohols and esters together, while aldehydes and acids each represented about 6–7% of the total chromatographic area.

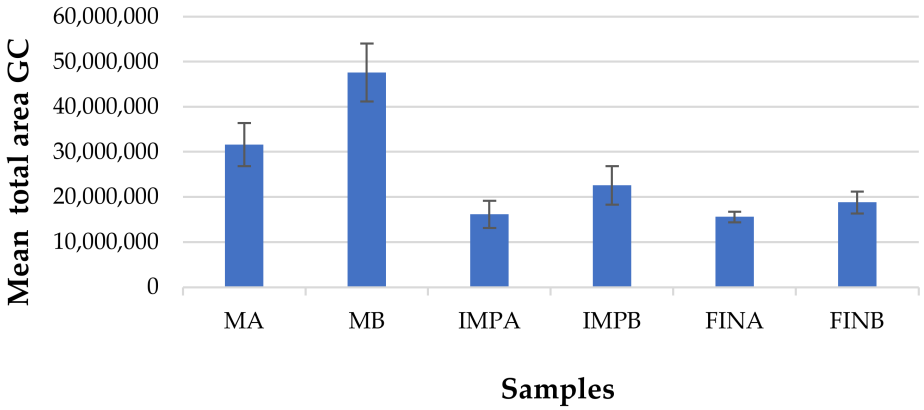


Figure 6. Mean total chromatographic area of the identified volatile organic compounds (VOCs) in sourdoughs and dough samples A and B.

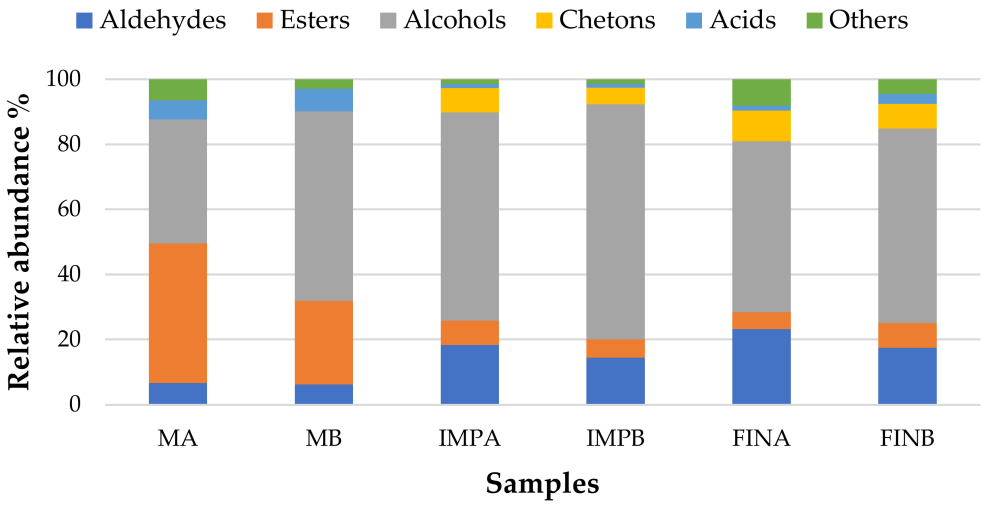


Figure 7. Relative abundance (%) of the chemical classes of volatile organic compounds (VOCs), determined in sourdough and dough samples at different steps of *Panettone* preparation.

Interestingly, the VOCs profile of MA was characterized by a balanced level of esters and alcohols, while in MB samples the alcohols prevailed; this finding agrees with the presence of *S. cerevisiae* in B samples. The relative composition of the volatile fraction changed after formulation and leavening, the most noteworthy changes being the increase in aldehydes from 6–7% to 17–23% of the total VOCs, a reduction in esters to 5–7%, and the development of ketones, represented by acetoin (3-hydroxy, 2-butanone), which was not detected in the sourdough samples.

The main aliphatic aldehydes found were acetaldehyde, characterizing the VOC profile of all samples and showing an evident increasing trend from MB to FINB samples (Table 5). To a lower extent, there were 2-butenal, only found in fermented dough samples, hexanal, the product of lipid oxidation by the lipoxygenase pathway [44], and heptenal, retrieved only in MA and MB samples; they accounted for about 4% and 0.3–0.7%, respectively. These aldehydes could not be detected in further steps, according to previous reports [16]; however, the hexanal loss was not followed by a corresponding 1-hexanol increase during maturation.

Table 5. Relative volatile organic compound (VOC) composition of sourdough and dough samples.

Chemical Compounds	Samples *					
	MA	**MB**	**ImpA**	**ImpB**	**FinA**	**FinB**
Acetaldehyde	2.22 ± 0.11	1.62 ± 0.13	8.43 ± 0.19	3.28 ± 0.01	8.31 ± 0.10	6.27 ± 0.05
2-Butenal	-	-	2.44 ± 0.16	1.56 ± 0.23	8.61 ± 0.65	3.80 ± 0.26
Hexanal	4.13 ± 0.17	3.86 ± 0.37	-	-	-	-
Heptenal	0.31 ± 0.04	0.66 ± 0.06	-	-	-	-
Benzaldehyde	-	-	0.55 ± 0.06	1.96 ± 0.12	0.86 ± 0.21	1.37 ± 0.05
Phenylacetaldehyde	-	-	6.80 ±0.47	7.62 ± 0.18	5.52 ± 0.33	6.08 ± 0.03
Aldehydes	*6.34*	*6.14*	*18.22*	*14.43*	*23.30*	*17.52*
Ethyl Acetate	39.73 ± 1.51	20.78 ± 0.43	7.17 ± 0.45	5.43 ± 0.23	4.08 ± 0.04	5.85 ± 0.15
Ethyl butanoate	-	-	-	-	0.86 ± 0.12	0.76 ± 0.13
Isoamyl acetate	2.03 ± 0.14	2.17 ± 0.23	-	-	-	0.39 ± 0.12
Ethyl hexanoate	-	0.38 ± 0.07	0.31 ± 0.00	0.20 ± 0.03	0.32 ± 0.02	0.64 ± 0.07
Hexyl acetate	-	0.26±0.01	-	-	-	-
Ethyl lactate	1.44 ± 0.04	2.14 ± 0.01	-	-	-	-
Esters	*43.19*	*25.74*	*7.48*	*5.64*	*5.26*	*7.26*
Ethyl alcohol	31.19 ± 0.22	44.05 ± 0.00	50.77 ± 0.66	53.19 ± 2.50	41.09 ± 2.65	40.51 ± 1.46
Isobutanol	-	-	1.40 ± 0.17	1.75 ± 0.11	-	2.51 ± 0.05
Isoamyl alcohol	4.30 ± 0.25	6.80 ± 0.32	6.42 ± 0.56	8.92 ± 1.06	6.70 ± 1.32	7.77 ± 0.25
1-Hexanol	1.96 ± 0.15	3.48 ± 0.42	1.60 ± 0.23	0.95 ± 0.12	1.12 ± 0.01	1.25 ± 0.06
2-Hexanol	-	-	0.64 ± 0.11	-	-	-
1-Octen-3-ol	0.30 ± 0.03	0.69 ± 0.03	-	-	-	-
Phenethyl alcohol	0.51 ± 0.01	3.27 ± 0.45	2.70 ± 0.38	7.44 ± 1.09	3.90 ± 0.62	8.22 ± 1.96
Alcohols	*38.27*	*58.30*	*63.52*	*72.26*	*52.82*	*60.25*
Acetic acid	5.34 ± 1.44	5.93 ± 0.09	1.23 ± 0.05	1.19 ± 0.09	1.11 ± 0.37	2.76 ± 0.35
Hexanoic acid	0.43 ± 0.07	0.94 ± 0.04	-	-	0.45 ± 0.12	0.31 ± 0.01
Acids	*5.76*	*6.87*	*1.23*	*1.19*	*1.11*	*2.76*
Limonene	2.49 ± 0.07	0.74 ± 0.01	1.08 ± 0.24	0.75 ± 0.11	0.96 ± 0.20	0.67 ± 0.06
Acetoin	-	-	7.42 ± 0.37	5.05 ± 0.33	9.39 ± 0.26	7.64 ±0.91
3,3-dimethyl hexane	-	-	-	-	2.48 ± 0.36	1.47 ± 0.19
1-butyl-2-propyl cyclopentane	-	-	-	-	3.24 ± 0.67	1.00 ± 0.40
2-Pentylfuran	0.92 ± 0.00	0.72 ± 0.02	-	-	-	-
Benzene,1,3-bis(1,1-dimethylethyl)	3.02 ± 0.28	1.03 ± 0.14	0.38 ± 0.06	0.69 ± 0.12	1.44 ± 0.02	1.44 ± 0.04
Others	6.43	2.49	8.89	6.49	17.51	12.21

* Data are expressed as % GC area ± standard deviation. The chemical classes of VOCs and their corresponding amounts are indicated in italics.

Among aromatic aldehydes, phenylacetaldehyde accounted for about 5.5–7.6% of total VOCs in dough samples, while benzaldehyde accounted for 0.5–2%. The B samples always showed higher relative abundances of these compounds, while none of them were detectable in sourdough samples, suggesting the contribution of dough ingredients in their formation.

Among esters, ethyl acetate represented about 40% and 21% of the total VOCs of sourdoughs MA and MB, respectively, followed by isoamyl acetate (about 2%), ethyl lactate (1.5–2%), and ethyl hexanoate, while their concentration was dramatically reduced in the leavened products. This finding agrees with previous data on the natural fermentation of bakery products [16], which report ethyl acetate relative abundances ranging from 16 to 27% in sourdough, falling to about 3% in leavened products.

Ethyl butanoate, which was not detected in sourdoughs, was determined in both leavened products, at about 0.8-0.9% of total VOCs.

VOCs belonging to the alcohols group were dominant, with ethanol being the most concentrated, followed by isoamyl alcohol and phenethyl alcohol. Isoamyl alcohol, ranging from 6.4 to 8.9% of total VOCs in the dough samples, was also present at comparable levels in the sourdough samples and always at higher relative abundance in B samples: this alcohol is considered the most impacting aroma component produced by yeasts, together with isobutanol [45]. Phenethyl alcohol represented only 0.5% of VOCs in MA, while it was

more concentrated in MB (3.3%); the relative abundance of this alcohol increased with the production steps, representing 3.9 and 8.2% of total VOCs in FINA and FINB, respectively. As for ethanol, phenethyl alcohol was always more concentrated in B samples. Isobutanol was not detected in sourdough samples but represented 2.5% of total VOCs in FINB, while it was not retrieved in FINA.

Interestingly, MA and MB samples did not show ketones among VOCs, while IMPA and IMPB, as well as FINA and FINB, revealed the presence of acetoin (3-hydroxy-2-butanone) up to about 8% of total VOCs: this compound can be attributed to both fermentation by *S. cerevisiae* and homofermentative LAB [46] and to the contribution of butter in the dough formulation. According to Montanari et al. [16], acetoin was not retrieved in sourdough, but could only be detected in the leavened dough at higher levels in dough samples A.

3.5. Correlation of VOCs and Species Detected in Sourdough and Dough Samples

To understand the correlation among the detected VOCs and bacteria or yeasts isolated a Pearson's correlation index was calculated. The correlation plot is reported in Figure 8. Considering bacteria isolates, the major contributor to the aroma composition was attributed to *Fl. sanfranciscensis*, which was shown to be positively correlated ($R^2 \geq 0.6$; $p < 0.05$) with a wide range of VOCs belonging to four classes, such as aldehydes, esters, alcohols, and acids, as well as with a minor compound as limonene. Two other species, specifically *Leuc. citreum* and *Lacp. plantarum*, contributed in a moderate way to the aroma production, resulting in each of them being positively correlated to four different compounds. In particular, *Leuc. citreum* was correlated to acetaldehyde, ethyl butanoate, ethyl hexanoate, and acetoin ($R^2 \geq 0.6$; $p < 0.05$). In the case of *Lacp. plantarum*, the major contribution was related to 3,3-dimethyl hexane, ethyl butanoate, 2-butenal, and 1-butyl-2-propyl cyclopentane. Interestingly, *Furl. rossiae* was shown to have a good correlation to ethyl alcohol production ($R^2 = 0.83$; $p < 0.05$). The other two species detected, *Lenl. parabuchneri* and *Leuc. mesenteroides*, were significantly correlated with just one compound, precisely 2-hexanol ($R^2 = 0.87$; $p < 0.05$) and isobutanol ($R^2 = 0.71$; $p < 0.05$), respectively. Regarding the yeasts detected, *K. humilis* and *S. cerevisiae*, their contribution in terms of VOCs was shown to be, generally, very low. In particular, *S. cerevisiae* was found to be positively correlated with isoamyl alcohol, ethyl hexanoate, phenethyl alcohol. No relevant correlation was highlighted for *K. humilis*.

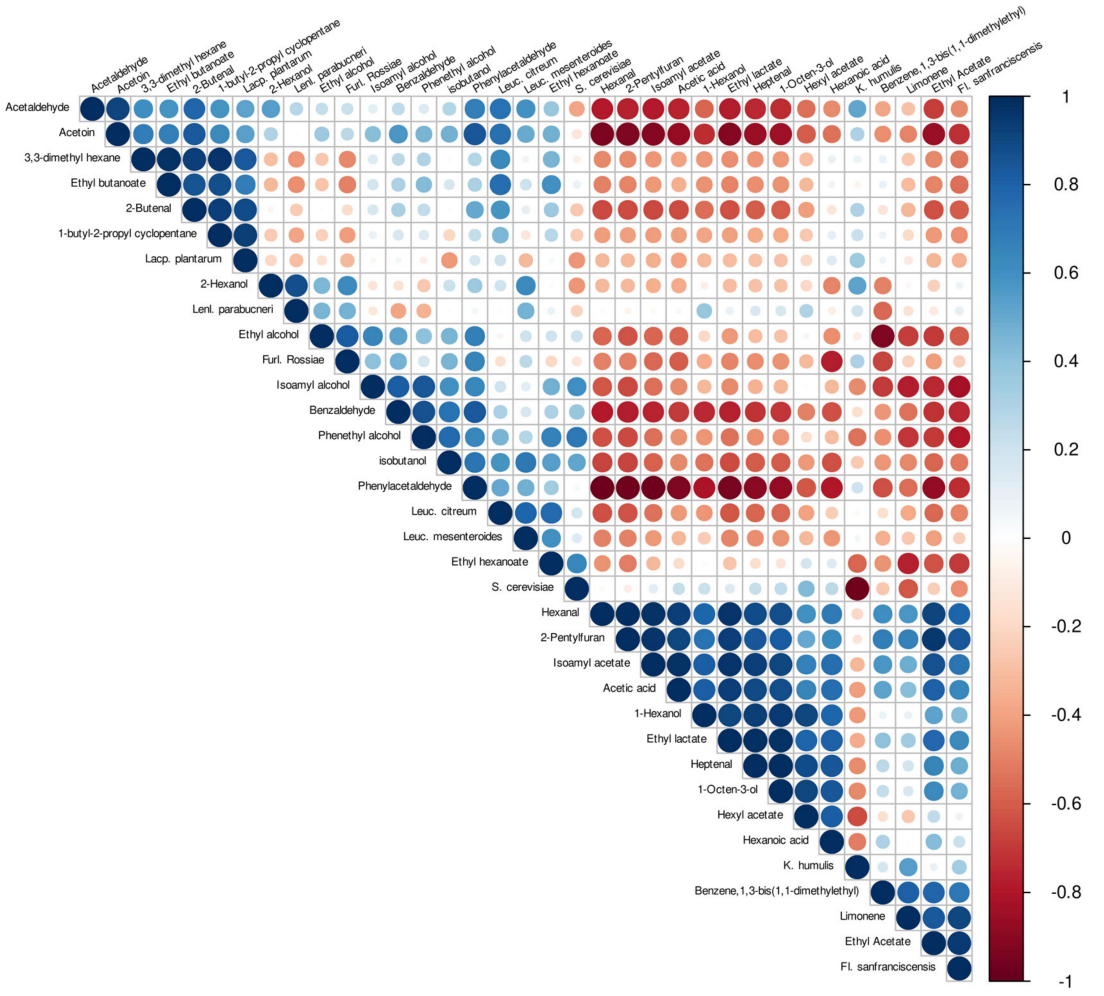


Figure 8. Correlation plot among discovered VOCs and isolated species, obtained using R package Corrplot and Pearson's correlation index. The positive and negative correlation were considered based on $R^2 \geq 0.6$ and $R^2 \leq -0.6$, respectively.

4. Discussion

In the present work, the viable microbial population of different dough samples collected during the production process of Panettone was assessed.

The sourdough MA showed a strong dominance of *K. humilis* species, with only one sample identified as a group-species *K. humilis/K. parahumilis.* This homogeneous result was already observed in traditional sourdoughs [8,10,13,47]. In sourdough MB, four sequenced strains belonged to the *S. cerevisiae* species, while the other strains belonged to the *K. humilis* species. The interactions of these two yeast species were also usually found in several traditional sourdoughs [8,48,49]. In our case, the minor number of refreshments of MB sample and the reduced fermentation step probably could have favored the dominance of *S. cerevisiae*, thanks to its rapid metabolism and growth rate.

Concerning LAB, the results showed the prevalence of heterofermentative-obliged bacteria, with the exception of *Lacp. plantarum*, which is a facultative heterofermentative bacterium, normally colonizing sourdoughs characterized by continuous back-slopping and low incubation temperature [3]. *Fl. sanfranciscensis* was found in both sourdoughs with a major occurrence in the A samples. Strains of this species are commonly reported as the main components of sourdoughs due to their ability to adapt to the highly selective conditions of this environment [4,6,18,49]. Moreover, *Fl. sanfranciscensis* strains utilize preferentially maltose and are generally unable to ferment fructose; therefore, there is a non-competitive association of this species with other different LABs. For instance, a stable association is reported between *Fl. sanfranciscensis* and *Lacp. Plantarum*, as the latter species

preferentially ferments glucose and fructose rather than maltose, whose metabolism is subject to carbon catabolite repression [3,50,51]. Regarding maltose utilization, another stable association is described between *Fl. sanfranciscensis* and maltose-negative yeasts such as *K. humilis*. On the other hand, the interactions of *Fl. sanfranciscensis* and *S. cerevisiae* are reported to be strain-specific and evidently competitive [52].

Regarding *Furl. Rossiae*, its presence in both sourdough samples is quite interesting. This species was described by Corsetti et al. [53] and subsequently studied for its fermentative properties by Di Cagno et al. [54]. *Furl. Rossiae* was found in both samples A and B, mostly in association with other LAB species, as shown in IMPA. The species *Leuc. Citreum* and *Leuc. Mesenteroides*, which were also found in both dough types, are typical of the last stages of the Panettone process [18]. Their presence, along with *Lacp. Plantarum*, *Furl. Rossiae*, and *Fl. Sanfranciscensis*, could be explained by the low temperature of the process [3]. The occurrence of *Lacp. plantarum* in sourdough is also widely described as a both sub-dominant and dominant species [18,55]. Less common but explainable is the presence of *Lenl. parabuchneri*, a species commonly found in beer and dairy products [55].

Concerning the VOCs detected and their correlation to the yeasts and LAB species found in the samples, the contribution of the microbial activity to the production of the different metabolites was evident.

Noteworthy are the VOCs belonging to the alcohols group. Ethanol, isoamyl alcohol, and phenethyl alcohol were much higher in MB compared to MA and, even though their amount in IMP and FIN samples decreased because of a dilution effect (due to addition of ingredients), they kept higher concentrations in the different stages of method B, with a final difference by around 37% between the two methods. This result may be ascribed to the presence of *S. cerevisiae* in the B samples. Hansen and Schieberle [56] reported that the content of some volatile compounds in baked leavened products such as bread is related to the concentration in the corresponding sourdough, where they are usually more concentrated, since dough recipes imply the use of a fraction of sourdough added to other ingredients. Ethanol represented about 31% and 44% in MA and MB, respectively, thus confirming itself as the principal alcohol in the volatile fraction. Similarly, Montanari et al. [16] reported a relative composition ranging from 37 to 42% in the volatile fraction of sourdough samples, which were of the same origin but obtained with different maturation conditions.

In the present study, the different origins and compositions of the two sourdoughs gave rise to higher differences and to a wider range of variability. In particular, the comparison between the two methods for sourdough preparation highlighted higher total esters content in MA samples; however, isoamyl acetate and ethyl lactate were significantly higher in MB samples (Table 5). Ethyl acetate was detected, though to a lower extent, in the leavened products, decreasing from IMP to FIN samples, while ethyl lactate and isoamyl acetate were not detected; this trend also agreed with the literature [16]. The decrease in ethyl acetate through the production steps was limited in method B, which revealed the occurrence of *S. cerevisiae*.

According to De Luca et al. [57], alcohols, acids, and aldehydes represent the most characteristic VOC families influencing the fruity, green, floral, sweet, alcoholic, and fatty acid odors in bread. The LAB species detected in our samples were differently correlated to the specific VOCs, showing a complementary behavior in some cases. This supports the use of a defined combination of selected strains to achieve the desired aroma production.

5. Conclusions

In the present work, the molecular characterization of the dough's population revealed the presence of the dominant yeasts, *K. humilis* and *S. cerevisiae*, as well as the LAB species, namely *Fl. sanfranciscensis*, *Lacp. plantarum*, *Furl. rossiae*, *Lenl. parabuchneri*, *Leuc. mesenteroides*, and *Leuc. citreum*.

Results highlight the relevance of refreshment procedures as the main factor in maintaining a stable and active microflora in industrial conditions.

Moreover, the correlation among the selected strains and the VOCs produced in the different samples allowed us to estimate the species contribution to aroma formation.

Therefore, the research of sourdoughs' selected strains, with known metabolic properties and high technological performances, is fundamental for their exploitation as starter cultures capable of controlling the manufacturing of sourdoughs. A pool of LAB and yeasts of this study deposited at the UMCC culture collection comprises candidate strains for the development of single and multiple selected starters for Panettone production.

Author Contributions: Conceptualization and original draft preparation: L.D.V.; investigation and visualization: L.D.V., G.I., S.L.C., F.L.; writing—review and editing: L.D.V., S.L.C., M.G., F.L., A.P.; supervision, project administration and funding acquisition: A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Panificio Fantuzzi s.r.l. (Reggio Emilia, Italy) for providing us the two sourdoughs samples in the different stages of the process. A special thanks to Katia D'Ambra and Andrea Feroce for their contribution in laboratory experiments. The JRU MIRRI-IT (http://www.mirri-it.it/) and IS_MIRRI21 (https://ismirri21.mirri.org/) are greatly acknowledged for scientific support.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Chapter III

This chapter is an introduction to the following papers **"Anti-Spoilage Activity and Exopolysaccharides Production by Selected Lactic Acid Bacteria"** published in *Foods* on 27 June 2022; **" Valorization of Bread Waste and Cheese Whey through cultivation of Lactic Acid Bacteria for Bio-preservation of Bakery Products"** published in *LWT* on 29 January 2023.

3.1 Lactic Acid Bacteria and their expanding application in the Food industries

In the last years, due to its crucial role in the food industries, LAB metabolism has collected an increasing interest from the scientific and industrial community [1-2]. In fact, as a fermentative species, LAB hold different metabolic features comprising the ability to generate organic acid and aromatic compounds, hydrolyse protein, produce exopolysaccharides, and anti-spoilage/inhibitory metabolites active against food-borne and spoilage pathogens both at bacterial and fungal level [1-5]. Owning to the aforementioned metabolic characteristics LAB represent a key instrument in different food sector [1-10].

According to the Food and Agriculture Organization (FAO), due to the predicted 35% increase in food consumption by 2030, food industries will be hardly challenged in terms of food quality, safety, and healthiness [4]. In particular, the ongoing outbreaks of foodborne illness in recent years, and the rising customer demand for organic and preservative-free foods, more susceptible to spoilage agents and short-term shelf life, create imperative action to find new strategies to preserve consumers' health and decrease contamination [9-10]. According to the Council for Agricultural Science and Technology, between 6.5 and 33 million occurrences of human illnesses are related to food, and *Salmonella enterica*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Staphylococcus aureus* are the five most prevalent foodborne pathogens in the European Union [3]. The World Health Organization (WHO) predicts that by 2050 if innovative solutions are not employed,

there would be an estimated 10 million more deaths worldwide due to foodborne infections [3]. In this contest, an efficient natural tactic for the eradication, prevention, and decrease in pathogenic/spoilage microorganisms in foods through the application of innovative antimicrobial agents should be employed. Various studies have demonstrated that several LAB-released metabolites, including organic acids, alcohols, phenolics, EPS, bacteriocins, and bioactive peptides, have anti-spoilage and antioxidant properties [8]. Furthermore, in a period distinguished by land degradation, climate change, and a growing population, guaranteeing high-yield production with restricted resources is crucial [11-12]. In this regard, bread and bakery products account for up to 20% of all daily food waste produced, and only a small amount gets recycled, primarily for use as animal feed [13-15]. LAB fermentation can valorise food wastes through the release of bio-actives compounds. Leftover bread and other bio-waste, such as cheese whey, can be employed as a source of selection and adaptation for LAB starter culture or used as amendments for plants, after LAB fermentation, reintroducing the waste in a circular economy, developing a more sustainable production [11]. Furthermore, their peculiar metabolic activity not only can be employed to ensure the safety and the improvement of bakery and gluten-free (GF) bakery products but also in innovative applications such as the creation of bio-edible coating [7, 16]. Bioactive packaging systems (coating/films) are a new technology to support the production of functional foods, in which bioactive compounds or LAB are contained within coatings or coating materials in order to have a direct impact on consumer health, creating healthy foods packed for a specific bioactive attribute [7,10]. The employment of LAB in the food sector and industrial biotechnology offers them an exceptional position in the perspective of the green economy and clean-label products [2-9, 16] .

3.2 LAB Antimicrobial compounds

The combined effect of a variety of antimicrobial metabolites generated throughout the fermentation process is thought to be the source of LAB and LAB starter culture's preservation activity in various food products. These include several organic acids, such as lactic, acetic, propionic, and succinic acids generated as end products [1-5]. The action of the antimicrobial metabolites produced by LAB during their metabolic activity is based on the disruption of the membrane, inhibition of the synthesis of cell-wall enzymes, decrease of intracellular pH, interference with proton gradients, and induction of reactive oxygen species, which increases oxidative stress within the cell [17]. Several authors have shown that LAB has a suppressive impact on the growth of foodborne pathogens such *Salmonella* spp., *L. monocytogenes*, and *E. coli* [18-20]. According to the vast majority of scientific publications, the key tactic applied to prevent spoilage/pathogenic microflora is to build a hostile environment for their growth, mainly due to lowering pH levels caused by the significant amounts of organic acid produced during LAB fermentation [21]. Organic acids are capable of a broad inhibition activity, both Gram-positive and Gram-negative bacteria, yeast, and moulds can be affected [3-4]. The ability of organic acids to kill microorganisms has been linked to their fragmented molecules, which deprotonate when they enter biological membranes. Additionally, organic acids are recognized as safe for human usage [4]. LAB cultures can produce various antimicrobial metabolites in addition to acids, including ethanol from the heterofermentative route, H_2O_2 from aerobic growth, and diacetyl from extra pyruvate from citrate [8]. Specifically, the flavoproteins oxidoreductases NADH peroxidase, NADH oxidase, and α-glycerophosphate oxidase may create H_2O_2, which exerts a potent oxidizing impact on cellular proteins and membrane lipids [4]. Hydrogen peroxide is instantly removed intracellularly in aerobic and facultative aerobic bacteria by the enzyme catalase. However, in species lacking the primary enzymes that scavenge hydrogen peroxide

or if their activity is inadequate, hydrogen peroxide can accumulate [22]. In 1936, the chemical diacetyl generally well recognized for giving its distinctive buttery scent, was also identified as an antimicrobial compound particularly active against Gram-negative bacteria [4]. Furthermore, other metabolites generated by LAB which have antagonistic activity include peptidic and proteinaceous bacteriocins and other small molecules such as 2,3-butanediol, acetoin, acetaldehyde, esters, amines and gamma-aminobutyric acid (GABA) [2, 23]. Antimicrobial peptides such as bacteriocins are produced by a variety of bacteria, including LAB [8]. Although there are examples of bacteriocins with a wider spectrum of activity, they are often active against bacteria closely related to the generating strain [9]. Generally, they are defined as GRAS or QPS, odourless and colourless, displaying no effect on the organoleptic and sensorial characteristics of food, and unlike traditional antibiotics, bacteriocins are cleared by the digestive system by proteolytic enzymes, making LAB-produced bacteriocins ideal for use in food [4].

3.3 LAB Antifungal compounds

LAB also display an inhibiting activity against moulds, responsible for food spoilage and mycotoxin production [21]. In fact, LAB bacteriocin-like substances and organic acids may exhibit fungistatic and/or fungicidal properties inhibiting both fungi and yeast [4]. Diacetyl, hydrogen peroxide, acetate, propionate, caproate, 3-hydroxy fatty acids, phenyl lactate, cyclic dipeptides, reuterin, phenyllactic acid (PLA), cyclic peptide, and fungicidal peptides are only a few of the antifungal compounds produced by LAB isolated from sourdough [24-25]. According to several investigations, the antifungal properties of sourdough LAB significantly extend the shelf life of bread and bakery product avoiding the development of moulds [25-28]. According to Axel et al. [29], the synergistic interaction of various antifungal metabolites produced by LAB with the pH drop caused by the formation of organic acids

results in a final antifungal activity that is more effective. Moreover, LAB's antifungal metabolites, in particular organic acids, have also demonstrated synergy with yeasts' proteinaceous compounds. Organic acids, in particular lactic and acetic acid, are assumed to ascertain LAB's mycotoxigenic inhibition properties [30]. While acetic acid presents a stronger antifungal activity due to its higher pKa, which results in a larger amount of dissociation within the cell, lactic acid was found to be the major metabolic antifungal compound linked with the decreasing pH and efficacy against different moulds species such as *Aspergillus, Fusarium*, and *Penicillium* [30-32]. Organic acids' effect is due to their undissociated form, able to diffuse in the moulds membrane and consequently degrade the microbial cell through the discharging of hydrogen ions and pH reduction [33]. Moreover, organic acid production is responsible for the triggering of other antifungal compounds prompted by the acid pH [33]. Another, crucial synergic activity is represented by the acetic acid and PLA combination. PLA results from the degradation of phenylalanine shares a metabolic route with lactic acid and exhibits particular activity against *Penicillium, Fusarium, Aspergillus*, and *Eurotium* species [34-35]. In addition to these compounds, also fatty acids and cyclic peptides are reported to be powerful antifungal metabolites contained in LAB-CFS [36]. Smaller peptides with a low molecular weight (10 KDa), generally show antifungal properties, disrupting structural integrity. Specifically, peptides disrupt the function of lipid bilayers by adhering to them in a carpet-like manner and puncturing their channels [36-37]. In different studies, fatty acids have been stated as the main antifungal compounds acting as preservatives in fermented food and are known to exhibit inhibition in the range of 10 to >100 µg/mL [38-40]. DL-hydroxyphenyl, 3, 3-(4-hydroxyphenyl) propionic, 4-dihydroxyhydrocinnamic, and 3-(4-hydroxy-3-methoxyphenyl) propanoic acids are only some of the active fatty acid produced by LAB. Antifungal fatty acids break down membrane lipid bilayers, resulting in the breakdown of membrane integrity, which causes

cells to disintegrate and release intracellular proteins and electrolytes [39, 41]. Additional targets of fatty acids include topoisomerase activity, and protein synthesis, which may be hindered by, among other things, acetylenic fatty acids, fatty acid metabolism, and myristic acid analogues [41]. Though, the structure has a considerable impact on the fatty acid's antifungal efficacy. In fact, unsaturated monohydroxy fatty acids resulted to be antifungally active, whereas saturated hydroxy fatty acids and unsaturated fatty acids don’t display any activity, suggesting that minimum one double bond and one hydroxyl group along a C18 aliphatic chain need be present in the structure of the molecules to display an anti-moulds activity [40-41].

3.4 LAB exopolysaccharides

Exopolysaccharides (EPS) are exogenous microbial metabolites generally produced and accumulated after the development of bacteria, yeast and microalgae [42]. Bacteria commonly possess the ability to generate polysaccharides with a variety of biological activities and their production in LAB has long been associated with the technical, functional, and health-promoting advantages of these microorganisms [43]. In particular, traditional fermented foods, such as sourdough, can be regarded as a valuable source of homopolysaccharides (HoPS)-producing LAB along with different polymers [44]. EPS can be created as tightly bound capsular forms on the cell surface or released into the extracellular space as slime material. According to their chemical arrangement and biosynthetic method, EPS generated by LAB are classified as HoPS and heteropolysaccharides (HePS). HoPS are formed of only one type of sugar and produced in the extracellular medium, while HePS are produced intracellularly from two or more types of sugar and subsequently transferred from the cell [45]. According to different studies the *in situ* production of polysaccharides by LAB has been strongly linked to the various technological, functional, and health-promoting

properties exhibited by these bacteria [46-48]. Specifically, the use of EPS-LAB producer's strains can drastically improve the sensory and quality of GF bakery products, generally characterized by poor nutritional aspects and tasteless [49-50]. Coeliac disease is a chronic autoimmune enteropathy triggered by gluten ingestion with a strict GF diet as the only medical therapy [46,50]. Though, available GF bakery products lack in terms of shelf-life, balanced nutritional value, taste, loaf volume, crumb structure, and storage stability. Due to these defects, alongside the new consumer's requirements, increase attention has been focused on sourdough LAB starter culture and their ability to replace high-priced hydrocolloids and additives through EPS *in situ* production [46]. LAB are known to be produced different cell surface-associated polysaccharides including peptidoglycan (PG), teichoic acids (TA) wall polysaccharides, lipoteichoic acids (LTA), pellicles, EPS and capsular polysaccharides (CPS) [48]. EPS and CPS in particular have been linked to the increase of viscosity, syneresis, stability, and sensory aspects of LAB fermented products, improving their rheological characteristics. They are both exocellular polysaccharides and only differ in how firmly they adhere to the cell surface. EPS are secreted into the extracellular matrix or loosely coupled with the cell surface via electrostatic interactions, frequently producing a slime layer, whereas CPS are strongly bonded, frequently covalently, to the cell surface and form a capsule surrounding the cell [48]. However, the location of the EPS or CPS, their structural characteristics including monosaccharide type and configuration, glycosidic linkage, non-sugar decorations, charge, conformation and molecular weight, quantity, and interactions with other system components are all elements influencing the actual impact on the techno-functional properties of the products [48]. Regardless of the considerable structural diversity, bacteria assemble via four different pathways (Figure 1): the Wzy-dependent pathway, the ATP-binding ABC transporter pathway, the synthase-dependent pathway and extracellular synthesis using a single glycosyltransferase (sucrase) enzyme [43,

45, 48].

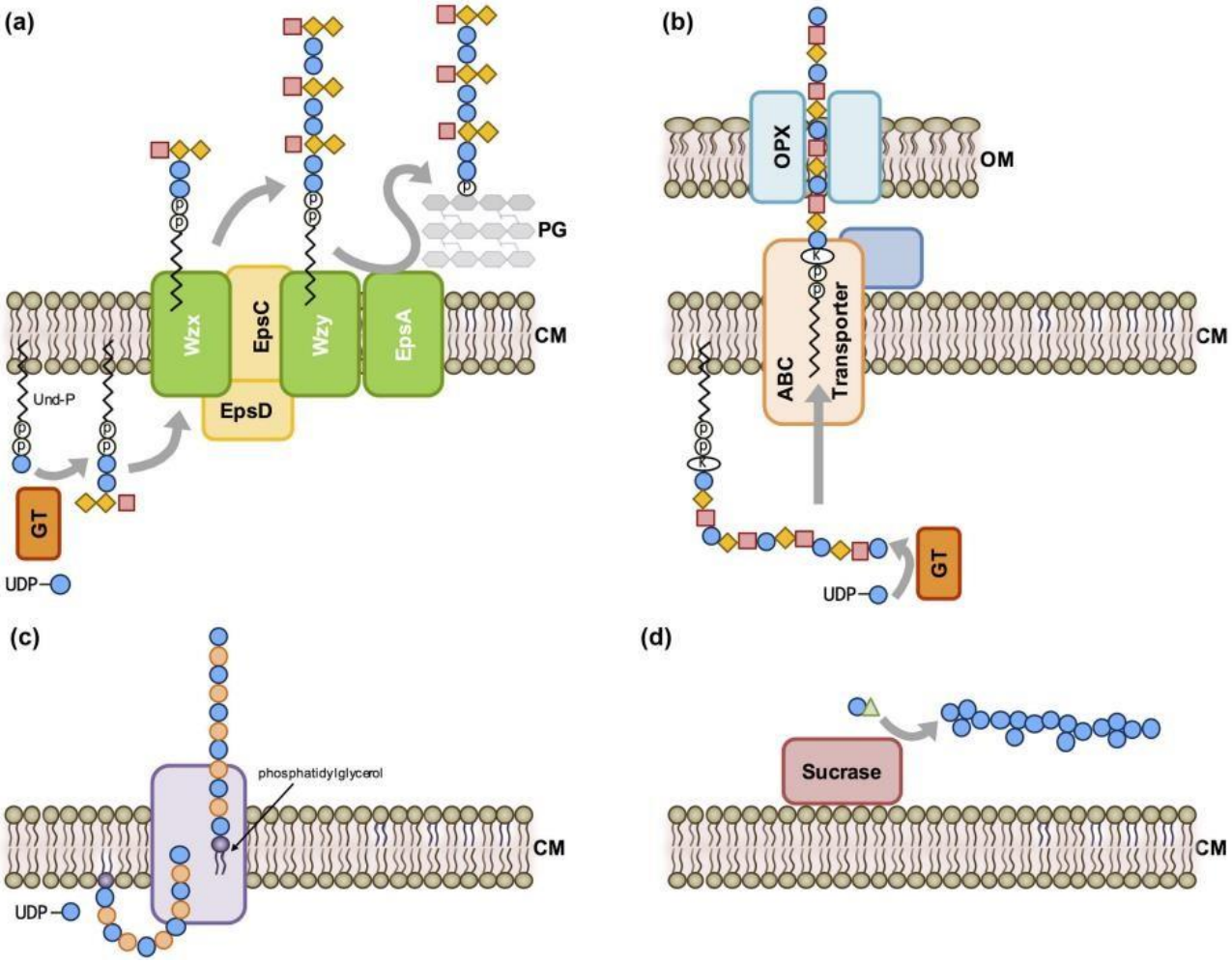


Figure 1. Pathways for the production of bacterial polysaccharides. a: representation of The Wzy-dependent pathway for the synthesis of CPS and EPS in Gram-positive bacteria. Primary players are the Wzy polymerase and Wzx flippase; b: ABC transporter-dependent, for the CPS in Gram-negative bacteria; c: synthase-dependent pathway for CPS and EPS; d: extracellular synthesis of HoPS by the use of a single GT (sucrase) protein.

Representation of: glucose as blue circle; galactose as yellow diamond; rhamnose as pink square; fructose as green triangle. CM: cytoplasmic membrane, OM: outer membrane; PG: peptidoglycan; GT: glycosyltransferase; Und-P: undecaprenyl phosphate; P: phosphate; k, poly-2-keto-3-deoxyoctulosonic acid linker. Image from [48].

3.4.1 EPS food-related function

Antibacterial activity

Numerous tests of EPS bioactivities have revealed a general inhibiting or resisting effect to pathogens [51]. Majority of these tests show the ability of these biomolecules to exhibit antimicrobial properties to either Gram-positive and Gram-negative pathogens in vitro plates, or pathogenic bacteria in the gastrointestinal tract. As stated by various studies LAB's EPS are able to display bacteriostatic and/or bactericidal activity against *Salmonella enterica, S. enteritidis, Micrococcus luteus*, and *Listeria monocytogenes* [52-53].

Hydrocolloids

LAB's EPS, in particular HoPS, are well-known to function as hydrocolloids, and their use is extremely valuable in sourdough [43, 54-55]. In fact, their ability to improve bakery products and GF bakery products is becoming crucially significant from the perspective of clean-label food [43, 50]. Hydrocolloids are commonly used to alter starch gelatinization, replace fat sources and add fiber to GF products, replacing external chemical additives [50]. Moreover, other technical aspects such as bread staling, dough rheology and machinability, dough stability during frozen storage, and loaf volume can be positively altered by EPS from LAB [55]. In the bakery context, both *ex situ* manufacture and the addition of EPS as well as *in situ* production through the use of selected starter cultures during fermentation are considered valuable techniques to improve the final products [44, 57-58]. For instance, dextran from *Leuc. mesenteroides* provide the long-term storage stability of panettone, and enhance the general shelf-life and volume of bread and milk bread [59]. However, production of HoPS by heterofermentative LAB might be associated with a boosted production of acetate causing a lower final volume and acidic taste, while HePS, on the contrary, do not affect acetate level and enhance the diversity in the starter culture [42].

3.4.2 Homopolysaccharides

Lactobacillus, Pediococcus, Weisella, Streptococcus, Oenococcus, and *Leuconostoc* are the genera producing HoPS [45]. HoPS are typically produced by an extracellular glycosyltransferase (GT) in the extracellular matrix and sucrose is often used as the donor of the equivalent monosaccharide by glucansucrases and fructansucrases, which then transport this residue to the reducing end of the developing HoPS [60].

They are constituted of D-glucose or D-fructose and are consequently denominated as glucans (α or β) and fructans [43, 48]. Glucansucrases and fructansucrases catalyse the polymerization of the glucans and fructans usually using sucrose as the donor of the corresponding monosaccharide and transferring this residue to the reducing end of the growing HoPS [60]. HoPS can also be classified based on the nature of the monosaccharide constituting their structure: dextran is a glucan only including glucose, while levan, inulin, and polygalactan are fructans that only contain fructose and galactose, respectively [43, 45]. Further classifications are based on their glycosidic linkages, branching, chain length, and molecular weight. Glucans can be sub-classified into α-glucans [dextran: α-d-Glc(1,4); mutan: α-d-Glc(1,3); alternan: (α-d-Glc(1,6)/α-d-Glc(1,3); and reuteran: α-d-Glc(1,4)/α-d-Glc(1,6) with α-d-Glc(1,4)/α-D-Glc(1,6) branching points], and β-glucans [β-d-Glc(1,3) with side chain linked (1,2)], whereas fructans can be classified into levan-type: β-d-Fru(2,6), and inulin-type: β-d-Fru(2,1), being both β-fructans [44-45]. HoPS molecular weight generally scales between 10^4-10^8 Da and is bacterial strain-dependent [61].

3.4.2.1 HoPS extracellular synthesis production pathway

The HoPS extracellular biosynthesis is basically detached from core metabolic functions, making it less energy-intensive for the cell and less susceptible to intricate regulatory mechanisms (Figure 2).

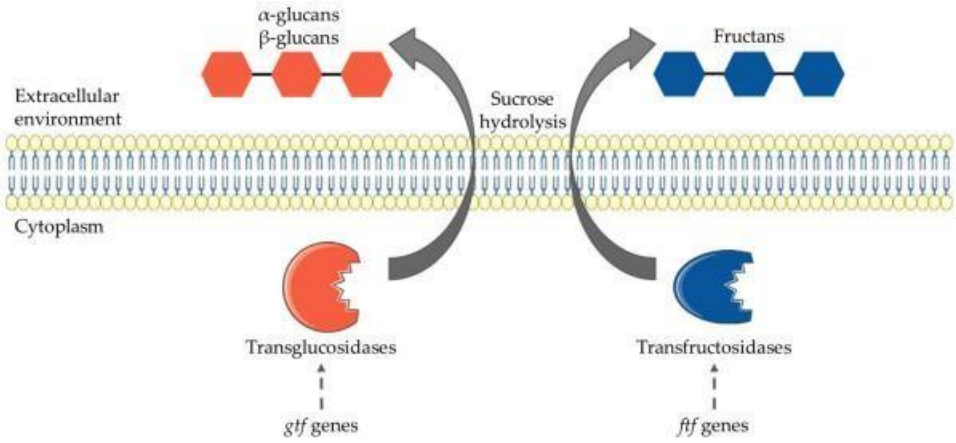


Figure 2. Representation of HoPS biosynthesis pathway. Imagine from [45].

In fact, only the synthesis and export of the GT need cellular energy. Indeed, the energy produced during the cleavage of the substrate's glycosidic bond is utilised to create new glycosidic bonds in the developing HoPS. Glucansucrases or fructansucrases are the extracellular enzymes involved in the biosynthesis of HoPS, and are described in the genera *Weissella, Leuconostoc, Lactobacillus,* and *Pediococcus* [44]. A monosaccharide is transferred from a particular substrate (generally sucrose, raffinose, or other oligosaccharides) to the expanding polysaccharide chains by fructansucrase and glucansucrase. The hydrolysis of sugars is catalysed by these enzymes, which are members of the glycosyltransferase (GTF, E.C. 2.4x.y) group, and the monosaccharide residues produced are then linked to a glycan acceptor chain [44-45]. Transglucosidases (E.C. 2.4.1.y; glucan-synthesizing dextran-sucrases, mutansucrases, and reuteransucrases) and transfructosidases are two subgroups of these enzymes (E.C. 2.4.1.y or 2.y; fructan-catalyzing transfructosidases levansucrases and inulosucrases). The *gtf* and *ftf* genes, respectively, are responsible for encoding these proteins (Figure 3).

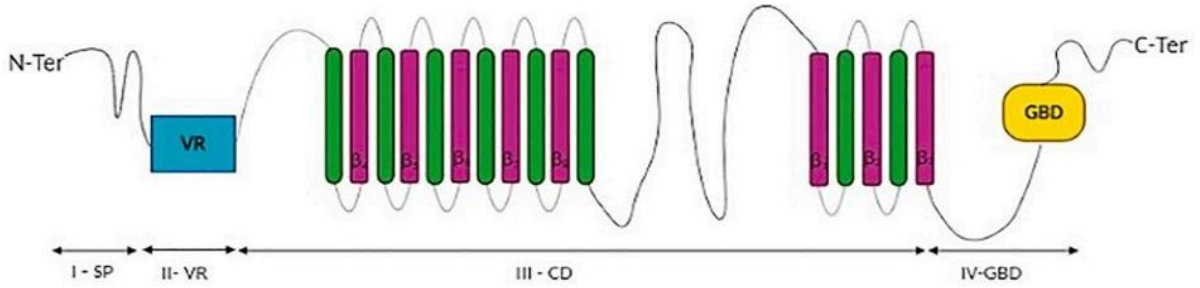


Figure 3. Schematic structure of GTF gene of LAB. The numerals at the top represent the amino acid residues. I: Signal peptide; II: Variable Region; III: N- terminal Catalytic Domain; IV: Glucan Binding Domain at C-terminal. Imagine from [62].

Glucansucrases are accountable for glucans and fructans synthesis and belong to the α-amylase superfamily as part of the glycosides hydrolases (GH). The catalytic (β/α)8-barrel domain of glucansucrases, which controls the reaction specificity, allows them to cleave the α-glycosidic connection between a glucose moiety and another monosaccharide moiety [62-63]. Additionally, minute variations in the amino acid sequence might affect product selectivity. In terms of the three-dimensional structures of glucansucrases, the catalytic core has three domains and two additional domains, the IV and V domains, are connected to it. Some of the domains might display a U-shaped structure due to their construction from two broken segments of the polypeptide chain [62-63].

3.4.2.2 Type of HoPS

Dextran

The word dextran indicates a large class of α-glucans in which the main backbone chain contains α-1→6 glycosidic linkages, although they might also be branched through various secondary linkages such as α-1→2, α-1→3, and α-1→4 [63]. The synthesis of dextran from sucrose was recorded from *Leuconostoc, Lactobacillus* and *Streptococcus*, nevertheless, the ability to produce this HoPS can be lost after serial refreshments are performed in matric rich

in salt [55]. Moreover, environmental factors, metabolic routes, and the velocity of microbial development display an impact on the dextrans' yield [64]. In industries, dextrans generally display a broad range of applications in different sectors, including food, drug, and oil drilling, based on their molecular mass, which affects the viscosity and rheological characteristics of the polymer solution [55, 63-64]. In the food sector, they are included in bakery products and confectionery to enhance viscosity, rheology, texture, volume, softness, or moisture retention, and prevent crystallization. In fact, in gluten-free products, a promising alternative to chemical hydrocolloids as structuring agents to improve the visco-elastic characteristic of the bread is represented by microbial dextrans. This natural hydrocolloid can be produced directly during sourdough fermentation, resulting in natural and clean-label products [64-65]. Furthermore, this hydrocolloid performs a major role in the maintenance of frozen foods and might be used as coatings to protect the surface of fish, meat, vegetables, or cheese from oxidation and chemical changes, and spoilage agents [65].

Alternan

Alternan is a branched glucan with a distinctive backbone structure of alternating α-1→6 and α-1→3-D-glycosidic links, generally produced by *Leuconostoc mesenteroides* strains. Due to this type of linkage high solubility, low viscosity, and remarkable resistance to microbial and enzymatic hydrolysis distinguish this HoPS from commercial dextran [62]. Its peculiar structure and characteristics made this polysaccharide suitable in food and cosmetics as a low-viscosity bulking agent and extender. It could be useful as a potential non-caloric, carbohydrate-based dietary ingredient in meals that are artificially sweetened. Due to its health-promoting qualities, the use of this HoPS as a culinary ingredient enhances bread quality and texture, functions as a bio-thickener, improves dietary fibre content, and makes both humans and animals feel full. Furthermore, as reported by Yilmazet et al. [66], silver nanoparticles with antibacterial and antifungal action against pathogenic strains have been

created using alternan. In addition, polydextrose, maltodextrins, and gum Arabic may be replaced by alternan [67].

Mutan

Different serotypes of *Streptococcus mutans* and many *Leuconostoc* and *Lactobacillus* strains have been shown the ability to produce the glucan known as mutan through the enzyme mutansucrase. According to Leemhuis et al. [63], Zannini et al. [55] and Ahmed at al. [68], the majority of mutans' α-1→3 glycosidic bonds prevent them from dissolving into water.

Levan

Levan is produced outside of cells, in acidic pH conditions, and is made up of d-fructofuranosyl residues connected by β -2→6 links [44]. Levan, is non-toxic and may be produced either enzymatically using saccharose as a substrate or by fermenting saccharose with bacteria like *Bacillus subtilis* [42]. Among LAB the levan producers can be ascribe to *Fructilactibacillus sanfranciscensis, Lactiplantibacillus plantarum, Leuconostoc mesenteroides, Pediococcus pentosaceus,* and *Limosilactobacillus reuteri* species [55]. Levan biosynthesis relies on an extracellular enzyme, levansucrase, (EC 2.4.1.10) also identified as sucrose 6-fructosyltransferase, β-(2–6)-fructosyltransferase, β-(2–6)-fructan: d-glucose 1-fructosyltransferase [69]. Levan synthesis from saccharose is catalysed by the extracellular enzyme levansucrase through the transfructosylation process. Prior to being expelled, the enzyme builds up in the periplasm where it also seems to take on its final configuration. The activity of the enzyme can be negatively influenced by glucose presence and temperature higher than 45°C. Furthermore, the optimal temperature for its production is strictly strain-related. Even if this HoPS is commonly present in some food and consequently often consumed in minimal quantity by humans, its beneficial features such as prebiotic effects, antitumor properties, cholesterol-lowering properties, and function as an eco-friendly adhesive have been evaluated only in the last years [42]. In the food industry, levan is used as

a source of di-fructofuranoses, fructose, and fructo-oligosaccharides as well as an emulsifying and encapsulating agent, colour and flavour vehicle, bio-thickener, fat substitute, and an alternative to produce GF products since levan improves rheology and texture, reducing the use of bread improvers [70].

Inulin

Inulin-type EPS are fructans including β-1→2 osidic bonds. *Leuconostoc, Streptococcus,* and *Lactobacillus* species have been reported to possess inulosucrase, indicating their ability to produce both inulin and levan. Long-chain inulin is used to generate gels, increase viscosity, and enhance organoleptic qualities, whereas short-chain inulin is specifically utilised as a sweetener (low calorie). It can be employed as a non-digestible fibre in dairy products and prebiotics to substitute sugar and fat and as a source of dietary fiber and fibre-enriched in bakery products where it can improve the nutritional quality of GF bread displaying also a prebiotic activity [68]. Furthermore, inulin has also shown antibacterial activity against *E. coli* and *S. aureus* in conjugated polyvinyl-alcohol composite nanofibers (inulin/PVA CNFs) [71].

3.4.3 Heteropolysaccarides

HePS are constructed of two to eight distinct monosaccharides, such as glucose, rhamnose, galactose, fructose, or mannose, and are arranged of repeating units with varying degrees of polymerization [44]. HePS structure is influenced both by the type of linkage and the variety and quantity of monosaccharides involved. The present linkage might be branched or linear with α and β connections. Generally, the sugars involved in *Lactobacillus'* HePS are represented by glucose, galactose, and rhamnose. However, HePS, due to their complicated and energetic expensive pathway, are commonly generated at low quantities in contrast to LAB's HoPS production [48].

3.4.3.1 HePS production through Wzy-dependent pathway

The Wzy-dependent pathway is a complex intracellular process responsible for the production of CPS and EPS in Gram-positive and Gram-negative bacteria (Figure 4). *Lactococcus, Lactobacillus,* and *Streptococcus* are the three LAB genera that are primarily studied for the Wzy-dependent *eps* gene clusters [48]. Genes that code for the Wzy-dependent exocellular polysaccharide biosynthesis proteins, in LAB, are often arranged in a cluster with an operon structure, and are typically chromosomal, however, they can also be found on a plasmid. The nucleotide sequences of *eps* gene clusters are among the most varied sequences in LAB genomes, and their diversity is often influenced by mobile genetic components.

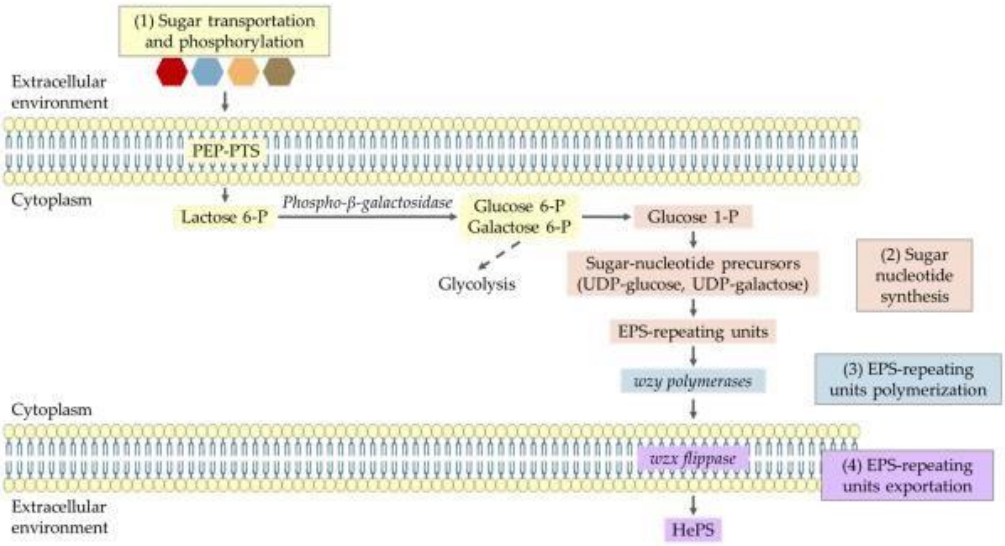


Figure 4. This picture illustrates a comprehensive generic Wzx/Wzy-dependent EPS biosynthesis pathway. EPS: exopolysaccharides; HePS: heteropolysaccharides; P: phosphate PEP-PTS: phosphoenolpyruvate-phosphotransferase system; UDP: uridine diphosphate. Image from [45].

In fact, the architecture of *eps* gene clusters frequently includes insertion sequence (IS) elements within or around the operon [72]. Nevertheless, *eps* gene clusters maintain their modular gene architecture. The entire biosynthetic process can be separated into five stages [73]: (1) Transportation and phosphorylation of mono- and disaccharides: The import of

monosaccharides or disaccharides like glucose, galactose, and lactose necessitates the use of one of two different routes, either phosphotransferase system (PTS)-assisted or permease-assisted. Permeases cannot synchronise the transport and phosphorylation like PTS can, thus any monosaccharides or disaccharides that are carried by them must be helped along by a particular kinase in order to undergo further phosphorylation. Galactosidase must also catalyse the conversion of glucose and galactose/phosphor-galactose from lactose/phospho-lactose; (2) activation of monosaccharides: phosphoglucomutase and galactose-1-phosphate uridylytransferase mediate the conversion of glucose-6-phosphate and galactose-1-phosphate to glucose-1-phosphate, and as a result, glucose-1-phosphate can be converted into UDP-glucose, UDP-galactose, and dTDP-rhamnose, respectively, with the help of several enzymes: (3) creation of repeated elements: numerous glycosyltransferases sequentially build individual repeating units by linking them to a undecaprenol diphosphate anchor (C55) present in the inner membrane surface; (4) repetition units moving from the intracellular surface to the extracellular surface: the flippase (Wzx) translocates repeating units into the periplasm or outer membrane; (5) release of lengthy chains and the polymerization of repetitive units: in Gram-positive bacteria, a chain-length regulation protein controlled the length of the EPS chain while repeating units are catalysed to form long chains by an outer membrane polymerization protein (Wzy).

3.5 LAB and Bio-edible coating

The food industry has applied traditional preservation methods like heat treatment, salting, or acidification for a long time to keep food from spoiling or developing pathogenic microbes, although these methods frequently cause unacceptably significant reductions in nutritional value [74]. In this setting, lately, innovative approaches for preservation techniques, capable of increasing the safety, quality, and functionality of food products while preventing the

growth of unfavourable microorganisms during storage, transportation, and handling, have been rising. Natural polymers, such as pectin, cellulose, and LAB EPS, can be employed as edible coatings and films to preserve the physical and chemical characteristics of various food products during their storage period [7, 75]. As already stated by different studies their use could avoid oxidation and moisture migration, as well as, spoilage control avoiding unfavourable microbial growth [75-80]. Coatings can be produced with a variety of structural components including proteins, lipids, and polysaccharides, or by combining two or more of these substances [77-80]. Since synthetic polymers are so inexpensive, biodegradable polymers have mostly gone unnoticed throughout the years, however, due to the over-solid packaging waste and depleting petroleum reserves, the usage of natural polymers derived from renewable sources has significantly expanded in recent years. Indeed, bioactive packaging systems can be considered a pioneering technology concept that can support the production of functional and clean-label foods [7]. Due to their palatability and biodegradability, biopolymers serve as an alternative environmentally acceptable and renewable source for the production of packaging, as opposed to synthetic films, which place a heavy burden on the environment as non-biodegradable waste [81]. Pectin is a white, amorphous, colloidal carbohydrate with a high molecular weight, generally found in ripe fruits. Due to its thickening, emulsifying properties, and ability to solidify into a gel is commonly used in food, medications, and cosmetics [82]. As a result of all these aforementioned characteristics and employments, this colloidal carbohydrate has entered the market of biopolymers with huge potential and opportunities for future advancements [79, 81-82]. Pectic compounds are typically linked to cellulose, hemicellulose, and lignin structures and are comprised of the most complicated family of polysaccharides. Pectin is primarily made of high molecular weight heterogeneous groups of glycano-galacturonans and acidic structural polysaccharides with different structures [82]. The backbone of pectin is

built up of molecules of (1→4)-α-D-galacturonic acid, joined with a few rhamnose residues in the main chain and arabinose, galactose, and xylose in the side chains [81-82]. Moreover, depending on the level of methylesterification (DM) of the carboxyl group, pectins can be classified as high methoxy pectins (HMP), with a DM 50%, and low methoxy pectins (LMP), with a DM 50% [81-82]. The latter is generally used for the production of food coating and can be applied using three different techniques (Figure 5) [83].

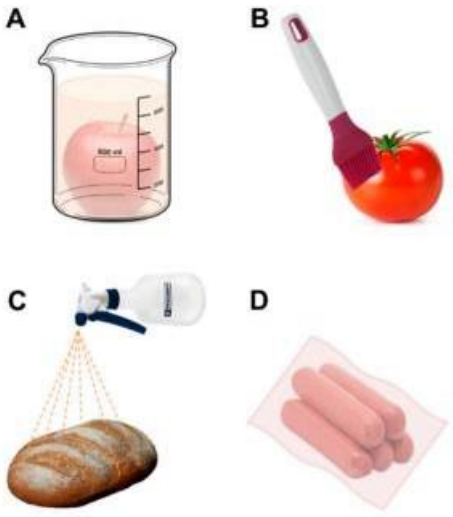


Figure 5. Main coating techniques used in Food industries. A: dipped; B: spread; C: sprayed: D: wrapped. Image from [82].

Desired LAB strains and bioactive compounds can be contained within the coating materials affecting directly the consumer's health and the food's nutritional aspects and shelf-life [74]. In fact, the main advantage of "active" films and coatings is that the inhibitory or quality-promoting agents can be specifically targeted to act on the food surface, where the majority of post-processing alterations occur (oxidation, microbial contamination). The anti-spoilage activity, in particular, benefits the presence of the active compounds/ microorganisms on the surface of the products directly affecting the spoilage flora [84].

The application of edible coatings has already been stated for various products, mostly fruits and vegetables, to shield them from physical, chemical, and microbiological degradation, but

also on bakery products. Since bread is a significant source of complex carbs, proteins, minerals, and vitamins, and is recognized as a staple food in the diet of many nations, the need for innovative preservative strategies has struck upon the baking sector, encouraging the use of live microorganisms in edible films [85]. In 2012 Altamirao-Fortoul et al. [85] already stated the importance of edible coating to include beneficial compounds and viable LAB along with microencapsulation technology. In recent years, according to Gregirchak et al. [86], edible coating for wheat bread containing LAB still represents the best alternative to avoid microbial spoilage without altering any organoleptic properties of the final products. Furthermore, the employment of edible coatings subsequently added to the final products, exerts a protection activity on the microbial cells allowing the use of viable LAB, avoiding the problem of the temperature of the product generally incompatible with microorganisms viability [80,83]. Bio-packaging has proven to be effective in protecting foods that have undergone the minimum amount of processing, and its use could result in financial savings by reducing the amount of food that is lost to natural ripening extending the shelf life of the final product [83, 86]. Specific attributes of coated products, such as sensory, physicochemical, and nutritional qualities, can be improved depending on the biomaterials employed and the kinds of biologically active chemicals [75-80, 83]. Although, several biopolymers and additives with high qualities to produce edible films and coatings have not yet been thoroughly investigated, and they may hold the key to successful insights into the protection and preservation of food products.

Despite the fact that LAB have been applied in fermentation products for a long time, their metabolic and functional features can still be exploited in a wide variety of ways in different industries. LAB species have the capability not only to ferment a wide range of raw materials into food products but also to create new food sources from industrial by-products.

Furthermore, the constant research on LAB's metabolic outcomes and innovative employment can be hired as industrial-grade bio-preservatives answering to the new clean-label trend, supporting a circular economy and environmental interest.

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Anti-Spoilage Activity and Exopolysaccharides Production by Selected Lactic Acid Bacteria

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Abstract: In this study, eight lactic acid bacteria (LAB) strains, previously isolated from traditional and gluten-free sourdoughs, and selected for their potential in improving the sensory and rheological quality of bakery products, were screened against some common spoilage agents. The anti-mould activity was tested using strains of the species *Fusarium graminearum*, *Aspergillus flavus*, *Penicillium paneum* and *Aspergillus niger*. Regarding the antibacterial activity, it was assessed against four strains of the species *Escherichia coli*, *Campylobacter jejuni*, *Salmonella typhimurium* and *Listeria monocytogenes*. Furthermore, LAB strains were evaluated for their ability to produce exopolysaccharides, which are gaining considerable attention for their functional properties and applicability in different food industrial applications. A strain-specific behaviour against the moulds was observed. In particular, *F. graminearum* ITEM 5356 was completely inhibited by all the LAB strains. Regarding the antibacterial activity, the strains *Leuconostoc citreum* UMCC 3011, *Lactiplantibacillus plantarum* UMCC 2996, and *Pediococcus pentosaceus* UMCC 3010 showed wide activity against the tested pathogens. Moreover, all the LAB strains were able to produce exopolysaccharides, which were preliminarily characterized. The assessed features of the LAB strains allow us to consider them as promising candidates for single or multiple starter cultures for food fermentation processes.

Keywords: sourdough; antimould activity; antibacterial activity; food safety; exopolysaccharides; starter culture

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Citation: Iosca, G.; De Vero, L.; Di Rocco, G.; Perrone, G.; Gullo, M.; Pulvirenti, A. Anti-Spoilage Activity and Exopolysaccharides Production by Selected Lactic Acid Bacteria. *Foods* **2022**, *11*, 1914. https://doi.org/10.3390/foods11131914

Academic Editor: Celia Costa Silva

Received: 30 May 2022
Accepted: 24 June 2022
Published: 27 June 2022

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1. Introduction

Lactic acid bacteria (LAB) have been used, since ancient times, to preserve a wide range of fermented foods, including bread, vegetables, and dairy products.

Several LAB strains can produce different kinds of metabolites, including active amino acids, peptides, sugars, and volatile compounds, that contribute also to the final flavour of fermented food products [1]. Moreover, some LAB have beneficial technological applications in fermented foods and beverages due to their functional activity [2].

Many researchers have focused on the health-promoting effects of LAB-driven fermentation, including probiotic, immunomodulatory, anti-hypertensive, anti-oxidant, anti-cholesterolemic, anti-inflammatory, and anti-diabetic effects, among others [3–5].

Recently, LAB have been investigated as preservative agents to control spoilage agents, which are commonly present in cereals and cereal-derived food products, causing quality and sensory deterioration [6]. Although chemical preservatives are still widely used, the food industry is forced to provide innovative products by reconsidering the microbial fermentation process and bio-compounds as an alternative to satisfy the consumer's demands for healthy and clean-label food [7].

Therefore, the research is pushed to obtain new starter cultures able to drive the fermentation process and enhance the microbiological safety and quality of foods.

Different LAB strains are known to produce antifungal compounds (including organic acids, peptides and fatty acids) able to control moulds, such as those belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera, that usually cause bakery product deterioration and are dangerous for health [8–10].

Additionally, several LAB may inhibit the growth of spoilage and common pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Campylobacter* spp., thanks to a synergistic effect of the pH decrease and production of different metabolites (e.g., lactic acid, acetic acid, bacteriocins and bacteriocins-like compounds) [11,12].

Above their antifungal and antibacterial activities, LAB are also able to produce exopolysaccharides (EPSs), which can strongly contribute to improving the shelf-life and palatability of food products. Generally, EPSs are synthesized and excreted by a variety of microorganisms, including LAB and acetic acid bacteria [13–15], and are classified into homo-EPS (HoEPS), such as glucans and fructans, composed of one repeated monosaccharide unit, and hetero-EPS (HeEPS), which are composed of several repeating units of sugars, such as pentose, hexose, or uronic acids, and may be branched or unbranched [16,17]. The use of EPSs in food production, as a natural alternative to commercial food additives, offers many advantages resulting in desirable rheological changes in foods, such as increased viscosity, reduced syneresis, and improved texture [18]. EPSs can act as emulsifying, thickening, and stabilising agents, providing firmness, creaminess, and mouthfeel, which are particularly desired for the improvement of gluten-free products [1,19]. Other positive attributes are related to their prebiotic, antitumor, immunomodulatory, and blood cholesterol-lowering activity [18,20,21].

In recent studies, the EPSs antagonistic roles to microbial pathogens, including viruses, bacteria and fungi, have also been investigated, although there is not a wide knowledge of the mechanisms responsible for this activity [13,22].

Since both the anti-spoilage activity and the EPSs production are features influenced by several factors, such as microbial growth and metabolic activity of each strain, microbial interaction, as well as the type of carbohydrates and enzymes [23,24], the screening of different LAB strains is a fundamental step for the design of ad hoc industrial starter.

Accordingly, culture collections are fundamental tools for innovative research and scientific services to customers and stakeholders as they can screen in-house the interesting candidates to select the best one with the specific features.

In this study, eight LAB strains of the Unimore Microbial Culture Collection (UMCC) were tested for their antimould and antimicrobial activity, as well as the production of EPSs. These strains were previously identified and characterised for different aroma compound production [25] and their applicability in gluten-free fermented products [26]. The final purpose was to consider their potential use as innovative starter cultures able to enhance the organoleptic properties, texture, palatability, and safety of different food matrices.

2. Material and Methods

2.1. Lactic Acid Bacteria Strains

The LAB strains used in this work (Table 1) are maintained in UMCC by cryopreservation at −80 °C, in MRS broth (Oxoid, Milan, Italy) and 25% (*v*/*v*) glycerol (Carlo Erba, Milan, Italy). LAB strains were grown overnight in MRS broth at 30 °C before being used for the anti-spoilage screenings or the EPSs production.

Table 1. Lactic acid bacteria strains from Unimore Microbial Culture Collection (UMCC) tested in the present study.

UMCC ID	Other Denomination	Species	Origin	
			Isolation Source	Region
UMCC 2990	BMA8	*Fructilactobacillus sanfranciscensis*	sourdough type I	Emilia Romagna (Italy)
UMCC 2992	BIA2	*Lentilactobacillus parabuchneri*	dough for Panettone	Emilia Romagna (Italy)
UMCC 2993	BIA6	*Leuconostoc citreum*	dough for Panettone	Emilia Romagna (Italy)
UMCC 2996	BFA1	*Lactiplantibacillus plantarum*	dough for Panettone	Emilia Romagna (Italy)
UMCC 3002	BIB7	*Furfurilactobacillus rossiae*	dough for Panettone	Emilia Romagna (Italy)
UMCC 3011	BFB3	*Leuconostoc citreum*	dough for Panettone	Emilia Romagna (Italy)
UMCC 3009	LC1RC13	*Lactiplantibacillus plantarum*	gluten-free sourdough	Sicily (Italy)
UMCC 3010	LL1C9	*Pediococcus pentosaceus*	gluten-free sourdough	Sicily (Italy)

Data source: UMCC Database, https://umcc.bio-aware.com/ (accessed on 26 May 2022).

2.2. Antimould Activity Screening

The LAB strains were tested for their antimould activity using a dual agar plate assay, according to Hassan et al. [27]. Four moulds provided by the Agro-Food Microbial Culture Collection (ITEM Collection, www.ispa.cnr.it/Collection, accessed on 24 June 2022) of the Institute of Sciences of Food Production, CNR (Bari, Italy), specifically *Fusarium graminearum* ITEM 5356, *Aspergillus flavus* ITEM 7828, *Aspergillus niger* ITEM 7090, and *Penicillium paneum* ITEM 13081, were used for the screening. Briefly, LAB strains were grown overnight in MRS broth at 30 °C, and 100 µL of each culture at 10^8 cfu/mL was mixed with 10 mL of MRS agar. Plates were incubated at 30 °C for 24 h and then overlaid with 10 mL of Potato Dextrose agar (PDA, Oxoid, Milan, Italy). The inoculum for testing antimould activity was prepared from spores of each mould, collected after 5 days of growing the cultures on PDA. 10 µL of spore suspension at 10^6 cfu/mL was placed in the middle of the PDA plates. All plates were incubated in aerobic conditions at 25 °C for 9 days and the radial inhibition of fungal growth was measured [28]. The experiment was repeated three times with three replicates each time.

2.3. Antibacterial Activity Screening

The antibacterial activity of LAB strains was assessed against four bacterial pathogens: *Campylobacter jejuni* ATCC 33250, *Escherichia coli* ATCC 43888, *Listeria monocytogenes* ATCC 19115, and *Salmonella typhimurium* ATCC 14028. The agar diffusion assay method was applied to evaluate the diameters of inhibition zones following the protocol of Arrioja-Bretón et al. [29]. Briefly, a layer of 15 mL of MRS agar was poured into the Petri dishes and, after solidification, overlaid by 10 mL of Brain Heart Infusion agar (BHIA, Oxoid, Milan, Italy). A total of 100 µL of each actively growing pathogen strain (10^6 cfu/mL) was streaked onto BHIA. Then, 20 µL of each LAB strain at 10^8 cfu/mL were loaded into wells of 8 mm in diameter and depth, up to the underlying MRS medium, obtained with the help of a borer. For each plate, two wells were set. The diameter of the inhibition zone was measured after cultivation for 48 h at 30 °C. The experiment was repeated five times with two replicates each time.

2.4. Screening for EPSs Production

A preliminary screening was performed to observe the ropy phenotype typical of the EPS-producing LAB [30]. In detail, active cultures of the LAB strains were streaked onto MRS-sucrose agar, prepared by supplementing MRS medium with 60 g/L of sucrose. The high sugar concentration was applied to produce a nutrient stress factor and increase the production of EPSs [31]. Cultures were incubated at 30 °C for 4 days. Subsequently, LAB exhibiting slimy surfaces and a ropy condition after touching the colony with a loop were reported as promising EPS-producing strains and transferred to the MRS-sucrose broth for the extraction procedure.

2.4.1. EPS Extraction Procedure

The extraction of EPSs from the selected LAB was performed by adapting the protocol reported by Wang et al. [32]. Specifically, 1 mL of each strain was used to inoculate flasks with 500 mL of MRS-sucrose broth. The flasks were incubated at 30 °C for 4 days and then heated at 100 °C for 30 min. The supernatant was recovered after centrifugation at 6000 rpm for 30 min and EPSs were precipitated overnight at 4 °C by addition of an equal volume of cold absolute ethanol. After, EPSs were recovered by centrifugation (6000 rpm for 120 min at 4 °C) and the pellet was redissolved in 10 mL of distilled water with gentle heating. Neutral sugars were eliminated by dialysis for 48 h, then all the samples were lyophilized for 48 h. To purify the samples from proteins, treatment with trichloroacetic acid (TCA) 14% (stirred overnight at room temperature) was performed. The proteins were removed by centrifugation at 6000 rpm for 30 min, and EPSs were precipitated using an equal volume of ethanol at −21 °C. The pellet was dissolved in double-distilled water and lyophilized again for 48 h. The final samples obtained were weighed and the values were expressed in g/L.

2.4.2. EPSs Characterization

To describe and evaluate the composition of the EPSs obtained, all the freeze-dried samples were characterised by attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy and liquid chromatography tandem mass (LC-MS/MS) spectrometry.

ATR-FTIR Spectroscopy

Some crystals of extracted EPSs were loaded on the ATR-FTIR. Spectra were collected using a Perkin Elmer Spectrum TWO UTR-FTIR with PerkinElmer® Spectrum IR software, version 10.6.0.893. To improve the signal-to-noise ratio for each spectrum, 32 interferograms with a spectral resolution of ±4 cm^{-1} were averaged. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra.

ESI-ION TRAP LC MS/MS (LCMSIT) Analysis

The EPSs samples were separated on a microscale Accucore TM HILIC column (2.6 µm; 50 mm × 2.1 mm; Thermo Fisher, Milan, Italy) using an LC-MS(n) Ion Trap 6310 A (Agilent Technologies, Milan, Italy). Solvent A consisted of H_2O with formic acid 0.1%; solvent B was acetonitrile. The following gradient conditions were used: t 0–3 min, 90% solvent B; t 3–6 min, 80% solvent B; t 6–10 min, 50% solvent B; t 10–15 min, 50% solvent B, and back to 90% solvent B until stop time of 25 min. A flow of 0.4 mL/min was applied [33]. For sample injection, the EPSs (6 mg/mL) were resuspended in H_2O. The samples were centrifuged for 1 min at 13,000 rpm before analysis to remove any particles from the sample solution. Aliquots of 15 µL were injected using an autosampler.

2.5. Statistical Analysis

Statistical analysis of data was determined by one-way analysis of variance (ANOVA), followed by Tukey's test, using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA); p-values ≤ 0.05 were considered statistically significant. All measurements were performed at least in triplicates. The results were expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Assessment of the Antimould Activity

The antimould activity of the LAB strains was assessed after nine days of incubation at the optimal growth temperature (25 °C) for the moulds (Table 2). A different strain behaviour was observed for each specific mould. *F. graminearum* ITEM 5356 and *A. flavus* ITEM 7828 were the most sensitive moulds, followed by *A. niger* ITEM 7090. Specifically, the *F. graminearum* strain was completely inhibited by all the LAB strains tested (Figure 1), while the *A. flavus* strain was inhibited only by *Lacp. plantarum* UMCC 2996 and UMCC 3009, *Furl. rossiae* UMCC 3002, *Lenl. parabuchneri* UMCC 2992, and *Fl. sanfranciscensis* UMCC 2990. Against the same mould, a slighter inhibition was detected by *Leuc. citreum* (UMCC 3011 and UMCC 2993) and *P. pentosaceus* UMCC 3010. In contrast, no inhibition was observed against *P. paneum* ITEM 13081. Regarding *A. niger*, a complete inhibition was reported with the strains of *P. pentosaceus*, *Furl. rossiae*, *Lenl. parabuchneri*, and *Leuc. citreum* (UMCC 3011). A distinctive antimould activity was observed by *Leuc. citreum* UMCC 3011 and UMCC 2993, previously isolated from Panettone doughs (Figure 2). This diverse activity could be correlated with the different origin of the two strains, since the Panettone doughs contained a different amount of sugars and fat, which affects the environmental conditions, such as the osmotic pressure and pH, causing different stress responses [25].

Table 2. Activity of eight lactic acid bacteria strains against the tested moulds.

MOULDS	CONTROL	*Leuc. citreum* UMCC 3011	*Lacp. plantarum* UMCC 2996	*P. pentosaceus* UMCC 3010	*Lacp. plantarum* UMCC 3009	*Leuc. citreum* UMCC 2993	*Lenl. parabuchneri* UMCC 2992	*Fl. sanfranciscensis* UMCC 2990	*Furl. rossiae* UMCC 3002
F. graminearum ITEM 5356	32.2 ± 0.66 ^{b}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}
A. flavus ITEM 7828	41.8 ± 0.32 ^{c}	35.7 ± 0.38 ^{b}	0 ± 0 ^{a}	27.1 ± 0.93 ^{b}	0 ± 0 ^{a}	34.5 ± 0.34 ^{bc}	0 ± 0 ^{a}	0 ± 0 ^{a}	0 ± 0 ^{a}
P. paneum ITEM 13081	38.5± 0.53 ^{ab}	35.5 ± 0.23 ^{ab}	39.4 ± 0.47 ^{b}	38.2 ± 0.21 ^{ab}	39.4 ± 0.13 ^{b}	37.0 ± 0.37 ^{ab}	34.1 ± 0.19 ^{a}	34.1 ± 0.19 ^{a}	33.5 ± 0.40 ^{a}
A. niger ITEM 7090	31.8 ± 1.03 ^{b}	0 ± 0 ^{a}	33.2 ± 0.66 ^{bc}	0 ± 0 ^{a}	32.1 ± 0.53 ^{b}	33.1 ± 0.38 ^{bc}	0± 0 ^{a}	39.4± 0.30 ^{c}	0 ± 0 ^{a}

The radial growth (in mm) of the moulds was detected after 9 days of incubation. Superscript letters represent the statistical differences within a row. Different letters are significantly different ($p < 0.05$). Data are presented as mean ± SD, $n = 9$.

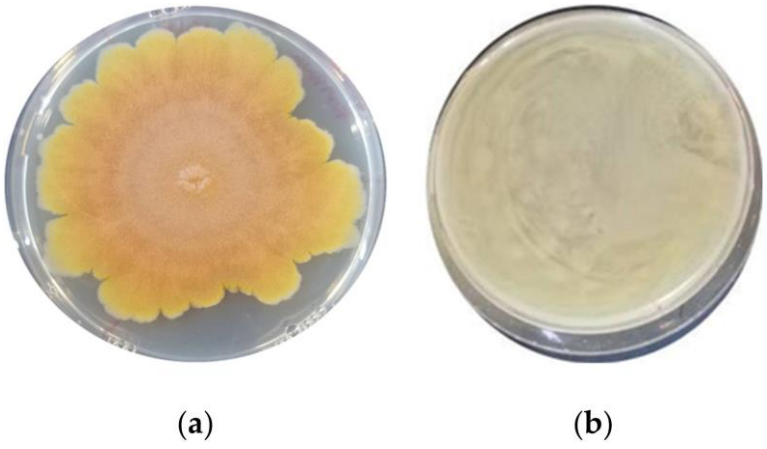


Figure 1. *Fusarium graminearum* on control plate (**a**) and on plate inoculated with *Leuconostoc citreum* UMCC 3011 (**b**).

Among the various LAB tested by several authors, those belonging to *P. pentosaceus* and *Lacp. plantarum* were reported to be the most prominent strains as bio-preservatives able to produce different compounds active against filamentous fungi [8,9,34,35]. In addi-

tion, other LAB species such as *Leuc. citreum* and *Furl. rossiae*, isolated from Italian *durum* wheat semolina, revealed a strong activity against *A. niger* [36]. Obligatory and facultative heterofermentative LAB such as *Lacp. plantarum*, *Fl. sanfranciscensis*, *Furl. rossiae*, *Leuc. citreum,* and *Lenl. parabuchneri* seem to display major antimould activity compared to the homofermentative bacteria such as *Lactobacillus delbrueckii* subsp. *Lactis*, *Lacticaseibacillus paracasei,* and *P. pentosaceus* [8]. Beyond lactic and acetic acid, LAB produce several metabolites which have antifungal activity, such as formic acid, propionic acid, butyric acid, n-valeric acid, hexanoic acid, diacetyl, hydrogen peroxide, caproic acid, 3-hydroxy fatty acids, phenyllactic and 4-hydroxy-phenyllactic acids, compounds of proteinaceous nature, and reuterin matrices [37–41].



(**a**) (**b**) (**c**)

Figure 2. *Aspergillus niger* on control plate (**a**) and on plate inoculated with *Leuconostoc citreum* UMCC 2993 (**b**) and UMCC 3011 (**c**).

3.2. Assessment of the Antibacterial Activity

To evaluate the antibacterial capacity of the LAB strains, a well diffusion assay was performed and the diameter inhibition zone (DIZ), expressed in mm, was measured after 48 h of incubation. The strains *Furl. rossiae* UMCC 3002, *Lenl. parabuchneri* UMCC 2992, and *Fl. sanfranciscensis* UMCC 2990 did not show activity against the selected pathogens, and consequently no inhibition zone was detected (Table 3). Regarding the other five strains, great variability of the inhibitory action was observed. The strains *Leuc. citreum* UMCC 3011, *Lacp. plantarum* UMCC 2996, and *P. pentosaceus* UMCC 3010 showed wide antimicrobial activity on all the pathogens. In particular, the strain *Leuc. citreum* UMCC 3011 was effective on all the pathogen strains tested, with non-significant differences. Its activity on *E. coli* ATCC 43888 is shown in Figure 3. The highest activity was detected in the case of the *P. pentosaceus* strain against *L. monocytogenes* (DIZ 18.00 ± 0.16) and *C. jejuni* (DIZ 15.6 ± 0.44).

Table 3. Antimicrobial activity showed by some of the lactic acid bacteria strains tested in this study.

PATHOGEN STRAIN	*Leuc. citreum* UMCC 3011	*Lacp. plantarum* UMCC 2996	*P. pentosaceus* UMCC 3010	*Lacp. plantarum* UMCC 3009	*Leuc. citreum* UMCC 2993	*Lenl. parabuchneri* UMCC 2992	*Fl. sanfrancis-censis* UMCC 2990	*Furl. rossiae* UMCC 3002
E. coli ATCC 43888	12.6 ± 0.17 ^{Ad}	7.2 ± 0.27 ^{Ac}	11.3 ± 0.24 ^{Ad}	4.4 ± 0.16 ^{Bb}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
S. typhimurium ATCC 14028	13.4 ± 0.43 ^{Ab}	13.4 ± 0.43 ^{Bb}	11.3 ± 0.60 ^{Ab}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
L. monocytogenes ATCC 19115	12.3 ± 0.09 ^{Aa}	11.8 ± 0.14 ^{Ba}	18.0 ± 0.16 ^{Bb}	10.9 ± 0.22 ^{Ca}	10.7 ± 0.14 ^{Ca}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
C. jejuni ATCC 33250	13.1 ± 0.21 ^{Ab}	14.6 ± 0.34 ^{Bb}	15.6 ± 0.44 ^{ABb}	11.6 ± 0.45 ^{Cab}	8.2 ± 0.25 ^{Ba}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}

Diameter inhibition zone (DIZ) is expressed in mm. $^{a–c}$ Mean values with different letters are significantly different ($p < 0.05$). Capital letters represent the differences within a column, while small letters represent differences within a row. Data are presented as mean ± SD, $n = 10$.



Figure 3. Inhibition zones due to the activity of *Leuconostoc citreum* UMCC 3011 against *Escherichia coli* ATCC 43888, observed by a dual plate agar assay.

On the contrary, *Leuc. citreum* UMCC 2993 demonstrated antimicrobial activity only on strains *C. jejuni* (DIZ 8.2 ± 0.25 mm) and *L. monocytogenes* (DIZ 10.70 ± 0.14 mm), respectively. Regarding the strains *Lacp. plantarum* UMCC 3009, no activity was observed on *S. typhimurium* ATCC 14028. The different behaviour of LAB strains, belonging to the same species but isolated from different samples, highlights that the antimicrobial activity is a strain-specific trait, as already observed by Divyrashee et al. [42].

The antibacterial activity of LAB has been studied during the last years by using different strains isolated from different substrates [43,44]. Strains of *Pediococcus* were reported to exhibit strong inhibitory ability against pathogenic bacteria, along with useful features such as high tolerance to sodium chloride, bile salt, and low pH [43]. Other studies have described the efficient antibacterial activity of *Leuc. citreum* and *Leuc. mesenteroides* strains against spoiling bacteria, including *L. monocytogenes*, in meat [45] and fruit [46]. The inhibitory capacity of LAB is generally ascribed to the final products of their fermentative metabolism. Organic acids, especially lactic and acetic acids, formic acid, free fatty acids, ammonia, ethanol, hydrogen peroxide, diacetyl, acetoin, 2,3-butanediol, acetaldehyde, benzoate, bacteriolytic enzymes, and bacteriocins, are the main compounds involved in the inhibitory activity against various spoilage bacteria [47].

However, as recently stated, the number of individual compounds produced by LAB is often insufficient to sustain the inhibitory phenotype, which most likely arises from the synergistic or additive bioactivity of a combination of compounds [48].

3.3. Characterization of the EPSs Produced by LAB Strains

The preliminary screening of the eight LAB on the MRS-sucrose plates, by picking-up colonies grown with an inoculation loop, allowed observation of the formation of filaments; thus, all the tested strains were considered as potential EPS-producers.

The EPSs extraction protocol included several steps for the removal of protein content, small neutral sugars, and salts. The pure EPSs obtained showed a spongy texture of about 1.4 g/L, which was a good amount considering that generally the EPSs production of different LAB strains is reported to vary between 0.01 g/L and 0.4 g/L under non-optimized culture conditions, and can reach multiple of this amount under ideal conditions [18,49]. In our tested condition, the high amount of sugar (60 g/L of sucrose) may have contributed to the increased production of EPSs. The possible explanations for the increased EPS synthesis under the stress of high sugar concentration include osmosis, the unlimited supply of sugar building blocks, and high energy availability [31,49]. Generally, the EPSs yield produced by LAB mainly depends on several parameters, such as temperature, pH, oxygen tension, incubation period, and microbial growth conditions [50].

3.3.1. ATR-FTIR Analysis

The LAB's ability of producing EPSs was firstly tested by ATR-FTIR, exploiting the method recently used to detect active groups of hydroxyls, carboxylics, and carbonyls [51], and identify chitin oligomers from enzymatically digested samples [52–54].

All the samples had similar spectra, in which the presence of polysaccharides was evident (Figure 4). The IR spectra of sugars from the literature were used for comparison.

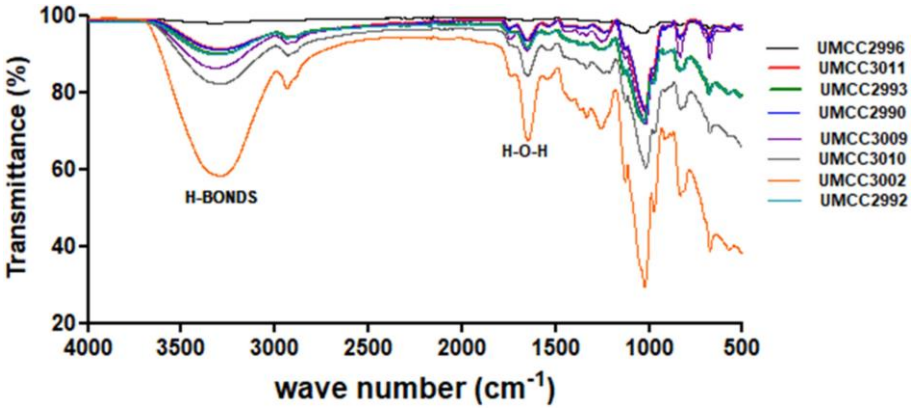


Figure 4. ATR-FTIR spectra of the freeze-dried samples obtained from the tested lactic acid bacteria strains.

The two different regions of the spectrum analysed are the 'sugar fingerprint' region (1500–950 cm^{-1}), which is fundamental for the identification and the structural characterization of polysaccharides, and the region of the functional groups (2500–3600 cm^{-1}), which gives information on the crystallinity of the sample (Figure 4). In particular, the first region contains bands assigned to sugar ring C–C and C–O vibration modes (1104 cm^{-1} and 1030 cm^{-1}, respectively) in the case of cellulose, but they could vary by about 20–30 cm^{-1} depending on the different kind of sugars, as well as on the antisymmetric stretching of the C1–O–C4 moiety of the glycosidic linkage (1160 cm^{-1}). The bands between 3400 cm^{-1} and 3270 cm^{-1} are attributed to O–H and stretching of the C=O—-H–O moieties for the intra-sheet hydrogen bond in crystalline polymer, together with the bands at 700 cm^{-1} (out-of-plane vibrations of O–H groups or rotational vibrations of the whole water molecule), 1640 cm^{-1} (H–O–H angle vibrations), and 2100 cm^{-1} (vibrations from the scission and rocking of water) [53–55]. The differences in the intensities and shape in the bands at 3300 and 1670 cm^{-1} are due to the content of water within the sample and the lengths of the polymers [53].

To note, the sample related to *Furl. rossiae* UMCC 3002 (orange line in Figure 4) displays very intense OH signals indicative of a considerable amount of water entrapped in the sample, leading to a less crystalline shape. For instance, oligomers consisting of fewer monomer units (5–7 glucose units) are more hydrophilic, and this is consistent with the broadening of the band at 3300 cm^{-1}. Finally, the different intensity could also depend on the number of oligomers in the samples, but we were able to recognise EPSs in all the samples. Moreover, considering that in chito-oligosaccharides there are bands at 1637 and 1545 cm^{-1} that can therefore be assigned to the acetyl groups of chitin, in particular to C=O and N–H vibrations, respectively, the absence of these bands indicates that no acetyl amidate EPSs are present in the blend [56].

3.3.2. LCMSIT Analysis

The LCMSIT analysis was performed to further assess the presence of EPSs and preliminarily identify their composition. By calculating the m/z ratios of oligomers of different length and monomer combinations, and by searching in the MS spectra of eluted peaks of all samples, it was possible to confirm the presence of EPSs (Figure 5).

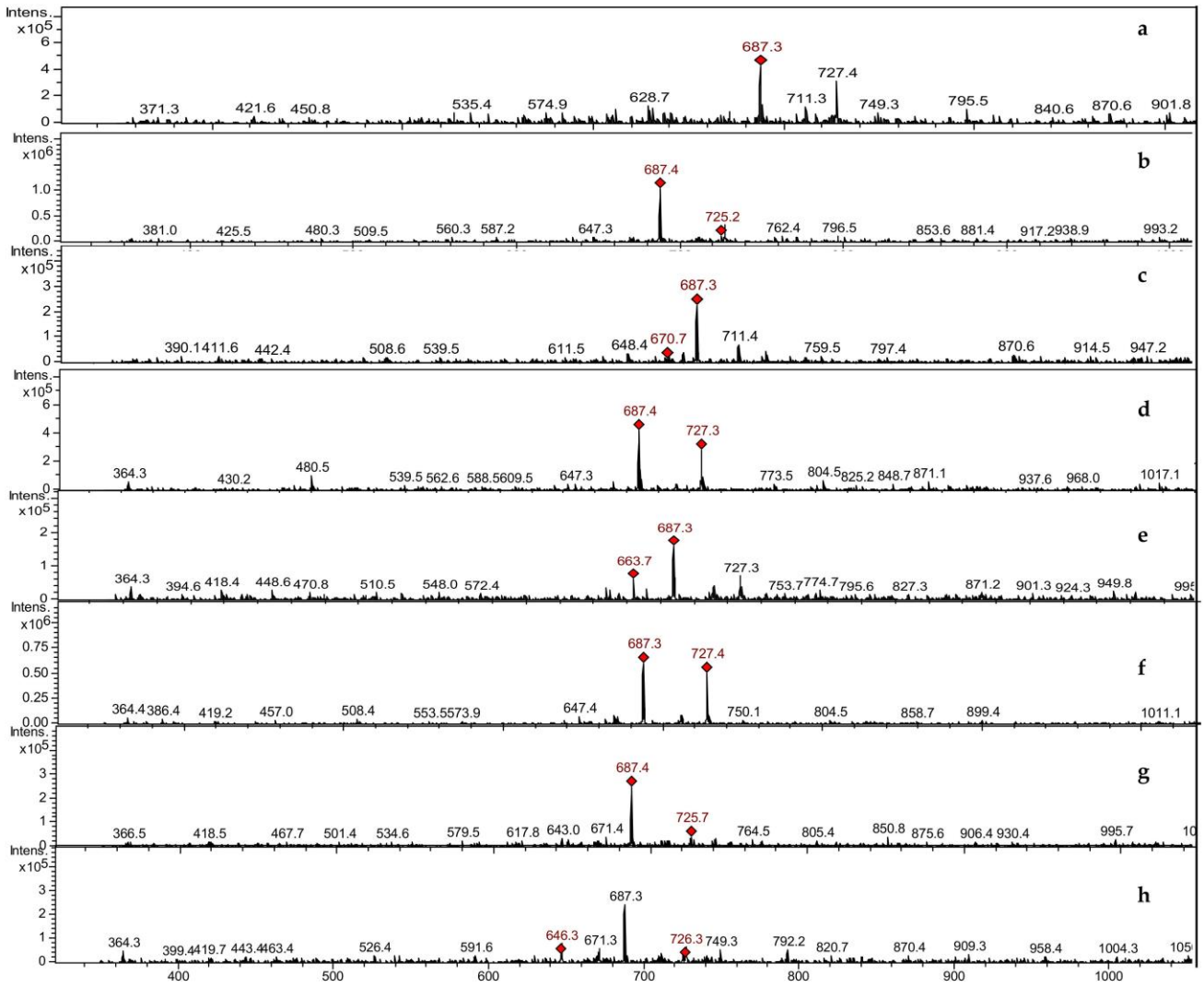


Figure 5. MS spectra recorded in positive mode for the samples obtained after EPSs extraction from the eight lactic acid bacteria strains tested: *Fructilactobacillus sanfranciscensis* UMCC 2990 (**a**), *Lentilactobacillus parabuchneri* UMCC 2992 (**b**), *Leuconostoc citreum* UMCC 2993 (**c**), *Lactiplantibacillus plantarum* UMCC 2996 (**d**), *Furfurilactobacillus rossiae* UMCC 3002 (**e**), *Lactiplantibacillus plantarum* UMCC 3009 (**f**), *Pediococcus pentosaceus* UMCC 3010 (**g**), *Leuconostoc citreum* UMCC 3011 (**h**). Highlighted in red are the masses that have been subsequently fragmented for MS/MS analysis.

It is known that the majority of LAB are able to synthesise different types of EPSs, producing oligomers of glucose, galactose, dextran, levan, rhamnose, arabinose, and mannose [17,57,58]. These oligomers are composed of repeating units of glucose (180.06 g/mol) and rhamnose (164.06 g/mol).

The most abundant MS signals for all samples fall at 687.4 m/z and at 727.2 m/z, which correspond to the mass of a tetra-glucose (666.24 g/mol) with one or two water molecules, or a sodium atom coordinated, with or without a water molecule. The details for the sample obtained from *Leuc. citreum* UMCC 2993 are reported in Figure 6, which includes the MS spectrum (in the first row), together with two MS/MS spectra of the peak at 727.4 m/z (tetra-glucose + $2H_2O$) and the peak at 1012.8 m/z, respectively. The identified masses are highlighted in red in the figure. Similar data were observed by the MS/MS analysis of all the other samples (Figures S1–S7).

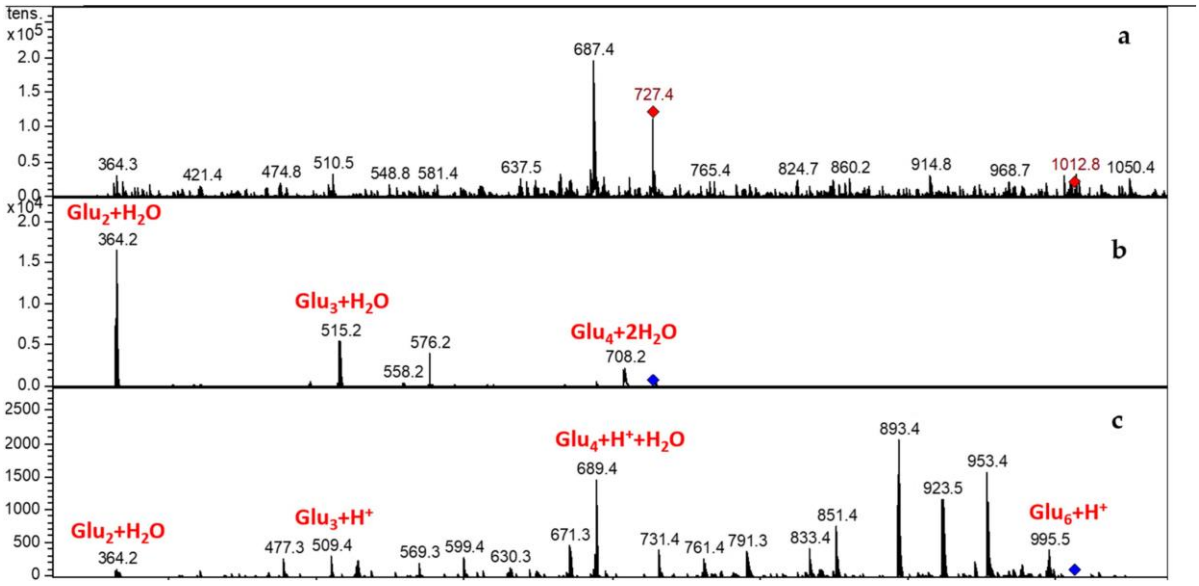


Figure 6. MS spectrum (**a**) and MS/MS spectra of the peak at 727.4 m/z (**b**) and 1012.8 m/z (**c**) of the sample obtained from *Leuconostoc citreum* UMCC 2993. Highlighted in red are the masses corresponding to the loss of a glucose unit.

The EPSs detection and characterization have shown an abundant production of glucose oligomers, along with levans, dextrans, and rhamnose oligomers. These oligomers are different in structure, but their homo-polymeric or hetero-polymeric character cannot be achieved by a simple MS or a tandem approach. Nevertheless, our analyses unambiguously ascribe the EPSs products to the glucans and fructans families. Generally, the production of HoEPS from LAB is markedly higher than that of HeEPS. This is because the former does not require active transport steps and energy consumption, except for the biosynthesis of the extracellular enzymes [17,59].

A complete EPS composition could be achieved by combining different analytical methods to detail monosaccharide composition, ring conformation, linkage, degree of branching, and molecular weight [17].

4. Conclusions

Spoilage agents represent a severe threat to food industries, driving them to use chemical preservatives to control the contaminations. However, consumers are increasingly attracted by natural and healthy food, rejecting more elaborated products containing chemicals. Therefore, the selection of LAB strains with high efficacy against spoilage agents is of great interest for building up innovative starter cultures able to produce high quality and safe products with also appreciated flavours, which satisfy the new market demand.

The eight UMCC LAB, screened in this study, demonstrated antimould and antibacterial activity, even if a strain-specific behaviour was observed. In particular, *F. graminearum* ITEM 5356 was completely inhibited by all the LAB strains. Regarding the antibacterial activity, the strains *Leuc. citreum* UMCC 3011, *Lacp. plantarum* UMCC 2996 and *P. pentosaceus* UMCC 3010 showed wide activity against the tested pathogens. Moreover, all the LAB strains were able to produce EPSs, which were preliminarily ascribed to the family of glucans and fructans. The assessed features of the LAB strains tested, enriched also by their ability to produce different organic acids and aromas compounds, as stated in our previous work, allow us to consider them as promising candidates to be used as single or multiple starter cultures for food fermentation processes and clean-label products.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods11131914/s1, Figure S1: MS spectrum (**a**) and MS/MS spectrum (**b**) for the sample obtained from *Fructilactobacillus sanfranciscensis* UMCC 2990; Figure S2: MS spectrum (**a**) and MS/MS spectrum (**b**,**c**) for the sample obtained from *Lentilactobacillus parabuchneri* UMCC 2992; Figure S3: MS spectrum (**a**) and MS/MS spectrum (**b**,**c**) for the sample obtained from *Lentiplantibacillus plantarum* UMCC 2996; Figure S4: MS spectrum (**a**) and MS/MS spectrum (**b**,**c**) for the sample obtained from *Furfurilactobacillus rossiae* UMCC 3002; Figure S5: MS spectrum (**a**) and MS/MS spectrum (**b**,**c**) for the sample obtained from *Lactiplantibacillus plantarum* UMCC 3009; Figure S6: MS spectrum (**a**) and MS/MS spectrum (**b**,**c**) for the sample obtained from *Pediococcus pentosaceus* UMCC 3010; Figure S7: MS spectrum (**a**) and MS/MS spectrum (**b**) for the sample obtained from *Leuconostoc citreum* UMCC 3011.

Author Contributions: Conceptualization, L.D.V. and G.I.; methodology, G.I. and G.D.R.; validation, G.P. and G.D.R.; investigation, G.I. and L.D.V.; resources, A.P.; data curation, G.I. and L.D.V.; writing—original draft preparation, G.I. and G.D.R.; writing—review and editing, L.D.V., G.P. and M.G.; supervision, A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding author.

Acknowledgments: A special thanks to Hossein Haghighi and Christian Martinelli for their contribution in laboratory experiments. The JRU MIRRI-IT (http://www.mirri-it.it/, accessed on 24 June 2022) and IS_MIRRI21 (https://ismirri21.mirri.org/, accessed on 24 June 2022) are greatly acknowledged for their scientific support.

Conflicts of Interest: The authors declare no conflict of interest.

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LWT - Food Science and Technology 176 (2023) 114524

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Valorization of wheat bread waste and cheese whey through cultivation of lactic acid bacteria for bio-preservation of bakery products

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ARTICLE INFO

Keywords:
Food waste
Cheese whey
Wheat bread
Lactic acid bacteria
Anti-Mold activity
Edible coating

ABSTRACT

In this work, three lactic acid bacteria (LAB) strains, specifically, *Lactiplantibacillus plantarum* UMCC 2996, *Furfurilactobacillus rossiae* UMCC 3002, and *Pediococcus pentosaceus* UMCC 3010, were tested in new bread-whey media composed by wheat bread and sweet cheese whey, designed as an alternative to the conventional MRS medium. The medium resulting from hydrolysis with amylase and neutrase (AN) was considered the best for the growth of all the strains. This medium was particularly optimal for the strain *F. rossiae* UMCC 3002, which showed an increase in growth of 114% compared to that in MRS medium. Additionally, the bio-preservative ability of the selected LAB was assessed in pectin-based coated sliced bread inoculated with *Aspergillus flavus* ITEM 7828, *Penicillium paneum* ITEM 1381, and *Aspergillus niger* ITEM 7090. Different LAB strain behavior was observed towards the specific molds. A good bio-preservation activity was shown from *F. rossiae* UMCC 3002 against *A. flavus* ITEM 7828 with results compared to the protection by ethanol treatment. The results obtained in this study suggest a novel strategy for the cultivation of selected starters with a bio-protection activity by valorizing bread waste and cheese whey by-products, in a circular economy perspective.

1. Introduction

Bread is one of the dominant food sources of the human diet (Hu et al., 2022), comprising several varieties with national and regional variants (Legan, 1993). It is an important calorie source, but also including protein, iron, calcium, and various vitamins (Zain, Shori, & Baba, 2021). The annual average consumption of bread in the world, is estimated to be around 70 kg per capita, and around 59 kg per capita just in Europe (De Boni, Pasqualone, Roma, & Acciani, 2019).

The bread loss of quality, caused by chemical, physical and microbiological agents, represents an important source of food waste both in the bakery sector and in the consumers' kitchen. Globally, around 10% of all manufactured bread is wasted (Narisetty et al., 2021). Mold and fungal deteriorations are the most important causes of bread loss. The high moisture content (about 40%) and water activity (about 0.94–0.97 a_w) of bread (Gobbetti et al., 2019) lead to mold attack susceptibility in 3–7 days.

Penicillium, Aspergillus, *Wallemia*, (Garcia et al., 2019; Quattrini et al., 2019), and other familiar molds such as *Rhizopus*, *Chrysonilia sitophila*, and *Mucor* are mainly involved in the spoiling of bakery products (Rahman et al., 2019). Chemical preservatives such as calcium propionate, ethanol, and sorbate are widely used to extend the microbiological lifetime of bread. However, in recent years, bio-preservation approaches have been evaluated to meet legislative issues, customer demand for clean-label products, and baking industry requirements (Akbar, Ali, & Anal, 2016; Fugaban, Holzapfel, & Todorov, 2022; Le Lay et al., 2016; Muhialdin, Hassan, & Saari, 2018; Nasrollahzadeh, Mokhtari, Khomeiri, & Saris, 2022; Russo et al., 2017; Sadiq et al., 2019). In particular, the sourdough technology, which involves yeasts and lactic acid bacteria (LAB), is reported to be a reasonable alternative to chemical preservatives while boosting flavor, texture, and nutritional characteristics (Axel, Zannini, & Arendt, 2017; De Vero, Iosca, Gullo, & Pulvirenti,

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https://doi.org/10.1016/j.lwt.2023.114524
Received 25 October 2022; Received in revised form 3 January 2023; Accepted 26 January 2023
Available online 29 January 2023
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2021; Hussein, 2022). The *in vitro* antifungal activity of various LAB strains has been widely assessed, however, only a few commercial products are available for bakery bio-preservation; an example is Hi Shield P from the company HI-FOOD S.p.A. (Leyva Salas et al., 2017). The limited number of solutions for bio-preservation on the market is mostly owing to several difficulties in developing effective and competitive solutions. The incorporation of live microorganisms in edible films and coatings has been recently reported as a promising strategy for bread bio-preservation (Guimarães, Abrunhosa, Pastrana, & Cerqueira, 2018). New developments in the usage of edible coatings include the conveyance of bioactive substances such as antioxidants, sensory enhancers, and antimicrobials (Ponce, Roura, del Valle, & Moreira, 2008) or the shelf-life extension, particularly for extremely perishable products. Coatings can also integrate live microorganisms to provide probiotic or antibacterial and -mold capabilities. Despite the numerous applications of antimicrobial edible coatings in meat, seafood, fruit, and vegetables, only some authors (Bartkiene et al., 2019; Ferreira Saraiva et al., 2016; Gregirchak, Stabnikova, & Stabnikov, 2020; Pruksarojanakul, Prakitchaiwattana, Settachaimongkon, & Borompichaichartkul, 2020) have utilized and highlight the potential of these new shelf-life extended techniques for bakery products.

Once the suitable strain with a desired bio-protective activity is selected, its maintenance at the industrial level, as a viable starter culture, requires a balance between cost and efficiency of cell production (Verni, Minisci, Convertino, Nionelli, & Rizzello, 2020).

Generally, conventional media, such as De Man, Rogosa and Sharpe (MRS) or M17, have been successfully used for LAB cultivation and ready-to-use commercial preparations are widely available. However, they are quite expensive, due to the high-priced components such as beef extract, yeast extract, and peptone, which are fundamental nitrogen sources for LAB. Moreover, to dissolve the powder it is necessary to use water, which is an important world resource.

The development of a zero-waste economy by 2025, including the increase of local profits and new technologies for waste valorization as tools of sustainable management, has been proposed by the European Union (EU) (Girotto, Alibardi, & Cossu, 2015). Like other countries, Italy is also making a great effort to support the bioeconomy by reconsidering the use of the main local wastes and by-products.

To reuse and valorize them, one of the strategies investigated recently, focused on the setup of culture media able to promote microbial growth (Benabda, Kasmi, Kachouri, & Hamdi, 2018; Boumaiza et al., 2018; Burns, Vinderola, Molinari, & Reinheimer, 2008; Johnson, Burgess, Shi, Li, & Blersch, 2022; Jung et al., 2022; Kusmiyati, Massora, & Wicaksono, 2022; Linares-Morales et al., 2022; Rahman et al., 2019; Verni et al., 2020; Yurliasni, Hanum, Firtiana, & Sy, 2022).

Regarding cheese whey, it is the most abundant dairy waste with a global production of around 10^8 tons/year. Approximately 10 L of cheese whey are produced to make 1 kg of cheese (Zotta, Solieri, Iacumin, Picozzi, & Gullo, 2020), with an estimated production of 40×10^6 tons/year in all Europe (with annual surplus calculated as on quarter of production) (Mollea, Marmo, & Bosco, 2013). Just in the Italian peninsula, cheese-whey production is estimated to be about 10^6 tons/-years with an annual surplus of more than 10^5 tons/years (Donzella et al., 2022). Despite being one of the most polluting products in the food industry, due to its high chemical and biological oxygen demands, as well as disposal issues (Sommella et al., 2016), cheese whey is considered a great source of vitamins and nitrogen that are essential for LAB development.

As a result of the previous statement, three LAB strains from the Unimore Microbial Culture Collection (https://umcc.bio-aware.com) were investigated in this study for the creation of an alternative growth medium capable of recycling wheat bread waste and Parmigiano Reggiano cheese whey by-product gathered from manufacturers in the province of Reggio Emilia (Italy). The strains' capacity to exert a bio-preservation activity on sliced bread, artificially contaminated with selected molds, was also evaluated and compared to the effect of ethanol, which is a typical exogenous chemical used in packaged sliced bread. In our earlier investigations, the employed strains, isolated from sourdough samples, were characterized for their ability to produce various aromas compounds (De Vero, Iosca, La China, et al., 2021). Furthermore, their potential *in vitro* anti-spoilage (bacteria and molds) activity, as well as their ability to produce exopolysaccharides was also assessed (Iosca et al., 2022). The final goal of the work is to develop a feasible strategy based on the use of specific waste for the cultivation of microbial starters with bioprotective activity. Both parts of the study aim to make new circular biological solutions for the bakery sector.

2. Materials and methods

2.1. Lactic acid bacteria strains

In this study *Lactiplantibacillus plantarum* UMCC 2996, *Furfurilactobacillus rossiae* UMCC 3002, and *Pediococcus pentosaceus* UMCC 3010, were used. The strains were revitalized from −80 °C conditions and grown on MRS broth (Biolife, Italy) at 30 °C for 48 h under microaerophilic conditions (using a jar).

2.2. Formulation of the alternative medium for LAB cultivation

Wheat bread waste, without signs of mold infestation, was provided by an artisan bakery in Emilia Romagna region (Panificio Fantuzzi, Montecchio Emilia, RE, Italy). Firstly, bread waste was finely ground to a particle size of about 2 mm and added to sweet cheese whey (pH 5.7 ± 0.01), resulted from Parmigiano Reggiano DOP production (Latteria Sociale di Migliara, Casina, RE, Italy), in a 1:4 ratio (w/w). The nutritional and chemical compositions of wheat bread and cheese whey are reported in Tables S1–S2. The pH of the cheese-whey was adjusted to 6.5 by using a pH-meter (XS Instruments mod. pH 6). Then, both proteolytic and amylolytic enzymes, singly or in combination were added. Specifically, neutrase, an endo-protease (1.5 AU/g) from *Bacillus amyloliquefaciens* (Novozymes, Denmark), was used at a final concentration of 1.5 mL/L, while amylase, a maltogenic enzyme (≥10000 MANU/g) from *Bacillus* sp. (Sigma-Aldrich®, Merck, Germany) was added at 0.5 g/L (final concentration). Some of the media were supplemented with yeast extract 1 g/100 mL (Oxoid, Thermo Scientific™, England) and 0.5 g/L of Tween 80 (Panreac Applichem, ITW Reagents, Germany). In detail, following a factorial experimental design 2^2, four different bread-whey media were prepared with addition of only amylase (A), addition of amylase and 1 g/100 mL yeast extract (AY1%), only amylase and neutrase (AN), addition of amylase, neutrase, and 1 g/100 mL yeast extract (ANY1%), respectively. To prepare the media, after adding all the components, they were incubated at 55 °C for 18 h, which are optimal conditions for the enzyme's activity according to the manufacturer's instructions. Then they were centrifuged at 6000 rpm for 20 min (centrifuge NEYA 16R, Remi Elektrotechnik LTD., India) to separate not hydrolyzed solid residues, and supernatants were collected. All the media were adjusted to pH 6.5 with NaOH (1 mol/L) before sterilization at 121 °C for 15 min. The precipitates were removed by filtering through a sterilized gauze followed by a centrifugation at 6000 rpm for 10 min. Aliquots of 5 mL of the different media were put in sterile tubes for the strains' inoculation. For each media, the chemical characterization and pH detection was done after autoclaving.

LAB strains were grown for 48 h on MRS agar plate at 30 °C, then cultures were suspended in saline solution (NaCl 0.9 g/L) and diluted up to 1 OD (optical density) detected at 600 nm by spectrophotometer UV/Vis UV-6300PC (VWR International Srl, Milan, Italy). The suspension was diluted at a ratio 1:10 in saline solution, and 100 μL were inoculated in tube test. The inoculum concentration (C_I) was assessed by CFU/mL count on MRS agar medium after incubation at 30 °C for 48 h, under microaerophilic conditions.

2.2.1. Bread-whey media characterization

Glucose content was determined using the Maltose/Sucrose/D-Glucose Assay Kit (Megazyme Int., Ireland) following the manufacturer's instructions, while the percentage of raw proteins was determined by Kjeldahl method. A nitrogen-to-protein conversion factor of 6.38 (Finete, Gouvêa, Marques, & Netto, 2013) was used to convert the total nitrogen in crude protein content following this formula:

$$\text{g} \Big/ 100\ \text{mL raw protein} = \frac{\text{mL HCl} \times \text{N HCl} \times 1.4007 \times 6.38}{\text{g of samples}}$$

The experiments were performed in triplicate.

2.2.2. LAB growth detection in the different media

The microbial growth and pH-value in the different bread-whey media was assessed after incubation at 30 °C for 48 h. Each strain culture was diluted in saline solution (NaCl 0.9 g/L) and serial dilutions from 10^{-1} to 10^{-7} were made. Appropriated dilutions were plated on MRS agar medium and incubated at 30 °C for 48 h under microaerophilic conditions. The results were converted in growth percentage (G%) with respect to those observed in the MRS medium, through the following equation:

$$\text{G}\ (\%) = \frac{(\log \text{C}_\text{I} - \log \text{C}_{\text{FBWx}})}{(\log \text{C}_\text{I} - \log \text{C}_{\text{FMRS}})} \times 100$$

where C_I was the inoculum concentration, C_{FBWx} was the final concentration in each type (x) of bread-whey medium and C_{FMRS} was the final concentration in MRS medium.

Similar to Verni et al. (2020), the effects of the independent variables, related to neutrase enzyme and yeast extract addition, on the microbial growth rate in each type of bread-whey medium, were evaluated by using a regression model with the equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$$

where y was the dependent variable (growth rate) to be modeled; b_0, b_1, b_2, b_{12} were intercept, linear and interaction coefficients of the model, respectively; x_1 and x_2 were the independent variables (neutrase and yeast extract). For the assessment of the regression model, the application tool in Excel was used.

2.3. Formulation of the edible coating with LAB

Edible pectin-based coating was prepared by using for 100 mL of sterilized distilled water, 0.2 g yeast extract (Oxoid, Thermo Scientific™, England), 2.5 g low methoxy amidated pectin (Herbstreith & Fox GmbH & Co. KG, Germany), 0.75 g glycerol (Sigma-Aldrich®, Merck, Germany), 1 g sorbitol (Sigma-Aldrich®, Merck, Germany), 1 g oleic acid (Sigma-Aldrich®, Merck, Germany) and 0,1 g Tween 80 (Panreac Applichem, ITW Reagents, Germany). Distilled water and low methoxy amidated pectin were mixed on a magnetic stirrer at 45 °C and 700 rpm for 20 min. Glycerol, sorbitol, and yeast extract were added at intervals of 10 min at the same temperature and rpm. Oleic acid and Tween 80 were added in the final step of edible coating preparations and the emulsion was left for 1 h at 30 °C and 450 rpm.

2.3.1. Inoculation of the coating with LAB and bread sliced treatment

For the bread sliced treatment, edible coating was inoculated with LAB strains (10^8 CFU/mL). Each type of the inoculated coating was sprayed (1 mL) with a compressor system (ABAC G-541A, Air Powered, Turin, Italy) on each side of a sandwich-like bread slice (about 5 g, 0.9 cm of thickness) and dried for 10 min (25 °C). Wheat bread previously prepared at Fantuzzi bakery was cut in a sterile environment to obtain slices. The ingredients used for preparing the bread were: 400 g wheat flour, 200 g durum wheat semolina, 350 g water, 10 g extra virgin olive oil, 10 g bakery yeast, 4 g table salt, and 5 g bread improvers (amylase and ascorbic acid). The ingredients were mixed in an automatic bread maker for 10 min and dough was placed to rest for 10 min in a proofing cabinet at 25 °C. After the first rise, the dough was divided and settled down in a specific baking mold with a size of 40 × 12 × 12 cm (Fig. S1). The dough piece was placed back into the proofing cabinet for the second rise, gradually reached overnight at a temperature of 22 °C. Finally, the bread was baked in the oven at 200 °C for 35 min.

After the complete treatment with the LAB coatings (CLAB), the bread slices were put into sterile petri plates and incubated at 30 °C with 75% relative humidity (RH), for 9 d.

2.3.2. LAB viability in pectin-based edible coating

The LAB microbial count was performed in triplicate after 1 d (t_0), 3 d (t_3), 6 d (t_6), and 9 d (t_9). The slice of bread, about 5 g, and corresponding saline solution (NaCl 0.9 g/L) for a dilution 1:10 was homogenized by using a laboratory blender Stomacher. Serial dilutions from 10^{-1} to 10^{-5} were made, 100 μL of appropriated dilutions were plated in triplicate on MRS agar and incubated for 48 h at 30 °C under microaerophilic conditions. The results were expressed as a percentage of the cell viability (CV%):

$$\text{CV}\ (\%) = \frac{\log\left(\frac{\text{CFU}}{\text{mL}}\right)\text{t}_{3;6;9}}{\log\left(\frac{\text{CFU}}{\text{mL}}\right)\text{t}_0} \times 100$$

2.4. Anti-mold activity screening on bread sliced

Three molds provided by the Agro-Food Microbial Culture Collection (ITEM Collection, www.ispa.cnr.it/Collection) of the Institute of Sciences of Food Production, CNR (Bari, Italy), specifically, *Aspergillus flavus* ITEM 7828, *Aspergillus niger* ITEM 7090, and *Penicillium paneum* ITEM 13081, were used for the contamination of the sliced bread. The coating with LAB and the bread slides treatment were performed as described before. Then the slices were put into sterile Petri plates and incubated at 30 °C with 75% RH for 24 h. The fungi inoculum was prepared from spores of each mold collected after 7 d growing of the cultures on Potato Dextrose Agar medium (PDA) (Oxoid, United Kingdom). A concentration of 10^5 spores/mL was set with the Burker cell-counting chamber (BLAUBRAND, Germany) and 100 μl of spores' suspension was used; for each mold, two inocula were performed on each bread sliced. All plates were incubated in aerobic conditions at 25 °C and 75% RH in a conditioned chamber for 9 d. Contaminated slices were detected at 3, 6, and 9 d after the inoculum and compared with two controls: one without LAB coating bread control (BC) and the other one treated with vanilla-flavored ethanol (EBC) (Voltolini, Brescia, Italy) applied at the same conditions discussed above. Data were measured by AUTOCAD software (version 2022) following the shape of the mold and expressed as mold quantification area cm^2 (Fig. S2) (Ferreira Saraiva et al., 2016). The experiments were performed in duplicate.

2.5. Statistical analysis

Statistical analysis of data was determined by one-way analysis of variance (ANOVA) followed by Tuckey's test using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). *P-values $\leq$0.05 were considered statistically significant. The results were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Bread-whey medium chemical composition

In this study, four bread-whey media for LAB cultivation were formulated, based on several evidences reported in the literature (Benabda et al., 2018; Burns et al., 2008; Verni et al., 2020). Each type of formulated bread-whey medium was characterized by the content of glucose (g/L), total nitrogen converted to percentage of raw proteins, and pH. The data were compared to those observed in the MRS medium

Table 1
Chemical composition of bread-whey media and MRS medium.

Samples	Glucose g/L	g/100 mL Raw Proteins	pH
MRS	19.01 ± 0.16^{a}	2.24 ± 0.02^{c}	6.29 ± 0.12^{a}
A	8.07 ± 0.38^{b}	n.d.	5.75 ± 0.03^{b}
AY1%	8.47 ± 1.04^{b}	1.34 ± 0.06^{d}	5.77 ± 0.01^{b}
AN	8.22 ± 0.34^{b}	3.49 ± 0.08^{b}	5.76 ± 0.01^{b}
ANY1%	8.75 ± 0.22^{b}	4.19 ± 0.04^{a}	5.77 ± 0.04^{b}

Values are given as the means ± SD (n = 3). Mean values with different letters are significantly different (*P < 0.05). The letters represent the differences within columns). (A, AY1%, AN, ANY1%: experimental conditions produced by bread-whey medium).

(Table 1). The four bread-whey media contained from 8.07 to 8.75 g/L of glucose without statistically significant differences. However, a relevant difference was observed comparing these values with the glucose content in MRS. Regarding the value of raw proteins, it was statistically different for all media. In the bread-whey medium A, proteins could not be detected, but when 1 g/100 mL yeast extract was added (medium AY1%), the proteins content resulted to be 1.34 ± 0.06 g/100 mL. In the MRS medium, the value of raw proteins was 2.24 ± 0.02 g/100 mL, a lower quantity compared to the values observed in the two bread-whey media treated with neutrase (3.49 ± 0.08 g/100 mL and 4.19 ± 0.04 g/100 mL in AN and ANY1%, respectively). These results suggest the use of protease (neutrase) to supply the coagulation of serum proteins and their complete loss during filtration and centrifugation, as observed in the experimental conditions A and AY1%. Regarding the final pH of the media, it was observed a decrease of the values after sterilization. In particular, pH was significantly lower in the bread-whey media (5.76 ± 0.01) compared to the MRS medium (6.29 ± 0.12), maybe due to whey buffering capacity.

Considering the high biological value and essential components contained in bread waste and cheese-whey, their utilization after enzymes treatments can support the LAB growth by providing important nutrients. From the standpoint of cost/energy analysis, enzymatic hydrolyzation to produce glucose from bread waste might be a viable and cost-effective option (Zhang et al., 2020). To reduce water usage while maintaining suitable growth compounds for LAB, which require rich media with amino acids and peptides (Hayek & Ibrahim, 2013; Mokoena, 2017; Sauer, Russmayer, Grabherr, Peterbauer, & Marx, 2017), cheese-whey was used. This dairy by-product is composed by lactose (45–50 g/L), soluble proteins (6–8 g/L), lipids (4–5 g/L), mineral salts (including mainly NaCl and KCl), calcium salts (mainly phosphate), lactic (0.5 g/L) and citric acids, non-protein nitrogen compounds (urea and uric acid), and group B vitamins (Zotta et al., 2020). The enzymatic parameters were chosen to release the major quantity of raw proteins from the cheese whey, while the glucose content was assessed to 8–10 g/L, which is relatively low in comparison with the LAB optimal requirement in 1 L of fermentation media estimated at 20 g/L (e.g., MRS). This consideration has been made because the cheese whey provides a high quantity of lactose, and an excessive amount of carbon source may inhibit the cells growth. Lactose's utilization by LAB is mainly related to their fermentation processes. After internalization into the cell, lactose can be metabolized thanks to two pathways: Leloir and Tagatose-6-Phosphate pathways (Iskandar, Cailliez-Grimal, Borges, & Revol-Junelles, 2019).

3.2. Evaluation of the LAB growth in the bread-whey media

The load of *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, and *P. pentosaceus* UMCC 3010 was measured after 48 h of incubation at 30 °C in the bread-whey media and compared to that obtained with MRS medium (Fig. 1, Table S3). The data were normalized and expressed as growth percentage (G%) with respect to MRS medium. For all the strains, the alternative media may be considered a good substitute of the

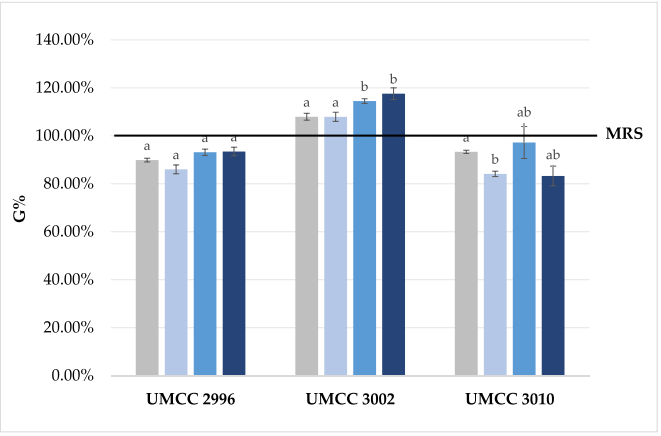


Fig. 1. Growth percentage (G%) of the strains *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010, in the different bread-whey media. The different columns correspond to the medium with: Amylase (A) (), Amylase and 1 g/100 mL yeast extract (AY1%) (), Amylase and Neutrase (AN) (), Amylase, Neutrase and 1 g/100 mL yeast extract (ANY1%) (), respectively. The line corresponds to G% = 100% assessed for the growth in MRS. Values are given as the means ± SD (n = 2). The different letters over the columns indicate statistically significant differences (*P < 0.05).

MRS. Specifically in the cases of *L. plantarum* UMCC 2996, the G% was very high in the four bread-whey media (A: 89.88 ± 0.79%; AY1%: 86.03 ± 1.85%; AN: 93.15 ± 1.32%; AN1%: 93.48 ± 1.78%), almost comparable to those observed in MRS. Also, *P. pentosaceus* UMCC 3010 demonstrated a high G% with no significant difference among the experimental conditions (range from 83.27 ± 4.10% to 97.21 ± 6.66%), even if the regression model showed a negative coefficient related to the addition of yeast extract ($y = 8.94 - 0.18x_2$) (Table 3). For *F. rossiae* UMCC 3002, all the alternative media promoted cells viability with optimal results. The cellular growth in the bread-whey media containing neutrase was higher compared to the other media including MRS. Specifically, the values observed were 114.49 ± 0.98% and 117.57 ± 2.44% in the media AN and ANY1%, respectively. The regression model applied confirmed that only the presence of neutrase had a significant effect on the response function values (Table 2), while the addiction of yeast extract did not affect the growth, resulting unnecessary ($y = 9.22 + 0.12x_1$).

Successful application of alternative media for LAB cultivation have been previously reported in literature. Burns et al. (2008), showed that a medium composed by cheese-whey + 0.3 g/100 mL of yeast extract and cheese-whey + 0.3 g/100 mL of yeast extract + 1 g/100 mL of glucose could promote the growth and the acidification kinetics of *L. acidophilus* H5 and *L. paracasei* LS with data comparable to those observed in MRS medium. Verni et al. (2020) valuated several different alternative media composed by bread waste. The best one was composed of glucose 24.53 ± 0.29 g/L, peptides 37.08 ± 0.39 g/L, and 10.76 ± 0.12 g/L of amino acids, respectively and no statistical differences (*P < 0.05) with the MRS medium was observed in relation to the LAB growth. In our study, the bread-whey media formulated, thanks to the simultaneous use of a carbon and protein source, were able to promote the LAB growth without the addition of other ingredients. In particular, the medium

Table 2
Regression model used to describe LAB growth rate as a function of the independent variables (y: cellular growth log CFU/mL; x_1: Neutrase; x_2: yeast extract).

Strains	Regression model
***L. plantarum* UMCC 2996**	$y = 9.11 + 0.12\,x_1$
***F. rossiae* UMCC 3002**	$y = 9.22 + 0.12\,x_1$
***P. pentosaceus* UMCC 3010**	$y = 8.94 - 0.18\,x_2$

Table 3
Quantifications of LAB antimold activity after 3, 6, and 9 d of incubation.

MOLDS	BC	EBC	*L. plantarum* UMCC 2996	*F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010
***A. flavus* ITEM 7828**					
t_3	2.43 ± 1.03^{c}	0 ± 0^{a}	1.26 ± 0.56^{c}	0.34 ± 0.16^{b}	2.37 ± 0.03^{c}
t_6	16.54 ± 1.82^{c}	2.18 ± 0.87^{a}	6.83 ± 1.03^{b}	7.65 ± 1.41^{b}	12.60 ± 2.83^{c}
t_9	30.86 ± 0.71^{b}	10.89 ± 0.49^{a}	33.11 ± 0.64^{b}	15.73 ± 2.60^{a}	33.57 ± 0.23^{b}
***A. niger* ITEM 7090**					
t_3	1.43 ± 0.11^{b}	0 ± 0^{a}	2.23 ± 0.40^{b}	2.55 ± 0.88^{b}	4.07 ± 0.28^{c}
t_6	15.40 ± 2.20^{c}	0 ± 0^{a}	10.22 ± 0.64^{b}	8.10 ± 0.21^{b}	11.71 ± 1.58^{bc}
t_9	33.94 ± 0.26^{d}	0 ± 0^{a}	32.93 ± 0.54^{d}	16.80 ± 2.16^{b}	22.68 ± 0.64^{c}
***P. paneum* ITEM 1381**					
t_3	3.54 ± 0.89^{b}	0 ± 0^{a}	1.66 ± 0.76^{b}	0 ± 0^{a}	3.13 ± 0.64^{b}
t_6	15.88 ± 1.41^{b}	1.59 ± 1.51^{a}	14.72 ± 1.40^{b}	7.40 ± 1.35^{a}	20.62 ± 0.52^{b}
t_9	28.16 ± 0.57^{b}	18.61 ± 1.48^{a}	27.48 ± 0.12^{b}	13.00 ± 2.21^{a}	28.72 ± 0.71^{b}

Values are expressed as mold propagation area $cm^2 \pm$ SD (n = 2). Mean values with different letters are significantly different (*P < 0.05). Small letters represent the differences within the rows.
BC: bread control; EBC: ethanol bread control; CLAB: coating with LAB.

resulting after hydrolysis with amylase and neutrase (AN) was considered optimal among the experimental conditions for all the strains, with likely results especially for *F. rossiae* UMCC 3002.

For each medium inoculated with a LAB strain, the pH value was detected after 48 h of incubation at 30 °C, and correlated to the strain's growth expressed as log CFU/mL (Fig. 2). The decrease of pH during fermentation is generally related to the production of organic acids, and values around 4 are archived for most of LAB (Wang et al., 2021). The strains showed a clustering on scatter plot, characterized by low pH values and high cellular concentration. The results highlighted the optimal fermentative process carried out by the LAB despite the substrate. The acidification detected for the strains was not statistically significant among the alternative media and MRS, despite the different initial pH values. For *L. plantarum* and *P. pentosaceus* the pH values detected were 4.08 ± 0.02 and 4.23 ± 0.04, respectively. Notwithstanding, only in the case of *F. rossiae* UMCC 3002, a linear behavior among pH values and cells concentration ($R^2 = 0.9239$) can be noted. In MRS the pH value was 4.65 ± 0.01, while in AN medium was 4.30 ± 0.02, with a correlation to a cell concentration of 8.86 ± 0.19 UFC/mL and 9.32 ± 0.04 UFC/mL, respectively.

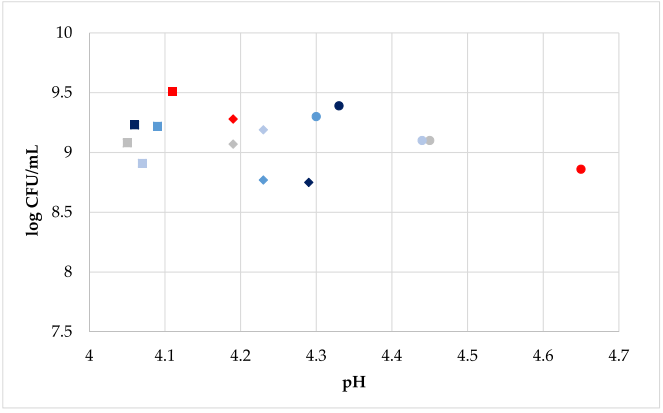


Fig. 2. Scatter plot of relations between cell concentration (log CFU/mL) and pH values detected after 48 h of incubation of *L. plantarum* UMCC 2996 (■), *F. rossiae* UMCC 3002 (●) and *P. pentosaceus* UMCC 3010 (◆) in the different media. The points with the same shape represent the growth on bread-whey (Amylase (A) (■), Amylase and 1 g/100 mL yeast extract (AY1%) (■), Amylase and Neutrase (AN) (■), Amylase, Neutrase and 1 g/100 mL yeast extract (ANY1%) (■)), and MRS (■) medium for the different strains. Values are given as the means ± SD (n = 3).

3.3. LAB cells viability in edible coatings

The viability of the three LAB strains, included into the edible coatings, was assessed during an incubation period of 9 d, in conditions which simulate a sliced bread storage at room temperature (25 °C; 75% RH) (Fig. 3). Cells viability was greater than 80% (corresponding to a concentration loss of ca. 1.4 log units) for all strains after 9 d despite the high moisture loss due to gas exchange. After three days each strain exhibited an unremarkable loss of viability. *L. plantarum* UMCC 2996 showed a decrease between the six and 9 d (from 100.00 ± 6.86% to 92.97 ± 6.55%), *F. rossiae* UMCC 3002 between three and six days (from 98.82 ± 7.43% to 79.76 ± 6.55%), instead *P. pentosaceus* UMCC 3010 did not show great variation and it exhibited the best viability at the end of the storage period (97.71 ± 3.18%). According to Rodrigues et al. (2011), high temperature and long storage periods are harmful to LAB survival. Pereira et al. (2016), reported a loss of viability of ca. 3 log units after 10 d of storage at 23 °C, while Gregirchak et al. (2020), stated a loss concentration of strains from ca. 1 to 3 after only 86 h compared four types of different coating. Nevertheless, in our study, the pectin-based edible coating contributed to have good viability for all the strains (greater than 80%) during the nine days of storage, therefore, it can be considered as a starting point for further optimization at an industrial scale.

Generally, pectin, as a polysaccharide, represents a suitable

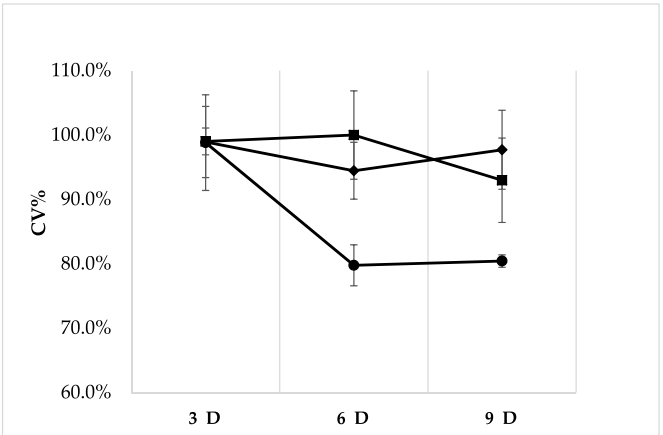


Fig. 3. Cells viability (CV%) of *L. plantarum* UMCC 2996 (■), *F. rossiae* UMCC 3002 (●) and *P. pentosaceus* UMCC 3010 (◆) incorporated in an edible coating of sliced bread during 9 d of storage (25 °C–75% RH). Values are given as the means ± SD (n = 3).

ingredient, due to its edibility and nutraceutical properties (Naqash, Masoodi, Rather, Wani, & Gani, 2017). Moreover, pectin can be easily extracted from fruit peels, and recent techniques have been developed to extract it with eco-sustainable processes and reagents (Cho et al., 2019). Pectics are polymers mainly composed of (1 → 4) α-D-galactopyranosyluronic acid units naturally esterified with methanol. According to their content of methyl esters or degree of esterification (DE), pectins are divided into high-methoxyl (HM, DE>50%) or low-methoxyl (LM, DE<50%). The DE has a decisive effect on pectin solubility and gelation properties (Campos, Gerschenson, & Flores, 2010). Pectin-based edible coatings have been already developed to improve the shelf-life of eggs (De Leo et al., 2018), different vegetables (Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2008; Ranjitha et al., 2017) or application on peaches (Hussain, Suradkar, Wani, & Dar, 2016), grapes (Yinzhe & Shaoying, 2013) and orange (Strano et al., 2021) showing good maintenance of the nutritional quality of products. Sorbitol may have a protective effect on bacteria incorporated, the mechanism by which it exerts its protective effect could be related to the interaction between sugars and phospholipid membranes, helping to maintain its fluidity (Santivarangkna, Kulozik, Kienberger, & Foerst, 2009). In the studies of Gialamas, Zinoviadou, Biliaderis, and Koutsoumanis (2010), when sorbitol was added to film formulation, the viability of the bacterial cells during storage at 25 °C over 30 d was increased. Moreover, in the study carried out by López de Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, and Montero (2012), from an initial concentration of 10^9 CFU/mL, both *L. acidophilus* and *B. bifidum* remained constant for 6 d in an edible coating containing sorbitol in its composition. Glycerol was added as a plasticizer to enhance the film flexibility, but it has also a role in cellular protection as sorbitol. Yeast extract was included as proteins and B vitamins source to enhance LAB viability, oleic acid was added as a surfactant to improve adhesion to the bread surface whereas Tween 80 as an emulsifying agent.

3.4. Assessment of the anti-mold activity on treated sliced bread

The preservation action of the edible coating containing *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, and *P. pentosaceus* UMCC 3010 was tested on sliced bread intentionally contaminated with three filamentous fungi: *Aspergillus flavus* ITEM 7828, *Aspergillus niger* ITEM 7090, and *Penicillium paneum* ITEM 1381. The anti-mold activity of LAB was assessed after 3, 6 and 9 d of incubation at the optimal growth temperature (25 °C) for the molds and 75% RH (Table 3). A different LAB strain behaviour was observed in relation to a specific mold (Figs. S4–6). Regarding *A. flavus* ITEM 7828, the strain *F. rossiae* UMCC 3002, showed the best bio-preservation activity. After 9 d, also bread treated with ethanol (EBC) (10.89 ± 0.49 cm^2) was contaminated and the difference was not statistically different between its preservation and the action of *F. rossiae* UMCC 3002 (15.73 ± 2.60 cm^2), but truly significant respect to BC (30.86 ± 0.71 cm^2) and other coated treated with LAB. The same behavior of this strain, with respect to bread sprayed with ethanol, was observed for *P. paneum* ITEM 1381. Nevertheless, the previous study *in-vitro* did not encounter an antagonism among the LAB strains tested and this particular mold (Iosca et al., 2022). No contamination was visible on bread coated with *F. rossiae* UMCC 3002 after 3 d. After 9 d the contaminated area was only half respect to those observed in BC (13.00 ± 2.21 cm^2 and 28.16 ± 0.57 cm^2, respectively) and no statistical difference compared to EBC (18.61 ± 1.48 cm^2) (Fig. 4). Iosca et al. (2022), reported an antimold activity by *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 against *A. niger* ITEM 7090, which was confirmed in this screening. After 3 d of storage, on coated bread was observed a yellow halo in correspondence of the inoculum, while in BC was present the typical black fungal mycelium. That difference could be caused by the low pH characterized by the metabolic actions of LAB that produce organic acids with bio-preservation action. After 9 d was observed a statistical difference between coated bread with *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 and BC, whereas the presence of ethanol inhibited *A. niger* completely (Fig. 5).

A considerable number of studies have tested LAB strains for their antifungal activity, but, to date, only a few commercial products are available (Leyva Salas et al., 2017). Bio-preservations by LAB are highly documented for preventing molds contamination of the wheat bread, which causes economic and health problems (Rahman et al., 2019). Multiple studies have shown effects of bio-preservation with LAB. The obligatory and facultative heterofermentative LAB, such as *L. plantarum*, *F. sanfranciscensis*, *F. rossiae*, *L. citreum* and *L. parabuchneri*, seem to display a major antifungal activity than the homofermentative bacteria, such as *Lactobacillus delbrueckii* subsp. *lactis*, *Lacticaseibacillus paracasei* and *P. pentosaceus* (Bartkiene et al., 2019). In this study, the anti-mold activity results highlight that *F. rossiae* UMCC 3002 has demonstrated a strong action against *A. niger* ITEM 7090, *A. flavus* ITEM 7828 and *P. paneum* ITEM 1381 reducing the contaminated area on bread surface in respect to BC, and in some instance respect to EBC. This strain specific activity might be related to the production of different organic acids and antifungal compounds working in synergy (data not shown).

4. Conclusions

In a circular food system, the formulation of new bread-whey media able to promote the growth of LAB represents a reasonable strategy for the cultivation of selected starters with a bio protection activity by valorizing bread waste and cheese whey by-product, also avoiding the use of an important resource like water. Due to their compatibility with food, the bread-whey media allow the direct use of the cultivated LAB cultures in edible coatings designed for bio preservation purpose. In our study, the positive effects of the alternative media on bacterial growth

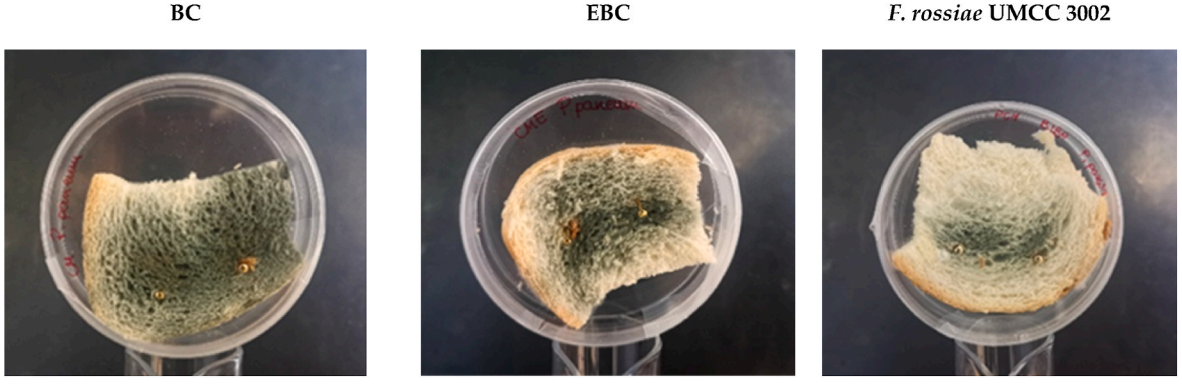


Fig. 4. Anti-mold activity against the simulation of microbial contamination of sliced bread with mycelium fungi of *Penicilium paneum* ITEM 1381 at the 9 d of storage (BC: bread control; EBC: ethanol bread control). The gold pin indicates the area where the mold was inoculated.

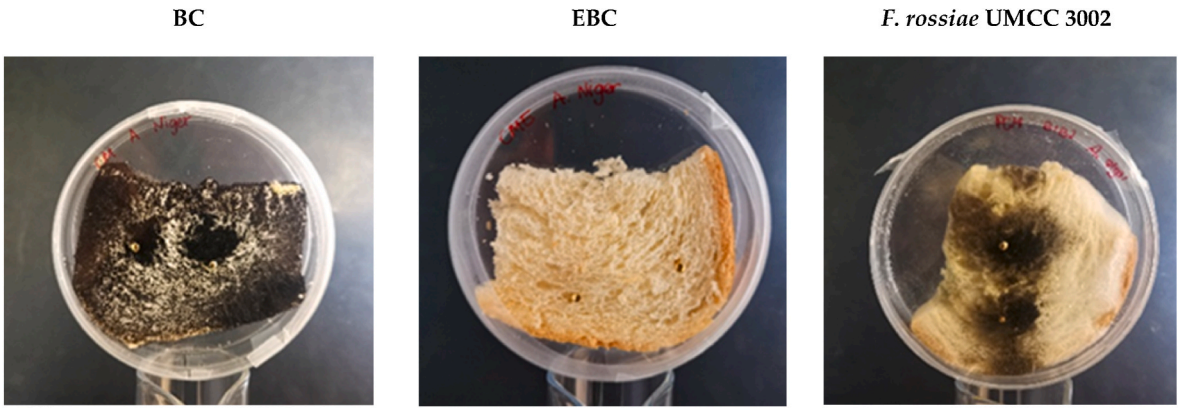


Fig. 5. Anti-mold activity against the simulation of microbial contamination of sliced bread with mycelium fungi of *Aspergillus niger* ITEM 7090 at the 9 d of storage (BC: bread control; EBC: ethanol bread control). The gold pin indicates the area where the mold was inoculated.

have been assessed. The medium AN, resulting from hydrolysis with amylase and neutrase, was considered the best for the growth of all the strains. In particular, the growth of *F. rossiae* UMCC 3002 was increased of 114% compared to that in the MRS medium. However, the microorganism metabolic activity in the alternative media could be affected by using different waste and by-product sources influencing the final growth ratio. Regarding the bio-preservation activity, some constraints also need to be assessed. Microorganisms' behavior might differ between the culture medium and the coated products. These differences could be linked to the complexity of the food or raw matrices, which are characterized by many abiotic and biotic variables that might affect LAB and mold development and metabolism, as well as the bioavailability of antifungal compounds. Furthermore, the maintenance of an available and stable microbial population during the whole storage period in the coating might be challenging due to variable environmental factors. Nevertheless, in the conditions described in this study, *F. rossiae* UMCC 3002 showed good bio-preservation activity against *A. flavus* ITEM 7828, suggesting an alternative to the use of chemical preservatives. Considering the evidence obtained from the reported results, the strain *F. rossiae* UMCC 3002 seems a promising candidate for the valorization and exploitation of food waste and by-products. Although, its bio-protection activity needs to be furtherly investigated, even against other spoilage agents, for the optimal employment of this strain in the manufacturing of clean-label bakery products.

Funding

Claus Heiner Bang-Berthelsen is funded by a Innomission pool 1 grant 1152-00001B called REPLANTED.

CRediT authorship contribution statement

Giovanna Iosca: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Monica Turetta:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Luciana De Vero:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision. **Claus Heiner Bang-Berthelsen:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision. **Maria Gullo:** Writing – review & editing, Project administration, Funding acquisition. **Andrea Pulvirenti:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Panificio Fantuzzi s.r.l. (Reggio Emilia, Italy) for providing the bread samples. A special thanks to Dr. Francesco Bigi of Packtin s.r.l. for supporting the edible coating preparation and Dr. Giancarlo Perrone of ITEM collection of Bari for providing the mold cultures. The JRU MIRRI-IT (http://www.mirri-it.it/) and IS_MIRRI21 (https://ismirri21.mirri.org/) are greatly acknowledged for scientific support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114524.

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Supplemental materials

Table S1. Wheat bread nutritional composition

Nutrients	100 g
Energy	219 Kcal
Carbohydrate	47.5 g
Sugar	1.1 g
Protein	7 g
Fat	1.4 g
Salt	0.8 g
Fiber	1.7 g

Table S2. Cheese-whey chemical composition

Chemical composition	g/L
Lactose	47
Protein	7,1
Lipid	0,5
Mineral Salts	5

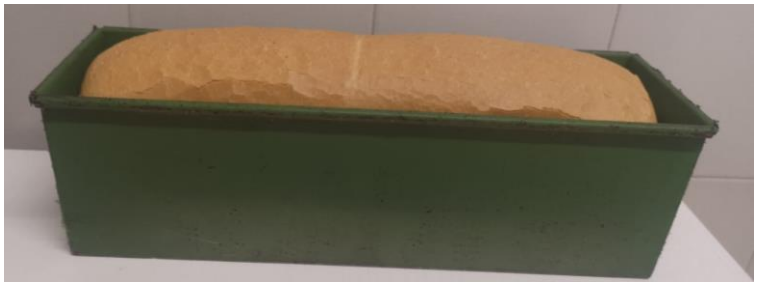


Figure S1. Bread made in a baking mold with size of 40x12x12 cm.

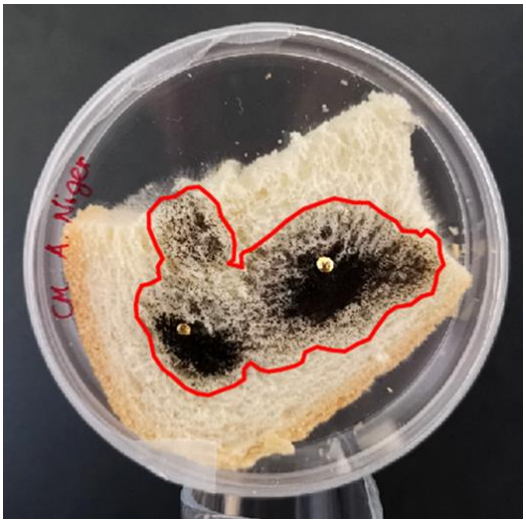


Figure S2. Quantifications of the area contaminated by applying AUTOCAD (2022) software.

Table S3. Growth percentage of the LAB strains tested in the alternative bread-whey media.

Strains UMCC ID	Growth %			
	A	**A1%**	**AN**	**AN1%**
***L. plantarum* UMCC 2996**	89.88 ± 0.79^{a}	86.03 ± 1.85^{a}	93.15 ± 1.32^{a}	93.48 ± 1.78^{a}
***F. rossiae* UMCC 3002**	107.96 ± 1.43^{a}	107.93 ± 1.92^{a}	114.49 ± 0.98^{b}	117.57 ± 2.44^{b}
***P. pentosaceus* UMCC 3010**	93.33 ± 0.66^{a}	84.13 ± 1.13^{b}	97.21 ± 6.66^{ba}	83.27 ± 4.10^{ba}

Values are given as the means ± SD (n = 2). Mean values with different letters are significantly different (*P < 0.05). The letters represent the differences within the rows. A: medium with amylase; AY1%: medium with amylase and 1 g/100 mL yeast extract; AN: medium with amylase and neutrase; ANY1%: medium with amylase, neutrase and 1 g/100 mL yeast extract.

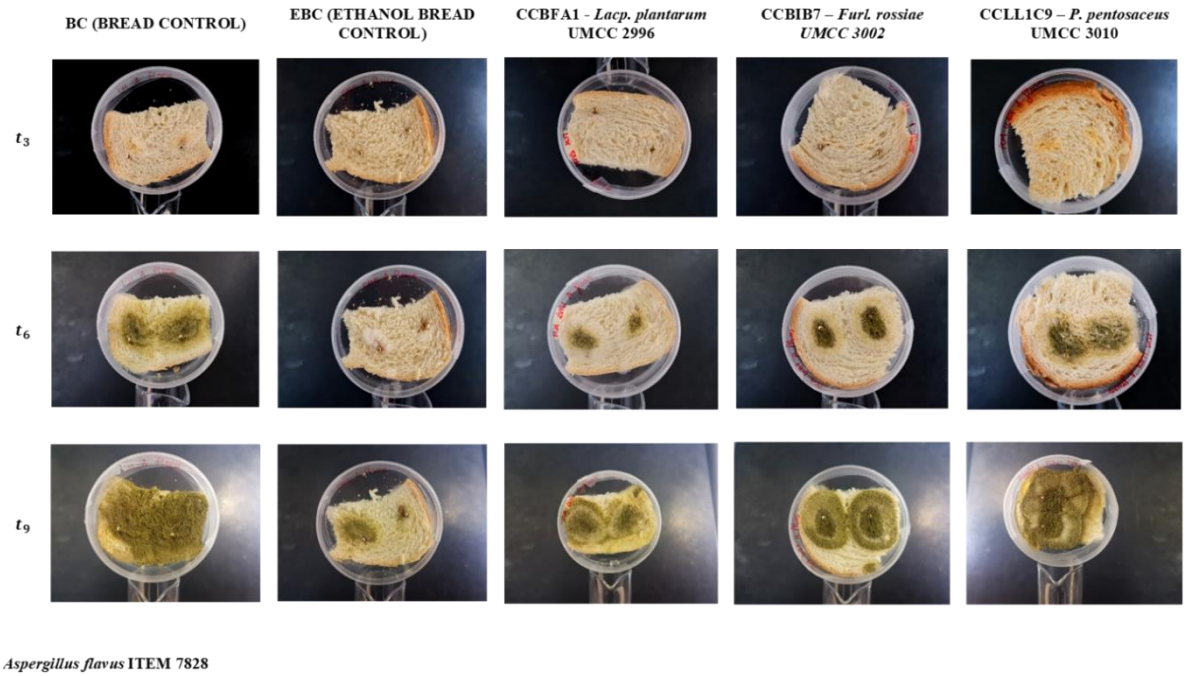


Figure S4. Anti-mold activity of the edible coating containing *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 against the simulation of microbial contamination of sliced bread with mycelium fungi of *Aspergillus flavus* ITEM 7828 during 9 d of storage. The gold pin indicates the area where the mold was inoculated.

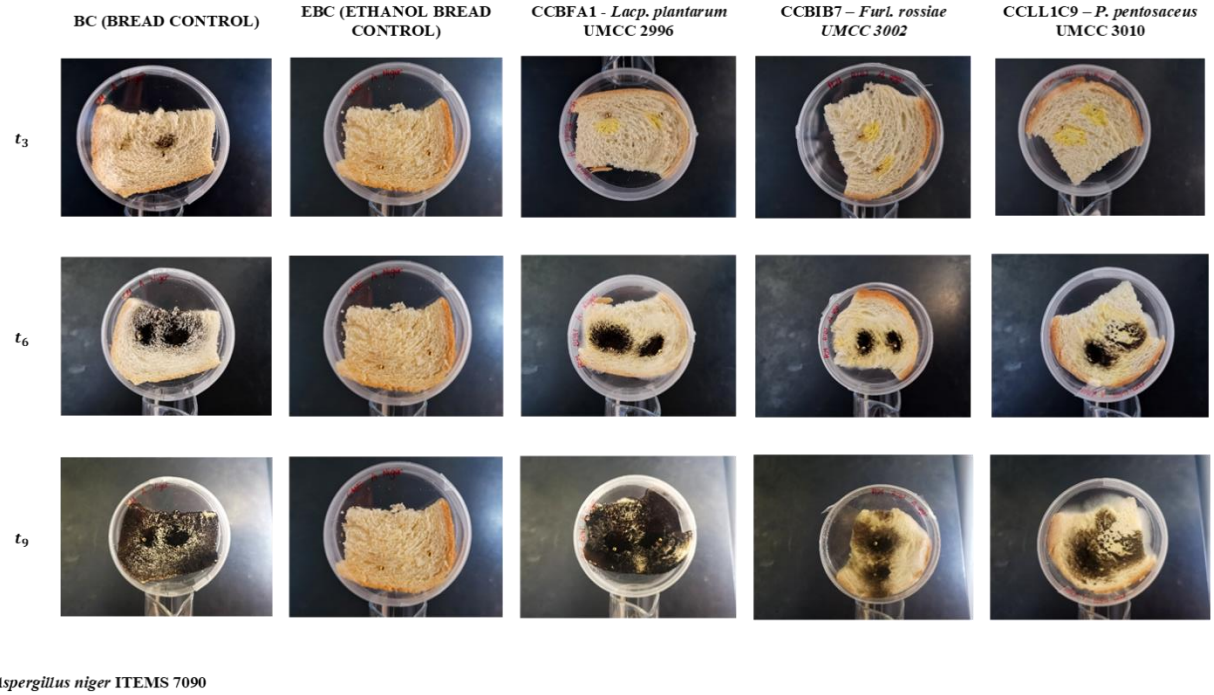


Figure S5. Anti-mold activity of the edible coating containing *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 against the simulation of microbial contamination of sliced bread with mycelium fungi of *Aspergillus n*iger ITEM 7090 during 9 d of storage. The gold pin indicates the area where the mold was inoculated.

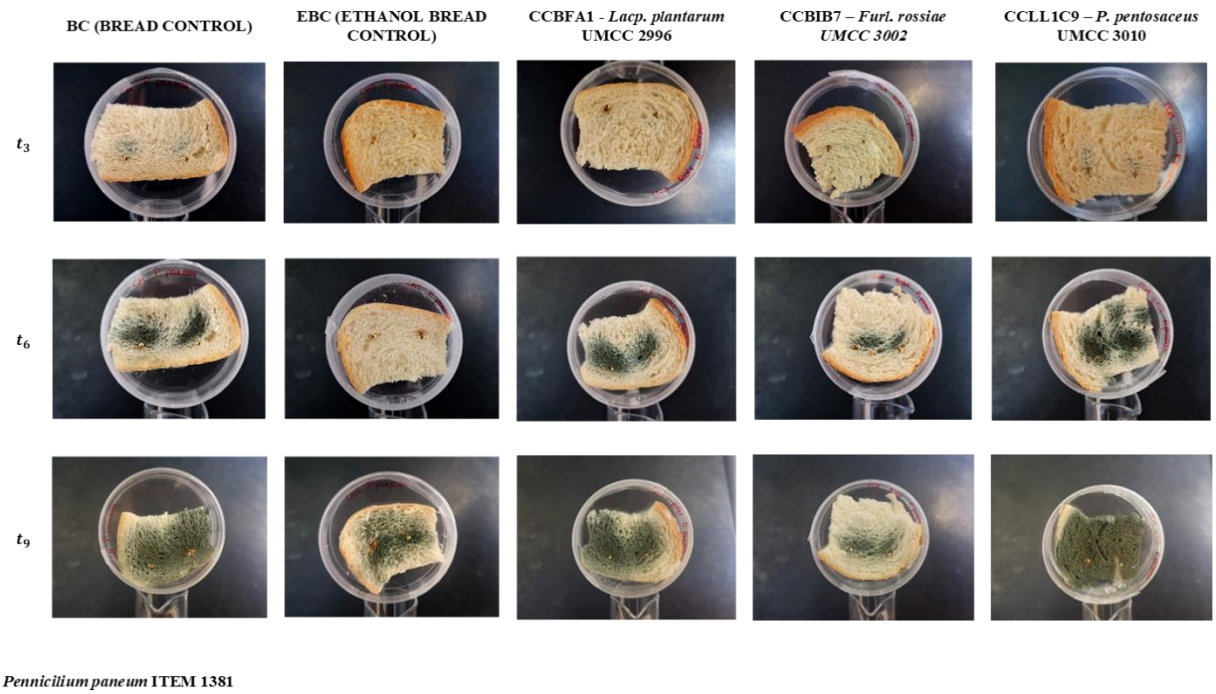


Figure S6. Anti-mold activity of the edible coating containing *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 against the simulation of microbial contamination of sliced bread with mycelium fungi of *Penicillium paneum* ITEM 1381 during 9 d of storage. The gold pin indicates the area where the mold was inoculated.

4. Chapter IV

This chapter is an introduction to the following paper **"Exploring the Inhibitory Activity of Selected Lactic Acid Bacteria against Bread Rope Spoilage Agents"** published in *Fermentation* on 16 March 2023.

4.1 Bread and Ropiness phenomena

Bread is recognized as an extremely significant staple food. As a result, cereals are among the most significant agricultural crops and monopolized around 60% of all farmed areas [1]. Bread and other products made from cereal account for more than 50% of the daily caloric intake worldwide [2]. According to different authors, by 2050, the world population is anticipated to increase by 50%, while grain demand is anticipated to more than double [2-5]. Consequently, meeting the world's growing population's demand for food is a massive challenge, notably in light of the fact that more than one-third of food is lost or squandered during postharvest processes [7]. Indeed, up to 50%–60% of cereals might be lost during processing due generally to the occurrence of microorganisms and insects, spoiling the cereals and making them inadequate for human and animal consumption [8]. The simplest type of bread is a dough made from a combination of water and flour, which can then be fermented before baking, frying, or steaming [4,5]. During bread or bakery goods production in general, grain seeds undergo various alterations in order to remove toxic or indigestible substances and accelerate the digestion of the beneficial components. In fact, processes such as dehusking, milling, high baking temperature, and subsequent Maillard reaction are applied [8]. The basis of cereals and bakery products contamination not only comprises different sources such as air, dust, water, soil, insects, birds, and rodents but also various stage processes from the storage and shipping containers to the processing facilities [1-2]. The nature of microbial contamination varies according to the

producing location and is greatly impacted by climatic factors including drought, rainfall, temperature, and sunshine, as well as unhygienic handling, soiled harvesting, processing tools, and unclean storage conditions [1]. Due to the disadvantaged conditions for their growth, bacteria are not generally involved as spoilage agents of dry grains, however, spore-forming bacterial species such as *Bacillus* spp. are able to endure and thrive later in the products [9]. In food coming from vegetable matrices, spore-forming bacteria are witnessed as a serious hazard [10]. In fact, spores exhibit usual resistance to physical thermal or non-thermal treatments normally applied in the food processing sector as well as to chemicals like disinfectants for later germinating and proliferating altering the final products [10]. Members of the genus *Bacillus*, and the related genus *Paenibacillus*, and *Lisynibacillus* are the main bacteria responsible for bread spoilage identified as rope or ropiness causing massive economic losses in artisanal and bakery industries [11-12]. Ropiness which is the most significant spoilage of bread and bakery products, alongside moldiness, occurs through bread crumb discoloration, and fruity sweetness that smells like ripe pineapple or melon [11-14]. Furthermore, when exposed to ropy agents, bread crumbs become mushy and sticky to the touch, and in more severe cases, can practically liquefy [12-14]. The primary characteristic of this rotting activity though is represented by the ropes or threads appearing when tearing two ends of bread apart (Figure 1)[13]. Though the baking temperature reaches 180–200 °C, the highest temperature attained in the crumb's core is about 97-101 °C for a short period, enabling the spore survival [12-13]. After successfully enduring baking, spores could easily germinate and thrive in environmentally favourable conditions, generally identified as temperatures over 25 °C, $a_w \geq 0.95$, and pH above 5.0 [12-15]. Additionally, only a modest concentration of spores in flour can lead to values of 10^7 CFU/g in bread crumb within 2 days, resulting in a consequent bread deterioration [12, 16]. Since ropiness

is often baffled with structure modification in bread, precise esteem of the economic losses due to these pathogens is challenging. However, economic issues are not the only concern, due to the toxigenic ability of some *Bacillus* species and the consequent threat to consumers' health leading to food poisoning [1-16]. On account of the aforementioned information, to prevent ropey spoiling, the baking business has relied on chemical preservatives such as sodium, potassium, or calcium salts of sorbic and propionic acid [17]. Nevertheless, persistent exposure to chemical preservatives may induce health problems. At the turn of the 21st century, the food sector stands a considerable challenge in providing foods that are not only efficient but also healthy and long-lasting relying on the use of organic preservatives such as LAB and their molecules [18-19]. In fact, to satisfy the new consumer's requirement and avoid the adverse effect caused by chemical preservatives, natural antimicrobial preservatives represent the core of the new food research [18].

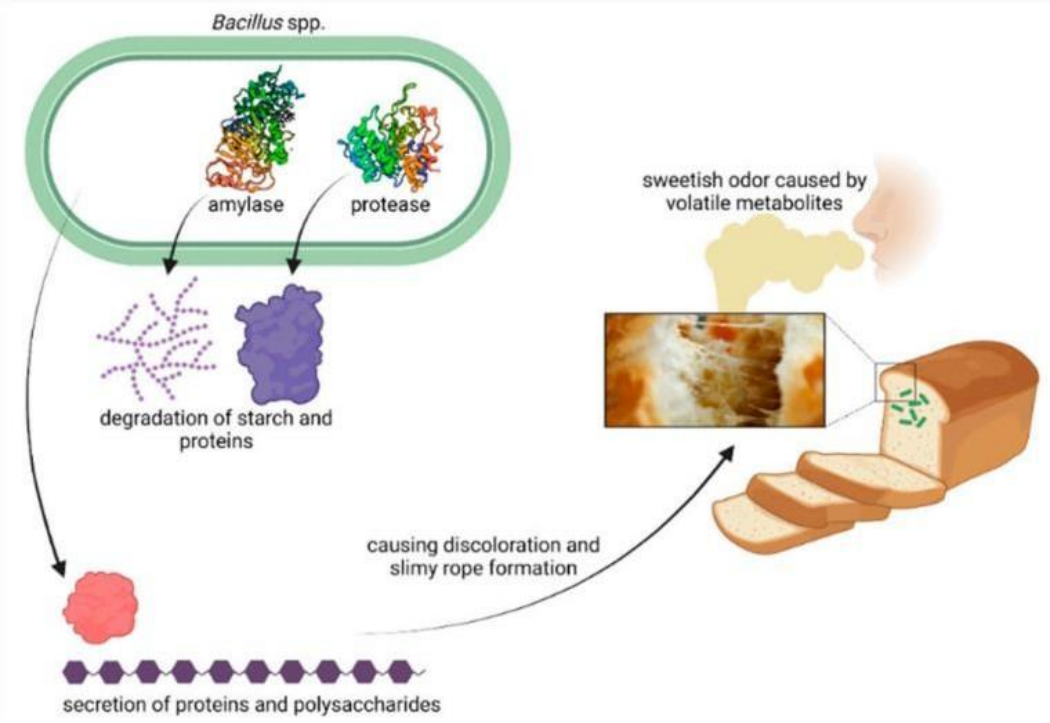


Figure 1. Mechanisms of alteration in bread due to ropy agents like *Bacillus* spp. Imagine from [15].

4.2 Rope species in bakery products and contaminant source

Starting from 1900 until now, several authors indicate *Bacillus* and the related genera *Paenibacillus* and *Lysinibacillus* as the major agents causing rope spoilage in bread [10-22]. These Gram-positive bacteria are widely distributed in nature and soil represented the primary endospore pool in the food chain [23]. During milling operations, microbial contaminants that are present in the raw material are frequently redistributed, leading to refined flour with poorer microbiological quality [24]. The number of microorganisms in raw cereals combined with inadequate milling hygiene directly impact the microbiological quality of flour since none of the milling activities contains a microbial-reduction stage. Due to the low a_w 0.60 flour is established as a microbiologically safe food unable to sustain bacterial growth, however, bacterial spore levels in flour can exceed 10^3 spores/g, and later jeopardize the quality of bread [23]. In addition, as a result of the research showing the impossibility to find commercial yeast devoid of bacteria, baker's yeasts are also identified as sources of putative spoilage agents [25-27]. Besides, rope-associated bacteria can be present also on equipment surfaces and the bakery environment.

Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Bacillus amyloliquefaciens, *Bacillus megaterium*, *B. cereus*, *Paenibacillus polymyxa* (previously *B. polymyxa*), and *Lysinibacillus* spp. such as *L. fusiformis* (previously *B. fusiformis*) are the major species accountable for rope spoilage in bakery products (Figure 2). However, the continuously changing within the *Bacillus* genus' taxonomy and the taxonomic collapse of the *B. cereus* group obstruct the accurate evaluation of species diversity causing rope deterioration [28-30]. Recently, according to various authors, the genus *Bacillus* only includes individuals from the Subtilis and Cereus clades, such as *B. subtilis, B. amyloliquefaciens,* and *B. pumilus,* and reclassification in 17 novel genera

and 11 existing one was made reclassifying various *Bacillus* in the *Paenibacillus* and *Lysinibacillus* genera [28-30].

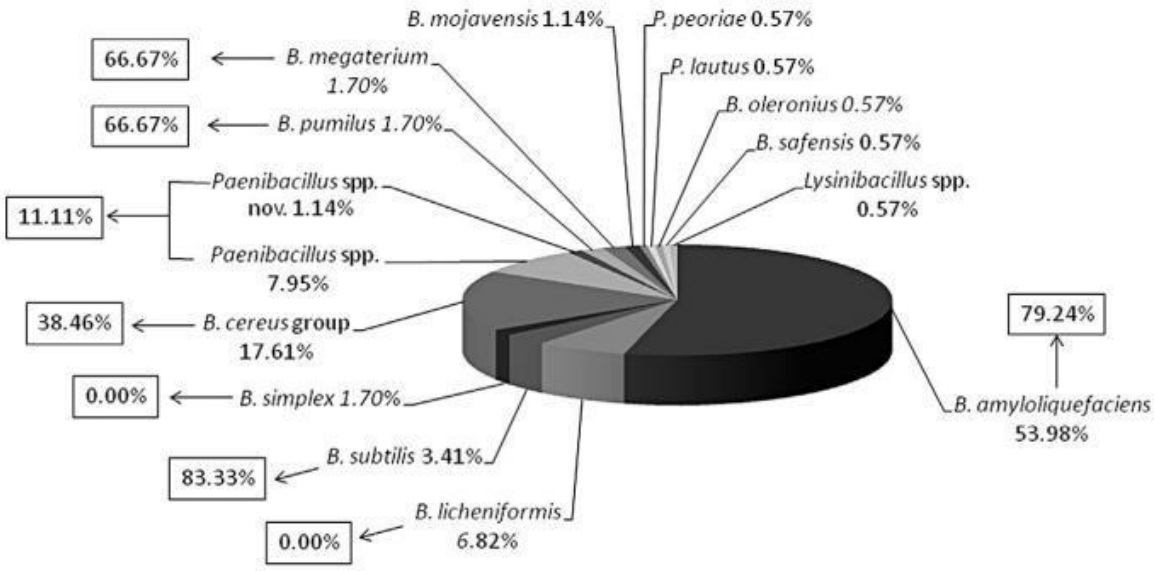


Figure 2. Incidence of spore-forming bacteria isolated from bread and bakery-related ingredients. The percentage reported in the square indicates the isolates able to cause rope spoilage following heat treatment at 100°C based on Valerio et al. [12]. Image from [31].

4.2.1 *Bacillus* spp.

The bacteria *Bacillus* spp. are Gram-positive, rod-shaped, facultatively anaerobic, motile, and capable of producing endospores [12, 14, 31]. Because *Bacillus* species are so common in nature, it is nearly difficult to prevent the presence of endospores in food products and raw components [31]. In addition to being heat stable and able to withstand baking their endospores are also extremely resistant to desiccation, radiation, and chemical agents. *B. cereus*, *B. subtilis*, *B. licheniformis, B. pumilus,* and *B. amyloliquefaciens* are the bacterial species most commonly linked to foodborne illness when it came to bakery items [12, 14, 18, 32-33]. Indeed, the

literature showed the presence of a wide variety of bacterial strains, mostly from the genus *Bacillus* and members of the *B. cereus* group [18, 32-34]. Due to the production of the hemolytic enterotoxin hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (CytK), which are heat-labile enterotoxins produced by *B. cereus* vegetative cells in the small intestine, diarrheal sickness an emetic syndrome can occur when ingested [31, 35-36]. Moreover, *B. cereus* strains can generate also hemolysins, collagenases, and phospholipases. In particular, the cyclic dodecadepsipeptide cereulide, responsible for the emetic symptom, showed resistance to high temperatures, proteolysis, and stability at pH between 2 -11 [31, 35-36]. The detection of this toxin was confirmed in different farinaceous products (bread, cakes, pasta) demonstrated also its insusceptibility to food processing and the gastrointestinal tract [31, 34]. Other species related to food poisoning are *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*. In fact, these species are responsible for the production of lichenysin A, heat-label enterotoxins, and amylosin, respectively [31, 35-38]. Furthermore also *B. pumilis* and *B. simplex* can cause foodborne illness due to the production of pumilacidin, a heat-stable cyclic lipopeptide and a heat-stable toxin comparable to cereulide [31, 38-40]. Therefore, as bread is ingested by individuals of all ages, as well as people affected by pre-existing illnesses and hospitalized patients, products having significant concentrations of *Bacillus* species, even not belonging to the *B. cereus* genus, should be deemed as dangerous [15].

4.2.2 *Lysinibacillus* spp.

The *Lysinibacillus* genus contains motile organisms with rod-shaped cells that generate ellipsoidal or spherical endospores. Members test positive for oxidase and catalase but negative for the synthesis of indole and H_2S [41]. As reported from different studies bakery products and

bread can be altered by *L. sphaericus*, *L. fusiformis*, and *L. boronitolerans* [12, 14-15, 42]. Due to the detection of heat-stable toxin production, clinical interest has been shown also for *Lysinibacillus* species, even though no association with foodborne illness has been found [43-44].

4.2.3 Paenibacillus spp.

The genus *Paenibacillus* was founded in 1993, and as indicated by their name (*paene*, in Latin, means nearly), they are considered almost-*Bacillus*. This genus contains around 30 species of facultative anaerobes, Gram-positive, low G+C, endospore-forming, neutrophilic, periflagellated heterotrophic bacteria [45]. One species, in particular, *P. polymyxa*, can be isolated from different environments such as soils, roots, and the rhizosphere of various crop plants, and it's considered a bakery product's spoilage agent [12-15, 31, 33, 45].

4.3 Ropiness prevention in bakery products by LAB

The use of LAB and sourdough, whose microbiota is dominated by these bacteria, is a commonly applied natural preservative against spoilage agents of bread and bakery products [15, 46-48]. Due to the production of lactic and acetic acid and the consequent pH dropping, LAB can largely prevent the development of ropiness agents [46-48]. Furthermore, other antimicrobial substances secreted by LAB, such as bacteriocins, ethanol, hydrogen peroxide, and fatty acids, contribute to the inhibition of ropy agents [15, 46-50]. As reported by various studies, the combination between acidic pH, organic acid production, and other antibacterial metabolites, derived from LAB, is crucial for the inhibition of *Bacillus* spp.. Furthermore, the different

studies emphasize the major inhibitory activity of the *in situ* metabolite production in contrast to the external addition of LAB antibacterial compounds, highlighting the importance of the application of functional LAB starter cultures as bio-preservatives [15, 18, 19, 46-53].

4.3.1 Organic acids from LAB

The presence of organic acids in food products is unquestionably the determining factor in increasing shelf life and safety. The amount of organic acid concentration is generally strain-dependent and can be affected by temperature, pH, and microbial interactions in the food matrix [54].

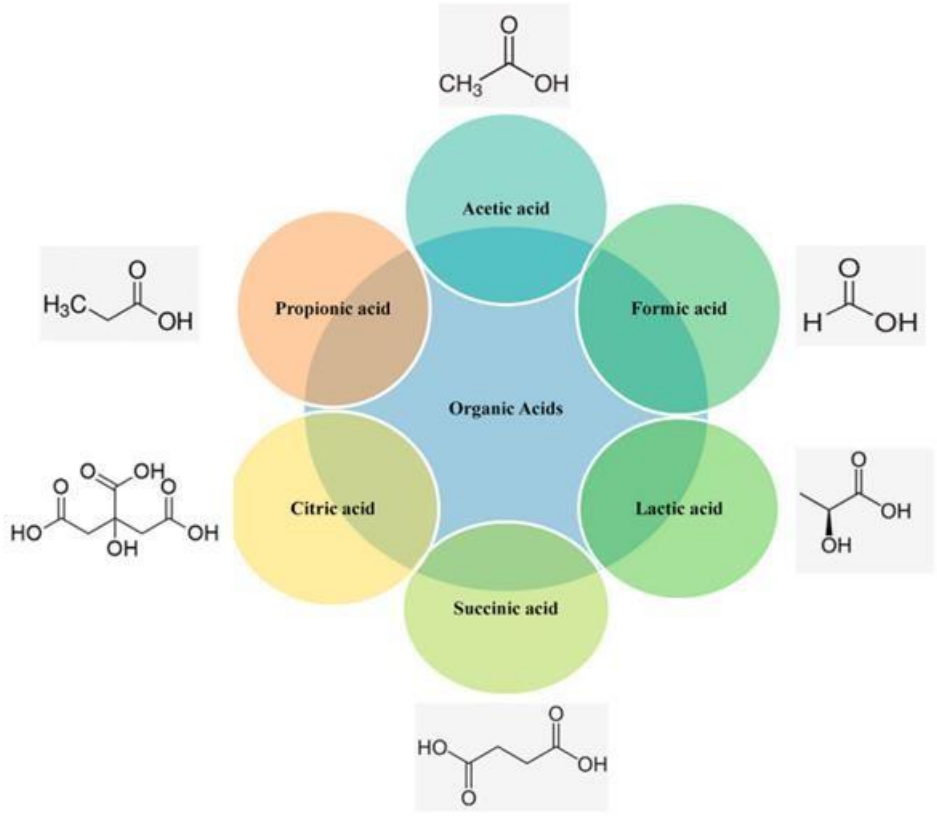


Figure 3. Organic acids produced by LAB. Image from [54]

Indeed, in-vitro synthesis of different organic acids by LAB (Figure 3) in MRS broth was studied by Ozcelik et al. [55] demonstrated how the generation of these compounds varies significantly depending on the bacterial strain and growing medium. Usually, short-chain organic acids, such as acetic acid, lactic acid, and succinate, exhibit antibacterial activity, while others like sorbic acid, also display antifungal properties [54]. Due to the aforementioned characteristics, the employment of LAB starter culture is a key tool for the enhancement of food safety and protection [51-57].

Lactic and Acetic Acid

The production of lactic acid (CH_3-CH(OH)-COOH) by LAB has a long history in the food business, and microbial technologies for its synthesis were developed around the turn of the 20th century [58]. This acid is used in a wide range of processed foods as an acidulating, flavouring, pH-buffering agent, and inhibitor of bacterial spoilage [56]. The solubility of non-dissociated lactic acid inside the cytoplasm membrane and the insolubility of dissociated lactate, which results in acidification of the cytoplasm and failure of proton motive forces, are the main causes of the inhibitory mechanism of this organic acid, affecting also the transmembrane pH gradient lowering the amount of energy available for cell growth [56]. CH_3–COOH commonly known as acetic acid, also demonstrated largely inhibition activity against both Gram-positive and Gram-negative pathogenic and spoilage bacteria, yeasts, and fungi. The particularly strong action of this acid is probably due to its low pKa [56]. In fact, the higher concentration of undissociated acid can travel through the cell membrane and cause acid stress as a result of dissociation [50-57]. Even if its effect is pH dependent and generally improved at acidic pH, commonly in the concentration of 0.2% a bacteriostatic effect can be seen while the bactericidal effect is displayed for concentrations exceeding 0.3% [56].

Succinic Acid

Succinate ($C_4H_6O_4$) is the last product of the fermentation process carried out by microorganisms and an intermediate product of the TCA cycle. Succinic acid can be produced both at chemical and natural levels with the use of LAB fermentation and its widely used in the food industry as a flavoring compound, acidulant, and anti-microbial agent [54, 59].

Propionic Acid

Propionic acid ($CH_3H_6O_2$) is a fungistatic agent used in food, that can also affect bacterial growth lowering viability in both Gram-positive and Gram-negative bacteria. Only very small amounts of propionic acid are produced by heterofermentative LAB. Propionic acids counteract the electrochemical proton gradient interacting with cell membranes. Although its impact is frequently influenced by the lactic acid-induced drop in pH [56].

Formic Acid

Formic acid (HCOOH) can be naturally present in several sources including insects such as ants and bees. Predominantly used as a preservative and antibacterial agent in livestock feed, formic acid in combination with other organic acids is known to display inhibitory activity against spoilage agents, moulds in particular also in bakery products [51, 54].

Citric Acid

Citric acid, a tricarboxylic acid ($C_6H_8O_7H_2O$) was discovered by Scheele in 1784 as a constituent of fruits, however, after the discovery of the TCA cycle is considered a common metabolite in all living systems. Citric acid is considered eco-friendly, economical, safe, versatile, and it's used as an antimicrobial compound in different food departments including bakery [60-64].

4.3.2 Bacteriocins

The term bacteriocin was formerly used to identify the *E.coli*'s colicin type of antimicrobial proteins and nowadays is used to define primary metabolites of polypeptides, proteins, or protein complexes produced by bacteria using ribosomes with antibacterial activity [50, 56]. Numerous bacterial species have been found able to produce bacteriocin, and various studies highlighted their employment in food preservation [65-67]. Bacteriocins are able to inhibit the reproduction and thriving of various bacteria, in particular, closely related species [68]. After many years of study and development, the molecular structure and antibacterial action of bacteriocins have been clearly documented. Bacteriocin has the ability to interact with the cell surface promoting cell permeability and impeding the development of cell walls, nucleic acid synthesis, and protein synthesis [50-56]. Following Klaenhammer [69] old division, bacteriocins can be categorized into four classes: class I of lantibiotics, class II divided into three subclasses: IIa, IIb, and IIc, class III and class IV represented by large peptides complexes with carbohydrates or lipids. However, due to the active research on them and the subsequent ever-changing classification, recent studies classified them only in three categories [70-71]. During these decades, due to their safety for human consumption and non-toxicity on eukaryotic cells, some bacteriocins have been employed in the food sector as bacteriostats and preservatives against a variety of foodborne pathogenic and spoilage bacteria [56, 65-71]. Furthermore, contrary to the bulk of pharmaceutical antibiotics, bacteriocins are proteinaceous molecules that the human digestive system's proteases promptly break down, and since their genetic determinants are usually plasmid-encoded, genetic manipulation is facilitated [56, 65-71].

4.3.2.1 Bacteriocins from Gram-positive Bacteria and their classification

Gram-positive bacteria's predominant bacteriocins fall into two broad categories [66-67, 70-71]. Class I consists of lantibiotics, whereas tiny, post-translationally unaltered bacteriocins are a part of class II [53, 66-71]. Lantibiotics are peptides with lanthionine and/or methyllanthionine residues undergoing substantial post-translational modifications. The diversity of this class of ribosomally produced and post-translationally modified peptides (RiPPs) has been demonstrated during the past ten years [66-68]. Bacteriocins can have very different chemical and spatial structures, as well as different biosynthetic characteristics. In Gram-positive bacteria, the synthesis of bacteriocins may be influenced by 2-21 genetic factors, and generally, different bacterial strains frequently synthesize many bacteriocins [71]. With the exception of a few classes, the majority of bacteriocins are synthesized as inactive precursors with an N-terminal leader peptide joined to the C-terminal peptide of the active centre later cut off by a protease in order to create a mature compound [71].

Class I

Lantibiotics are short peptides made up of a few uncommon thermostable amino acids. They may be created by combining two alanines to form lanthionine or by combining an amino butyric acid with an alanine to create b-methyl-lanthionine and they provide bacteriocin's structure stiffness and resistance to protease action.[65, 68]. This class generally includes 19 to 50 amino acids but may also contain non-standard amino acids such as lanthionine, -methyllanthionine, dehydrobutyrine, dehydroalanine, and labyrinthine because of substantial post-translational modifications [70]. Depending on the specifics of their production lantipeptides can be categorized into four groups, two of which comprise molecules having antibacterial action [67, 70]. Following the structural characteristics three forms of lantibiotic IA, IIA, and IB can be

distinguished, corresponding to bacteriocins with linear, combined, and globular conformation, respectively [71]. **Class IA** is represented by polycyclic antimicrobial peptide ribosomally synthesized by LAB during the fermentation process. Nisin, generated by some strains of *Lactococcus lactis* subsp. *lactis*, is the principal member of this class [56, 68]. The unusual amino acids lanthionine (Lan), methyllanthionine (MeLan), didehydroalanine (Dha), and didehydroaminobutyric acid (Dhb) are among the 34 amino acid residues composing nisin [72]. Nisin, regarded as safe from 1988 and commonly known in Europe as E234, inhibits various spoilage, spores, and pathogenic pathogens such as *L. monocytogenes*, *S. aureus*, *B. cereus*, and *C. botulinum* as well as LAB when associated with spoilage actions [72]. The **type IIA** lantibiotics vary from the aforementioned because of their thioether ring formation and the preserved consensus sequences in their leader peptides. Additionally, they have a "double-glycine" (GG/GA/GS) motif right before the cleavage site, which makes their leader sequences more similar to class II bacteriocins [65, 71]. Whereas, **class IB** is characterized by tetracyclic lantibiotics targeting only Gram-positive bacteria and displaying no activity against fungi or Gram-negative bacteria [72].

Class II

Class II, which is further classified into class IIa (pediocin-like bacteriocins), class IIb (two-peptide unmodified bacteriocins), class IIc (circular bacteriocins), and class IId (unmodified, linear, non-pediocin-like bacteriocins), comprises tiny heat-stable, unmodified peptides produced by several LAB [70, 73]. These peptides have hydrophobic and/or amphiphilic sections and are cationic with a neutral pH, and their action target is represented by Gram-positive bacteria cells resulting in damage and consequent death [72]. The subclasses division of these types of bacteriocins is primarily based on their amino acid sequence similarity, mode of action, and

target specificity [72]. **Class IIa**, also known as one-peptide pediocin-like bacteriocins, include Pediocin PA-1, Pediocin AcH, Lactococcin A, Lactococcin B, Sakacin A, and others [72]. The highly conserved amino acid motif Tyr-Gly-Asn-Gly-Val/Leu (also known as the YGNGV motif or "pediocin box") near their N-termini and the presence of two cysteine residues forming a disulfide bond are the two characteristics that distinguish pediocin-like peptides, which range in length from 37 to 58 residues [72]. In addition, the members of this category display a similar range of bacterial inhibition. Pediocin PA-1 is the most explored bacteriocin of this category and has been successfully utilised as a bio-preservative in food against the genus *Listeria, Bacillus, Clostridium*, and *Staphylococcus* [56, 72-73]. **Class IIb** consists of two complimentary cationic peptides and its antimicrobial activity is produced when both peptides are present in equal amounts, due to this mechanism of action it is also known as two-peptide bacteriocins [72]. Two main bacteriocins Lactococcin G and Enterocosins are part of this class IIb [67-72]. Generally, genes in the same operon are expressed at the same time, and the two peptides typically work together to provide significant synergistic effects. Their mechanism of action likewise involves a decrease in the intracellular ATP content and the loss of membrane potential. Individually, these peptides have relatively little action [72]. **Class IIc** comprises miscellaneous unmodified bacteriocins. They are generated by Gram-positive bacteria and are commonly known as circular cationic peptides [72]. All single-peptide non-modified bacteriocins that do not meet the requirements of type IIa or type IIb are automatically members of type IIc, as there are no restrictions imposed by either physical structure or traits of their genetic loci. Compared to other kinds of linear antimicrobial peptides, bacteriocins' circular properties increased their antibacterial efficacy and stability. This mechanism of action is due to the introduction of a class IIc circular backbone into the cytoplasmic membrane of the target bacterium cells resulting in the

creation of pores and cell death [67, 72]. An example of bacteriocins class IIc are sakacin Q and Enterocin AS-48 [67]. In **Class IId** the fundamental structures and modes of action of this family of bacteriocins against the intended Gram-positive bacteria are quite diverse. They are resistant to protease enzymes and are considered stable antimicrobial peptides under high acidity and high-temperature circumstances. Aureocin A53 is the greatest illustration of this class IId of bacteriocins [72].

Class III

Large, heat-instable proteins classified as class III bacteriocins have molecular weights of more than 30 kDa and are further separated into class IIIa and class IIIb bacteriocins [68]. Their mode of action differs from the other bacteriocins, in fact, this type of bacteriocin encourages the lysis of the target microorganism's cell wall. Their C-terminal part identifies the target cell, whereas their N-terminal component is homologous to an endopeptidase involved in cell wall production [68]. **Class IIIa** includes proteolytic enzymes (bacteriolysin) such as Lysostaphin, active against target Gram-positive bacteria destroying the peptidoglycan in the cell wall structure [72]. Whereas **class IIIb** is identified by non-lytic bacteriocins like helveticin J a 37-kDa bacteriocin that preferentially targets other *Lactobacillus* species when the pH is neutral and the environment is aerobic or anaerobic [68, 72]. The mechanism of action is able to break the target Gram-positive bacteria cell wall and the cytoplasmic inner membrane causing the outflow of cell contents [72].

Class IV

Complex bacteriocins with lipid or carbohydrate moieties are referred to as class IV bacteriocins [68]. Sublancin and Glycocin F, two bacteriocins that have been studied and placed in different subclasses, belong to class IV [72]. Sublancin is a glycopeptide utilised as an alternative to

traditional antibiotics against some strains of *S. aureus*, while Glycocin F, produced by *Lacp. plantarum* and generally active against Gram-positive bacteria is a 43-amino acid bacteriocin that possesses two beta-linked N-acetylglucosamine moieties coupled by side chain connections to a serine (Ser18) via oxygen and to a cysteine (Cys43) via sulphur [72].

Numerous studies have suggested how LAB metabolite outcomes such as organic acids and bacteriocins can substitute chemical preservatives in the food leading to broader use of bio-preservatives [15, 18-19, 46-62, 65-73]. Since environmental and growth factors control the production and effectiveness of the various metabolites, it is necessary to determine the ideal circumstances in which to use them and explore novel isolation niches. In conclusion, LAB can be considered an *in situ* living factor in food.

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Exploring the Inhibitory Activity of Selected Lactic Acid Bacteria against Bread Rope Spoilage Agents

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Abstract: In this study, a wide pool of lactic acid bacteria strains deposited in two recognized culture collections was tested against ropy bread spoilage bacteria, specifically belonging to *Bacillus* spp., *Paenibacillus* spp., and *Lysinibacillus* spp. High-throughput and ex vivo screening assays were performed to select the best candidates. They were further investigated to detect the production of active antimicrobial metabolites and bacteriocins. Moreover, technological and safety features were assessed to value their suitability as biocontrol agents for the production of clean-label bakery products. The most prominent inhibitory activities were shown by four strains of *Lactiplantibacillus plantarum* (NFICC19, NFICC 72, NFICC163, and NFICC 293), two strains of *Pediococcus pentosaceus* (NFICC10 and NFICC341), and *Leuconostoc citreum* NFICC28. Moreover, the whole genome sequencing of the selected LAB strains and the in silico analysis showed that some of the strains contain operons for bacteriocins; however, no significant evidence was observed phenotypically.

Keywords: lactic acid bacteria; starter culture; organic acids; rope spoilage; bread spoilage; bakery products

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Citation: Iosca, G.; Fugaban, J.I.I.; Özmerih, S.; Wätjen, A.P.; Kaas, R.S.; Hà, Q.; Shetty, R.; Pulvirenti, A.; De Vero, L.; Bang-Berthelsen, C.H. Exploring the Inhibitory Activity of Selected Lactic Acid Bacteria against Bread Rope Spoilage Agents. *Fermentation* **2023**, *9*, 290. https://doi.org/10.3390/fermentation9030290

Academic Editor: John Samelis

Received: 28 February 2023
Revised: 13 March 2023
Accepted: 14 March 2023
Published: 16 March 2023

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1. Introduction

Bread is considered an essential staple food in many cultures, being a valuable source of proteins, lipids, vitamins, and minerals [1]. Unluckily, bread and most of the bakery products available on the market are characterized by short shelf life. Generally, bread quality can be affected by physicochemical decomposition, known as staling, and microbiological contamination, with daily losses ranging from 9.7% to 14.4% [2]. Among bread-spoilage bacteria involved in bakery product loss, those belonging to *Bacillus* spp., including *B. subtilis*, *B. licheniformis*, *B. cereus*, *B. amyloliquefaciens*, *B. mycoides*, *B. pumilus*, and a few species of the genera *Paenibacillus* and *Lysinibacillus*, contribute to two-thirds of total food waste, along with fruit and vegetables [3–7]. Contamination by these microorganisms leads to "ropy" bread, which is characterized by an unpleasant ripe fruity odor similar to an overripe pineapple, melon, valerian, or honey, with a sticky, soft, and discolored crumb due to enzymatic degradation of starch, proteins, and exopolysaccharides (EPS) [8]. These bacteria are normally present in the bakery environment, surfaces, and atmosphere [9]. Furthermore, raw materials such as wheat, seeds, semolina, and brewer's yeast during harvesting and processing conditions are an optimal colonizing source for bacteria, which can prosper during storage periods. Humidity and heat enable bacteria growth to reach high levels of contamination in the flour after the milling process, leading to a higher speed of the rotting process in bread (24–48 h) [7]. The successful colonizing ability of these bacteria is associated with their endospores, a highly thermoresistant structure, which allows retention of their viability during the baking process [10]. The ubiquitous presence and the

spore-forming ability of these microorganisms, in combination with the increasing demand for eco-friendly ways of handling food and food products themselves, make the elimination of these spoilage agents a challenge, both at the artisanal and industrial levels. Furthermore, the increasing trend of "green consumerism" demands to be met particularly by the food industries. With this, chemical preservatives are now being rejected, which results in "new" standards considered for food safety and extended shelf-life [11,12]. Ideally, the need for alternative preservatives should be obtained from naturally occurring sources that can be achievable using microorganisms or/and their metabolites [13]. In this context, selected lactic acid bacteria (LAB) can be exploited to prevent spoilage of bread and contribute both to the production of sensory properties and microbial safety of bakery products [14,15]. The use of LAB as a starter culture has a long history in a variety of fermented foods. Particularly in sourdough, lactic acid fermentation is considered one of the most prominent antispoilage "technologies" due to the production of lactic acid, acetic acid, fatty acids, short peptides, and the pH reduction, which lead to the suppression of several spoilage agents [16–18]. Several authors have already investigated different LAB cultures for sourdough as an additive-free method to avoid rope development in bakery products [19–22]. In this study, a wide pool of LAB strains, provided by two recognized culture collections in Italy and Denmark, were tested against some common bread spoilage bacteria, specifically belonging to *Bacillus* spp., *Paenibacillus* spp., and *Lysinibacillus* spp. High-throughput and ex vivo screening assays were performed to select the best candidates. They were further investigated to detect the production of active antimicrobial metabolites and bacteriocins. Moreover, the technological and safety features of the selected strains were assessed. The final goal was the detection of candidate LAB strains with an inhibitory activity to be potentially used as biocontrol agents for the production of clean-label bakery products.

2. Materials and Methods

2.1. Microorganisms Used in This Study

2.1.1. Lactic Acid Bacteria Strains

A total of 18 LAB strains from the *Lactobacillus*, *Pediococcus*, and *Leuconostoc* groups were selected to be tested against bread spoilage agents (Table 1). Five strains were provided by the Unimore Microbial Culture Collection (UMCC), University of Modena and Reggio Emilia (Italy), and the remaining strains were provided by the National Food Institute Culture Collection (NFICC), Technical University of Denmark (Table 1). UMCC strains were identified and characterized by 16 s RNA gene sequencing in previous works and selected for their antispoilage activity [14,15]. Regarding NFICC strains, they were identified by using the MALDI Biotyper® sirius IVD System (BRUKER, Roskilde, Denmark) or through whole-genome sequencing, and selected for their suitability to ferment plant-based substrates [23]. The original culture of the strains is maintained in their respective collections by cryopreservation at −80 °C in cryovials containing De Man, Rogosa, Sharpe (MRS) broth (Oxoid, Milan, Italy) mixed with 25% (*v*/*v*) glycerol. An active culture of each strain was used for all the reported screenings.

2.1.2. Spoilage Bacteria Strains

According to previous works by Saranraj and Gheeta [24] and Valerio et al. [6], a total of 29 different bread spoilage bacterial strains were chosen for the screenings (Table 2). They were provided by the NFICC collection and the German Collection of Microorganisms and Cell Cultures (DSMZ).

Table 1. Lactic acid bacteria strains tested in the present study. They were provided by the National Food Institute Culture Collection (NFICC), Technical University of Denmark and by Unimore Microbial Culture Collection (UMCC), University of Modena and Reggio Emilia (Italy).

Strain Code	Species	Isolation Source
NFICC10	*Pediococcus pentosaceus*	Sourdough
NFICC19	*Lactiplantibacillus plantarum*	Dill
NFICC27	*Lactiplantibacillus plantarum*	Sourdough
NFICC28	*Leuconostoc citreum*	Sourdough
NFICC58	*Pediococcus pentosaceus*	Sourdough
NFICC72	*Lactiplantibacillus plantarum*	Gooseberry
NFICC87	*Leuconostoc citreum*	Beetroot
NFICC94	*Leuconostoc citreum*	Spinach
NFICC103	*Pediococcus pentosaceus*	Pumpkin
NFICC163	*Lactiplantibacillus platarum*	Field pea
NFICC207	*Lactiplantibacillus plantarum*	Glasswort
NFICC293	*Lactiplantibacillus plantarum*	Dragsholm plant
NFICC341	*Pediococcus pentosaceus*	Brewer's spent grain
UMCC 2990	*Fructilactobacillus sanfranciscensis*	Sourdough type I
UMCC 2996	*Lactiplantibacillus plantarum*	Dough for Panettone
UMCC 3002	*Furfurilactobacillus rossiae*	Dough for Panettone
UMCC 3010	*Pediococcus pentosaceus*	Gluten-free sourdough
UMCC 3011	*Leuconostoc citreum*	Dough for Panettone

Table 2. Selected bread spoilage bacteria tested in the present study. They were provided by the German Collection of Microorganisms and Cell Cultures (DSMZ) and the National Food Institute Culture Collection (NFICC).

Strain Code	Species	Isolation Source
DSM 2301	*Bacillus cereus*	Food poisoning incident
DSM 4222	*Bacillus cereus*	-
DSM 4312	*Bacillus cereus*	Vomit
DSM 22905	*Bacillus cytotoxicus*	Vegetable puree
NFICC119	*Lysinibacillus fusiformis*	Beetroot
NFICC432	*Paenibacillus polymyxa*	Walnut
NFICC503	*Bacillus mycoides*	Beech leaves
NFICC510	*Bacillus altitudinis*	Plant
NFICC526	*Bacillus mycoides*	Red fir
NFICC528	*Bacillus subtilis*	Sourdough
NFICC529	*Lysinibacillus sphaericus*	*Common Juniper*
NFICC530	*Bacillus pumilus*	*Common Juniper*
NFICC531	*Bacillus simplex*	*Common Juniper*
NFICC532	*Lysinibacillus fusiformis*	*Common Juniper*
NFICC740	*Bacillus cereus*	Plant
NFICC781	*Bacillus cereus*	Kombucha
NFICC816	*Bacillus thuringiensis*	Animal feces
NFICC855	*Bacillus weihenstephanensis*	Potato
NFICC869	*Bacillus amyloliquefaciens*	Pasteurized BSG
NFICC871	*Lysinibacillus sphaericus*	Pasteurized BSG
NFICC879	*Lysinibacillus boronitolerans*	Potato
NFICC882	*Lysinibacillus fusiformis*	Potato
NFICC889	*Lysinibacillus boronitolerans*	Potato
NFICC906	*Bacillus simplex*	Potato
NFICC1127	*Bacillus amyloliquefaciens*	Pasteurized BSG *
NFICC1130	*Bacillus amyloliquefaciens*	Pasteurized BSG
NFICC1525	*Bacillus subtilis*	Herring Garum
NFICC1534	*Bacillus subtilis*	Miso
NFICC1549	*Bacillus licheniformis*	Apple pulp

* BSG: brewers' spent grains.

2.2. Screenings for Antibacterial Activity of the LAB Strains

2.2.1. High-Throughput Screening Assay with LAB Cell-Free Supernatants (CFS)

The preliminary screening of the selected LAB activity against 29 bread spoilage strains was performed by using the LAB cell-free supernatants (CFS), following the protocol of Inglin et al. [25] with some modifications. The different spoilage strains were inoculated in a definite medium, specifically, brain heart infusion (BHI) broth (Oxoid, Milan, Italy) (*Bacillus* spp.), M17 broth (Oxoid, Milan, Italy) (*Lysinobacillus* spp.), and potato dextrose (PD) broth (Oxoid, Milan, Italy) (*Paenibacillus* spp.), and incubated at 30 °C for 24 h. The LAB strains were incubated in MRS broth at 30 °C for 48 h. LAB supernatants were obtained by centrifuge at 6000× *g* 15 min following sterile filtration (0.20 µm) in new Eppendorfs (ThermoFisher, Waltham, MA USA). After 50 µL of BHI/M17/PD containing 0.5% of an overnight culture of the chosen spoilage agent was transferred to a 200 µL clear-glass, flat-bottom 96-well microtiter plate (ThermoFisher, Waltham, MA, USA) using a multichannel pipette. Then, 30 µL of LAB supernatant was transferred to each well. Optical density at 600 nm (OD_{600}) was measured at time zero (t0) using a plate reader infinite M200PRO (Tecan, Männedorf, Switzerland), and results were analyzed using the formula 1.5 × OD_{600}t0. Then, the plates were incubated at 30 °C (optimal condition for the spoilage strains), and the OD_{600} was subsequently controlled after 24 h and 48 h. For each strain, OD_{600} value below the formula threshold indicated an inhibition activity.

2.2.2. Double-Agar-Layer Screening Assay

The double-agar-layer screening assay was performed by adapting the protocol described by Iosca et al. [15]. Briefly, spoilage strains were incubated in their respective optimal media mentioned previously (BHI broth, M17 broth, and PD broth) at 30 °C for 24 h. After incubation, initial OD_{600} was measured and adjusted to a final concentration of 10^5 CFU/mL. LAB strains were grown in MRS broth at 30 °C for 24 h, then OD_{600} was measured and adjusted as needed to obtain 10^8 CFU/mL. After, 1 mL of MRS agar was poured into 24-well plates (ThermoFisher, Waltham, MA, USA) and overlaid with 500 µL of the specific growth medium of the spoilage strains (BHI agar for *Bacillus* spp.; M17 agar for *Lysinobacillus* spp.; and PD agar for *Paenibacillus* spp.). After solidification, 10 µL of the spoilage agents was spread on each well and left to dry. Subsequently, a hole of 4 mm was made and 5 µL of each LAB strain was inoculated in the wells. Results were taken after 48 h of incubation at 30 °C, wherein growth inhibitions were noted. Scores were designated as follows: complete inhibition of the spoilage agents was scored 3, strong inhibition was scored 2, and moderate to weak inhibition was scored 1, while no inhibition was scored 0.

2.3. Confirmatory Assay of Antibacterial Activity in Bread Medium (BM)

To evaluate the ex vivo antibacterial activity of the LAB strains selected from the previous screenings, a culture medium from plain wheat bread was simulated following the protocol designed by Verni et al. [26] and Iosca et al. [17] with some modifications. Briefly, 600 g of wheat bread was homogenized using a blender to facilitate the enzyme treatment, and water was added in a 1:4 ratio (*w/w*). After the blended mixture, Neutrase, an endoprotease (1.5 AU/g) from *Bacillus amyloliquefaciens* (Novozymes, Bagsværd, Denmark) was used at 1.5 mL/L, while Amylase (Novozymes, Bagsværd, Denmark) was added at 0.5 g/L. Proteolytic and amylolytic enzymes were then added to facilitate nutrient compound availability. Bread suspensions were then incubated at 55 °C for 18 h, considered the enzymes optimal conditions. After the incubation, mixtures were centrifuged (6000 rpm for 20 min) and supernatants were collected. The medium pH was adjusted to around 5.7, filtered, and sterilized at 121 °C for 15 min. Preselected lactic acid bacteria CFS, obtained as previously described, were then tested in ex vivo conditions using BM to detect the best performers. As above, Inglin et al.'s [25] protocol was performed. LAB and selected *Bacillus* spp. spoilage agents were grown in BM. All screening assays were carried out in triplicate. Data were analyzed and compared using a one-tailed *t*-test. Strains with significant inhibitory activities were selected and further analyzed.

2.4. Assessment of the LAB Bioactive Compounds

2.4.1. High-Performance Liquid Chromatography (HPLC) Detection of the Main Compounds Produced in MRS Fermentation

To detect and identify the main compounds produced by the most prominent inhibitory LAB strains, the CFS of the evaluated strains were examined by HPLC after 14 h, 18 h, and 24 h of fermentation in MRS at 30 °C. HPLC was equipped with an Aminex HPX-87H column (300 × 7.8 mm column) (Bio-Rad, Hercules, CA, USA), and a Shodex RI-101 refractive index detector was used. The flow rate of the mobile phase (5 mM H_2SO_4) was 0.5 mL/min, and the column oven temperature was maintained at 60 °C. All reagents were analytically pure, standard curves were first identified individually, and retention times were calculated. Oxalic acid, tartaric acid, formic acid, lactic acid, citric acid, acetic acid, and succinic acid were the key acids chosen for the analysis, as suggested by Hui-Hu et al. [27]. All the samples were loaded in triplicate. MRS broth was also analyzed as a control. Statistical analysis was performed using Kruskal–Wallis nonparametric analysis. A p-value of <0.05 was considered statistically significant.

2.4.2. Bacteriocins Production

The potential bacteriocin production from the various LAB was evaluated by treating LAB's CFS with proteolytic enzymes, including proteinase K, trypsin, and α-chymotrypsin at a final concentration of 0.1 mg/mL. The preparation of CFS was performed as previously described. Subsequently, after 1 h incubation at 37 °C, heat treatment at 95 °C × 10 min was performed to terminate the enzymatic processes before spotting it on a plate [28].

Moreover, another assay of CFS spotted on a BHI was performed according to the method described by Fugaban et al. [28]. Briefly, the selected LAB were cultured in MRS broth at 30 °C for 18 h, and the CFS was obtained by centrifugation (8000× *g*, 10 min). Using sterile 1 M NaOH, supernatant's pH was adjusted to 6.5 and heat treated at 80 °C for 10 min to inactivate putative proteolytic enzymes and eliminated hydrogen peroxide. The supernatant was then filtered with 0.2 µm syringe filters (Sartorius Ministart Syringe hydrophilic Filter, Göttingen, Germany). Ten microliters of the CFS were then spotted on a BHI plate with 1% agar seeded with appropriative test organisms at a final viable cell count of ~10^5 CFU/mL and left to dry. Plates were then incubated for 24 h at 30 °C to observe the formation of inhibition zones. The experiment was conducted in triplicate.

2.4.3. Kinetic Screening Assay

To further confirm that the antibacterial activity of the selected LAB inhibits spoilage growth, the method proposed by Fugaban et al. [28] was followed. Test organisms, *B. cereus* DSM 2301, *B. thuringensis* NFICC816, *B. weihenstephanensis* NFICC855, *B. amyloliquefaciens* NFICC1130 and NFICC1127, and *B. licheniformis* NFICC1549, were grown individually in sterile 96-well flat-bottom plates. After 3 h, LAB-CFS, obtained as previously described, was added to the appropriate wells. Sterile BHI inoculated with 10% test organisms was dispensed in the first 10 columns of the plate, leaving the last two for sterility control and growth control. Experiments were performed in two independent set-ups for each LAB-CFS, with the test organism being treated. Plates were incubated at 30 °C for 18 h and, simultaneously, the OD_{600} nm were measured for 24 h.

2.5. Phenotypical Characterization of the LAB Strains

2.5.1. API Test

Carbohydrate utilization was characterized by using API 50 CHL panel test (bioMérieux, Marcy l'Etoile, France) according to the protocol suggested by the manufacturers. Briefly, all the LAB strains were initially grown in MRS broth for 18 h at 30 °C. Subsequently, cells were collected by centrifugation and washed twice in 0.85% NaCl saline solution. Cell concentrations were adjusted to 0.5 McFarland. Suggested volumes of inoculum were added to each corresponding well. Set-ups were incubated at 30 °C, and color

changes were monitored after 24 h and 48 h. Results were interpreted based on the manufacturers' recommendations.

2.5.2. Determination of Acidification Ability in BM USING ICINAC

Acidification profiles were generated for the antibacterial strain in BM. Exponentially growing LAB, previously incubated in MRS at 30 °C for 18 h, were washed as mentioned before, and 1 mL was inoculated in 40 mL of sterile BM, obtained as already described in the previous paragraph. The fermentation was performed in 40 mL of liquid Bread Media and monitored with the *iCinac* system (AMS alliance, Frepillon, France), which allows monitoring of acidification kinetics during fermentation [29,30]. The cultures were incubated in a water bath at 30 °C during all procedures, and sampling was recorded every 30 min for 48 h.

2.6. Safety Assessment

2.6.1. Hemolytic Activity

The hemolytic activity of the LAB present in this study was evaluated using Columbia Blood Agar (Oxoid LTD, Basingstoke, UK) with 5% defibrinized horse blood according to Fugaban et al. [31]. Strains grown for 18 h in MRS at 30 °C were spot-plated (10 µL) on the agar surface. Positive hemolytic activity was indicated by clear yellow zones around the bacterial growth (β-hemolysis). The reference strains used were *Staphylococcus aureus* NFICC1477, *B. cereus* DMS 2301, and *Lc. citreum* NFICC88 as controls for α-, β-, and γ-hemolysis, respectively. All experiments were performed in triplicate.

2.6.2. LAB Antimicrobial Susceptibility

The antimicrobial susceptibility testing (AST) was performed according to the suggestions set by the European Food Safety Authority (EFSA) in the set guidelines for the assessment of AST of human and veterinary significant microorganisms [32]. The assay was performed in a 96-well microplate using microbroth dilution with specific antibiotics from Sigma-Aldrich (ampicillin, chloramphenicol, ciprofloxacin, gentamycin, kanamycin, streptomycin, and vancomycin) on cation-adjusted Mueller–Hinton broth supplemented with MRS (5.0 g/L). The assay included 10 antibiotic dilutions in two-fold and controls (growth and sterility controls). Inocula were adjusted to 0.5 McFarland units and disseminated appropriately to obtain a final concentration of 10^5 CFU/mL. The plates were incubated following EFSA guidance (35 ± 1 °C for 18 h). The lowest concentration with complete bacterial inhibition was recorded as the MIC and analyzed according to the standards set for LAB.

2.7. Whole-Genome Sequencing Analysis: In Silico Screening for Functional Genes and Virulence Genes

Whole-genome sequencing of the selected LAB strains was performed using Illumina technology. Libraries for paired-end sequencing were constructed using the Nextera XT kit (Illumina, CA, USA) guide 15031942v01. The pooled Nextera XT libraries were loaded onto an Illumina NextSeq reagent cartridge using the NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) with a standard flow cell. Raw FASTQ files were trimmed using standard Trimmomatic settings, and genome assembly was performed using Unicycler [33,34]. Subsequently, annotation and conversion of the genome in protein sequence were obtained using the Bacterial and Viral Bioinformatics Resource Center (BV-BRCbeta, https://www.bv-brc.org/, accessed on 13 January 2023) [35,36]. To detect putative bacteriocins' sequence and ribosomally synthesized and post-translationally modified peptides (RiPPs), BAGEL4 (http://bagel4.molgenrug.nl, accessed on 15 December 2022) was used in combination with UniProt Consortium and the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/, accessed on 1 December 2022) using BLAST protein [37,38].

3. Results

3.1. Screening Assays

3.1.1. High-Throughput Preliminary Screening

The high-throughput screening assay suggested by Inglin et al. [25], which aims for an efficient screening for putative antispoilage LAB, was conducted using 96-well plates. The obtained results were analyzed based on the formula: $1.5 \times OD_{600}$ t0. Results are reported in Table S1. All the strains in which the OD_{600} value after 24 h and 48 h resulted to be under the cut-off derived from the formula were considered to be putative inhibitory candidates. All results obtained in this preliminary screening were further evaluated. In this assay, the best antagonistic strains were *L. plantarum* UMCC 2996 and NFICC 19, 27, 72, 163, 207, and 293. Additionally, *P. pentosaceus* UMCC 3010 and NFICC 10, 58, 103, and 341, along with *Lc. citreum* NFICC28 and NFICC94, demonstrated having the highest inhibitory activity in the typical growth media used.

3.1.2. Antibacterial Activity of LAB Assessed by Double-Agar-Layer Screening Assay

Additional assessment of the antibacterial activity of the LAB strains was assessed against *Bacillus* spp. using well diffusion assay in 24-well microtiter plates. Inhibitions were analyzed and graded based on the scores previously mentioned, and results are indicated in Table S2. Assessments of activities showed that the strain *P. pentosaceus* NFICC341 had the broadest inhibition (66% of the spoilage agents), followed by 66% for *P. pentosaceus* UMCC 3010 and 57% for *Lc. citreum* UMCC 3011 and *F. sanfranciscensis* UMCC 2990. A strong inhibition was also detected for *F. rossiae* UMCC 3002 and *L. plantarum* NFICC207, with a percentage inhibition of 52% for both strains. For the other evaluated strains, even though inhibitory abilities were detected, due to a percentage under 50%, no significant antagonistic activity was observed.

3.2. Antibacterial Activity of LAB by Confirmatory Assay in BM

To confirm the activities of the CFS of the selected LAB candidates, an ex vivo experiment was performed using liquid BM. This is to mimic the nutritional bread conditions to allow the evaluation of the actual antimicrobial potential of the candidates. Results obtained after 48 h were investigated with the use of one-tailed *t*-test as reported in Figures 1–6. Based on the results obtained by the first screenings, *Bacillus cereus* DSM 2301 was tested against the CFS of five LAB (Figure 1). The set-up treated with *L. plantarum* NFICC19 showed the strongest inhibitory activity, with a significant difference compared with the control DSM 2301 in BM.

Similar action was detected for *L. plantarum* UMCC 2996, *Lc. citreum* NFICC28, and *F. sanfranciscensis* UMCC 2990. The lowest activity was shown by *L. plantarum* NFICC293. *B. thuringiensis* NFICC816 was challenged with the CFS of six LAB strains, specifically, *L. plantarum* NFICC19, 27, 293, *Lc. citreum* NFICC28, and *P. pentosaceus* NFICC341 (Figure 2). Here, only *Lc. citreum* NFICC28 was able to significantly prevent the growth of the spoilage organism. Additionally, *B. weihenstephanensis* NFICC855 was challenged with various LAB-CFS, including *L. plantarum* strains (NFICC19, 27, 72, 207, and 293), *F. sanfranciscensis* UMCC 2990, *Lc. citreum* NFICC28, and *P. pentosaceus* strains (UMCC 3010, NFICC58, and 341). As reported in Figure 3, significant results were obtained. Candidates from all four species were able to inhibit pathogen growth, particularly *L. plantarum* NFICC72, NFICC163, and *P. pentosaceus* NFICC58.

Two strains of *B. amyloliquefaciens*, NFICC1130 and NFICC1127, were used in these experiments (Figures 4 and 5), recording completely different results even if challenged with the same LAB-CFS strains. The majority of the LAB were able to inhibit *B. amyloliquefaciens* NFICC1130; the highest inhibitory activities were recorded for *P. pentosaceus* NFICC10 and *L. plantarum* NFICC19. On the contrary, *B. amyloliquefaciens* NFICC1127 showed no inhibition with the same LAB-CFS. The CFS of different LAB strains, including *L. plantarum*, *Lc. Citreum*, and *P. pentosaceus*, were tested against *B. licheniformis* NFICC1549, as reported

in Figure 6. *L. plantarum* NFICC72 and NFICC293 appear to be the best inhibitory agents, along with *Lc. citreum* NFICC94.

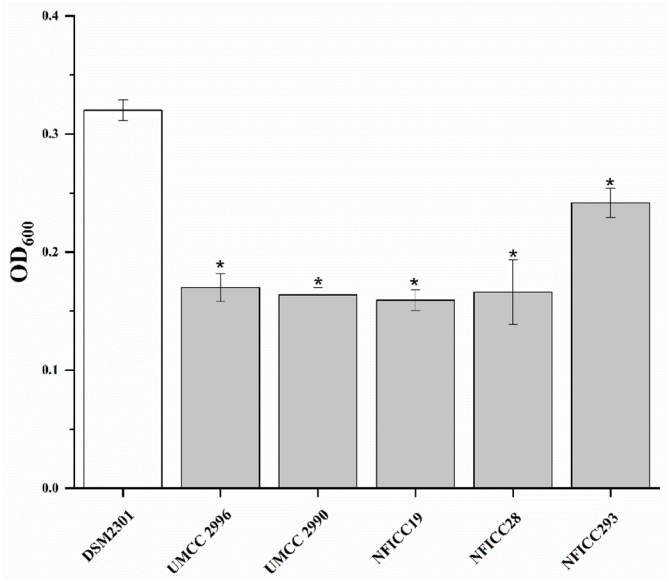


Figure 1. Antibacterial activity of the selected LAB' cell-free supernatant in Bread Media (BM) against *B. cereus* DSM 2301. One-tail *t*-test was performed comparing OD_{600} value after 48 h growth of *B. cereus* DSM 2301 in BM and the same pathogens challenged with CFS of *L. plantarum* (UMCC 2996, NFICC19, and 293), *F. sanfranciscensis* (UMCC 2990), and *Lc. citreum* (NFICC28). The mean value of all the treated samples was significantly lower than the control sample (untreated sample); t(2) = −2.92 and $p = 0.05$. Bars with * are significantly different.

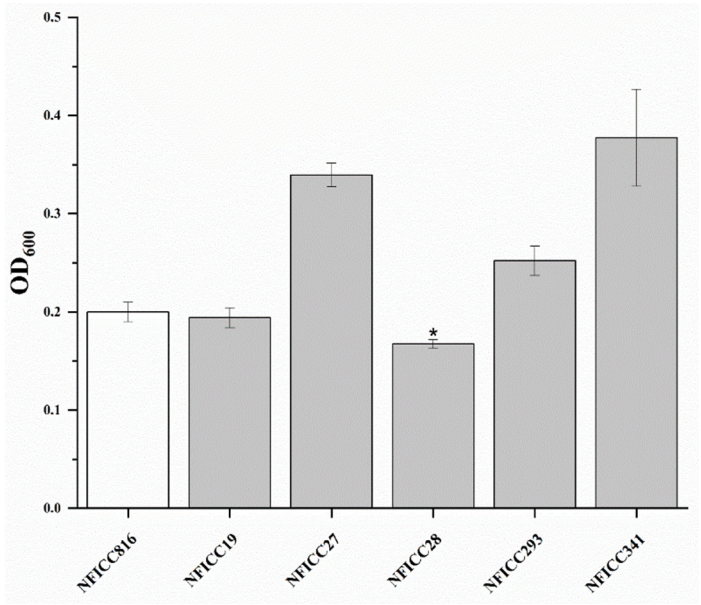


Figure 2. Antibacterial activity of the selected LAB's cell-free supernatant in Bread Media (BM) against spoilage agents *B. thuringensis* NFICC816. One-tail *t*-test was performed on the obtained triplicates comparing OD_{600} value after 48 h growth of NFICC816 in BM and the same pathogens challenged with CFS of *L. plantarum* (NFICC19, 27, and 293) and *P. pentosaceus* (NFICC341). The mean value of one treated sample of *Lc. citreum* (NFICC28) was significantly lower than the control sample (untreated sample), while the other samples respected the null hypothesis; t(2) = −2.92 and $p = 0.05$. Bars with * are significantly different.

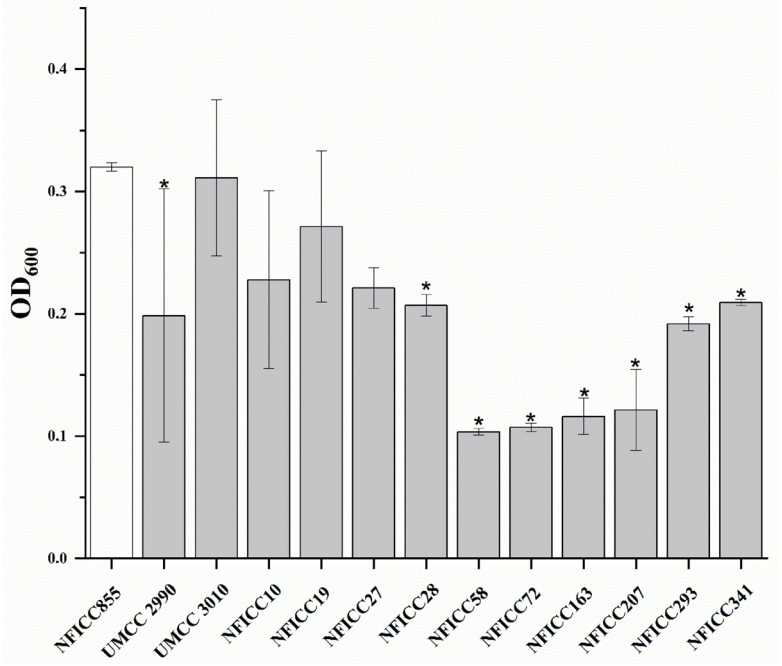


Figure 3. Antibacterial activities of the selected LAB's cell-free supernatant in Bread Media (BM) against spoilage agents *B. weihenstephanensis* NFICC855. One-tail t test was performed on the obtained triplicates comparing OD_{600} value after 48 h growth of NFICC855 in BM and the same pathogens challenged with CFS of *L. plantarum* (NFICC19, 27, 72, 207, and 293), *F. sanfranciscensis* (UMCC 2990), *Lc. citreum* (NFICC28), and *P. pentosaceus* (UMCC 3010, NFICC 58, and 341). The mean value of the samples represented by * was significantly lower than the control sample (untreated sample); t(2) = −2.92 and $p = 0.05$.

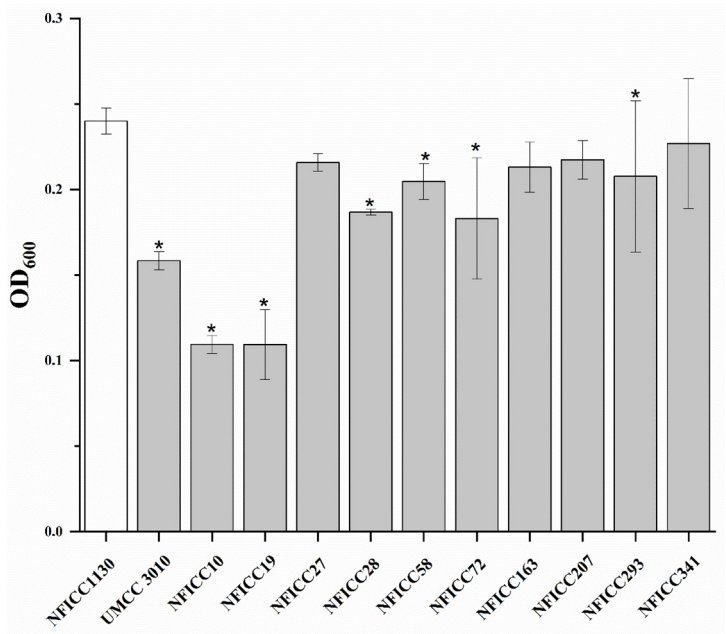


Figure 4. Antibacterial activity of the selected LAB's cell-free supernatant in Bread Media (BM) on spoilage agent *B. amyloliquefaciens* NFICC1130. One-tail *t*-test was performed on the obtained triplicates comparing OD_{600} value after 48 h growth of NFICC1130 in BM and the same pathogens challenged with CFS of *L. plantarum* (NFICC9, 27, 72, 163, 207, and 293), *Lc. citreum* (NFICC28), and *P. pentosaceus* (UMCC 3010, NFICC10, 58, and 341). Bars with * are significantly different, with a mean value significantly lower than the control sample (untreated sample), while samples NFICC 27, 163, 207, 293, and 341 respect the null hypothesis; t(2) = −2.92 and $p = 0.05$.

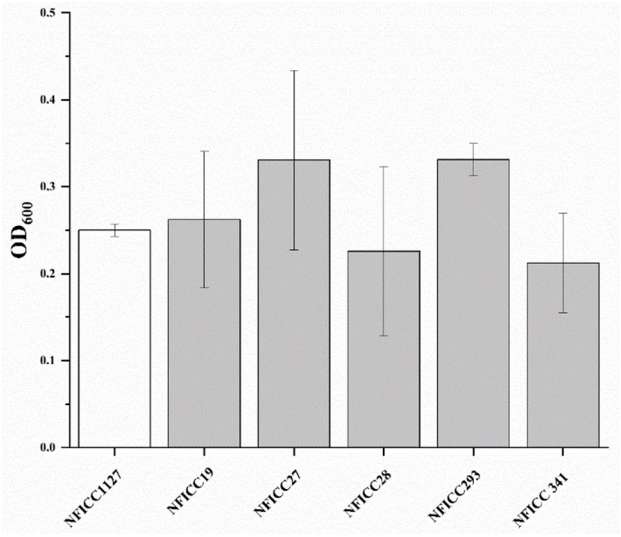


Figure 5. Antibacterial activity of the selected LAB's cell-free supernatant in Bread Media (BM) on spoilage agent *B. amyloquiefaciens* NFICC 1127. One-tail t test was performed on the obtained triplicates comparing OD600 value after 48 h growth of NFICC1127 in BM and the same pathogens challenged with CFS of *L. plantarum* (NFICC19, 27, and 293), *Lc. citreum* (NFICC28), and *P. pentosaceus* (NFICC341). No significant differences between the control and treated samples were observed at t(2) = −2.92 and $p = 0.05$.

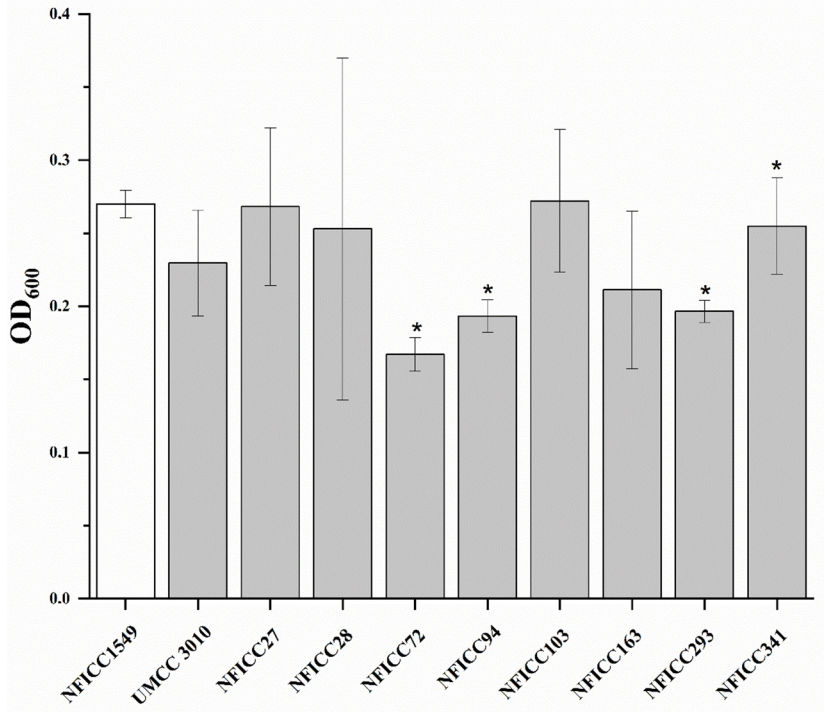


Figure 6. Antibacterial activity of the selected LAB's cell-free supernatant in Bread Medium (BM) on spoilage agent *B. licheniformis* NFICC1549. One-tail *t*-test was performed on the obtained triplicates comparing OD_{600} value after 48 h growth of NFICC1549 in BM and the same spoilage agent challenged with CFS of *L. plantarum* (NFICC27, 72, 163, and 293), *Lc. citreum* (NFICC28 and 94), and *P. pentosaceus* (UMCC 3010 and NFICC341). The * indicate measurements that are significantly different, with a mean lower than the control samples; t(2) = −2.92, $p = 0.05$.

3.3. Detection of Bioactive Compounds

3.3.1. Metabolites Detected by HPLC during Fermentation in MRS

LAB's ability to produce organic acids, other potentially antimicrobial compounds, and sugar consumption was assessed (Table 3). The CFS of the evaluated strains were examined by HPLC after 14 h, 18 h, and 24 h of fermentation in MRS. Observation of the results shows a corresponding ratio between amounts of lactic acid and acetic acid after 24 h. In congruence with the assessment for acidification, *L. plantarum* strains NFICC72, 163, 293, and 19 showed a strong and fast acidification activity. The present results also show that the ethanol detected is too low or not produced at all. Other compounds such as citric acid, oxalate, tartaric acid, and succinic were detected in trace amounts only.

3.3.2. Evaluation of the Bacteriocins Production and Kinetic Screening Assay

The screening of potential bacteriocin production revealed no antibacterial protein-associated inhibitions. Moreover, the assay of CFS spotted on a BHI confirmed the inhibitory activity of acids compounds and the absence of produced bacteriocins due to the nonformed inhibition zones. The results obtained from the kinetics assay highlight the bacteriostatic activity of the selected LAB. In fact, observations of the results demonstrates the presence of an extended lag phase, supporting the presence of organic acids in the cell-free supernatants and the absence of bacteriocins.

3.4. Assessment of Technological Features of the Candidate LAB Strains

3.4.1. Sugar Fermentation Profiling (API Test)

Sugar fermentation profiles obtained by API 50 CHL galleries were assessed for all the selected LAB, as indicated in Table 4, wherein the most common sugars in bakery products are highlighted (complete API profiles after 48 h are shown in Table S3). Results show that the most utilized sugars by the strains include glucose, fructose, maltose, and cellobiose.

3.4.2. Acidification Ability in BM

To further assess the strains' performance for dough fermentation, their acidification ability was tested in BM during a period of 24 h. In Figure 7, only the best performers are reported. A decrease in pH ≈ 4.5, which is considered to be safe, was noted. The strains considered to be the fastest acidifiers were *L. plantarum* (UMCC 2996, NFICC19, and 163), *Lc. citreum* NFICC28, and *P. pentosaceus* UMCC 3010, which reached a pH ≈ 4.5 with at least 10 h of fermentation.

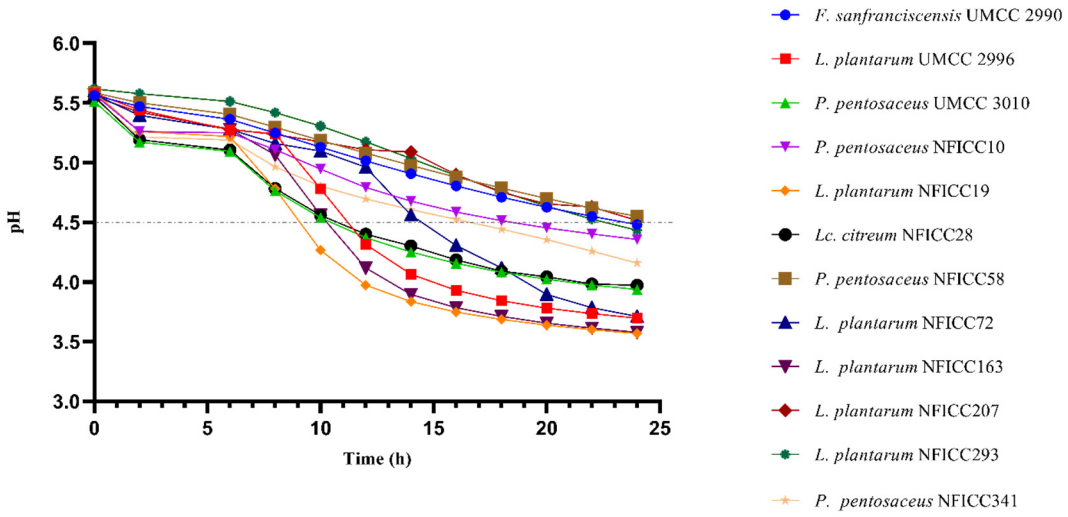


Figure 7. Acidification performance in Bread Medium showed by the best LAB candidates against bread rope-producing strains of *Bacillus* spp.

Table 3. Different compounds (g/L) detected by HPLC after 14, 18, and 24 h of fermentation in MRS broth. The creation of the heat map is based upon the detected MRS broth concentration: green color highlights the production, red color the consumption, and yellow color was used when the detected amount was equal to MRS broth. The measurement were with three independent experiments, and Kruskal–Wallis statistical analysis was performed to detect the significant strains with respect to plain MRS. The significance is marked with an *.

	Sample/time	MRS	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC2 996	*P. pentosaceus* UMCC 3010	*P. pentosaceus* NFICC10	*L. plantarum* NFICC19	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*L. plantarum* NFICC163	*L. plantarum* NFICC207	*L. plantarum* NFICC293	*P. pentosaceus* NFICC341
OXALATE	14H	0.31	0.37	0.36	0.38	0.39	0.95 *	0.34	0.32	0.39	0.57 *	0.39	0.30	0.34
	18H		0.37	0.33	0.34	0.40 *	0.93 *	0.39 *	0.38	0.38	0.29	0.36	0.63 *	0.39 *
	24H		0.37	0.44	0.44	0.48 *	1.38 *	0.57 *	0.41	0.70 *	0.69 *	0.32	0.55 *	0.37
CITRIC ACID	14H	1.86	0.00 *	0.00 *	0.00 *	0.00 *	1.77	1.04	1.18	0.00 *	0.00 *	0.00 *	0.00 *	0.38 *
	18H		1.13	0.00 *	1.06	1.12	1.05	1.05	1.86	0.92 *	0.09 *	1.04	0.00 *	0.00 *
	24H		0.99	0.90 *	1.04	0.96	1.60	0.40 *	2.15	0.00 *	0.00 *	0.81 *	0.00 *	1.10
TARTARIC ACID	14H	0.00	1.12 *	1.08	1.13 *	1.12 *	1.17	1.47 *	1.00	1.10 *	1.07	1.19 *	0.97	1.08
	18H		1.12 *	1.00	1.03	1.08 *	0.81	1.32 *	0.00	1.02	0.61	1.17 *	0.00	0.00
	24H		0.00	0.00	0.00	0.00	1.22 *	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GLUCOSE	14H	14.62	14.33	9.34 *	12.25	11.56	6.32 *	6.97 *	8.00 *	13.39	7.02 *	13.62	6.04 *	10.13
	18H		14.99	7.06 *	11.19 *	11.13 *	0.95 *	5.37 *	5.67 *	11.28	4.87 *	11.74	5.23 *	8.59 *
	24H		14.02	11.86	9.04 *	8.98 *	0.98 *	2.78 *	4.03 *	0.00 *	0.00 *	9.52	4.07 *	7.61 *
SUCROSE	14H	1.49	1.79	0.76	1.33	1.57	0.00 *	0.00 *	0.00 *	1.57	0.93	1.03	0.51	1.14
	18H		1.68	0.75	1.26	1.25	0.71	0.00 *	0.00 *	0.49 *	0.51 *	0.92	0.49 *	0.95
	24H		1.72	1.33	1.22	1.34	0.70 *	0.00 *	0.00	0.87 *	0.83 *	1.06	1.11	1.08
GLUTAMIC ACID	14H	0.23	0.08 *	0.25	0.06 *	0.07 *	0.00 *	0.23	0.15	0.08 *	0.10	0.00 *	0.17	0.18
	18H		0.13	0.13	0.11 *	0.12 *	0.21	0.10 *	0.12 *	0.13	0.11 *	0.09 *	0.13	0.18
	24H		0.09	0.00 *	0.00 *	0.00 *	0.23	0.00 *	0.00 *	0.20	0.20	0.00 *	0.00 *	0.00 *
SUCCINIC	14H	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	2.07 *	0.00	0.00
	18H		0.75 *	0.59	0.70 *	0.74 *	0.00	0.00	0.07	0.44	0.48	1.10 *	0.44	0.00
	24H		0.00	0.00	0.00	0.00	1.22 *	0.00	0.08	0.00	0.00	1.15 *	0.00	0.00
FORMIC ACID	14H	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.07 *	0.00	0.00	0.09 *	0.07	0.15 *
	18H		3.76 *	0.01	0.13 *	0.15 *	0.00	0.00	0.11 *	0.03	0.00	0.03	0.00	0.08
	24H		1.44 *	0.01	0.23 *	0.18 *	0.00	0.00	0.12	2.02 *	0.00	0.01	0.00	0.14
ACETIC ACID	14H	0.00	4.36	4.77 *	4.73 *	4.48	4.90 *	5.44 *	0.16	4.34	4.73 *	5.14 *	4.41	4.56
	18H		4.56 *	4.45 *	4.38 *	4.50 *	4.86 *	5.52 *	2.23	4.35	4.49 *	4.89 *	4.49 *	4.47 *
	24H		4.39	4.41	4.40	4.55 *	4.79 *	4.77 *	4.29	4.60 *	4.65 *	4.84 *	4.38	4.51 *
1,3 PROPANDIOL	14H	0.00	0.71	0.85 *	0.83 *	0.82 *	0.86 *	0.92 *	0.71	0.75	0.86 *	0.89 *	0.84 *	0.81
	18H		0.79	0.78	0.81	0.82	0.89 *	0.86 *	0.81	0.53	0.85 *	0.83 *	0.88 *	0.75
	24H		0.67	0.74	0.79 *	0.80 *	0.84 *	0.67	0.84 *	0.70	0.84 *	0.83 *	0.86 *	0.82 *
2,3 BUTANDIOL	14H	0.00	0.05	0.06	0.21 *	0.20 *	0.00	0.00	0.10	0.18 *	0.00	0.29 *	0.00	0.18 *
	18H		0.18 *	0.00	0.16 *	0.18 *	0.00	0.00	0.13	0.14	0.00	0.26 *	0.00	0.06
	24H		0.00	0.06	0.19 *	0.17 *	0.00	0.00	0.15	0.00	0.00	0.26 *	0.00	0.08

Table 3. *Cont.*

	Sample/time	MRS	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC2 996	*P. pentosaceus* UMCC 3010	*P. pentosaceus* NFICC10	*L. plantarum* NFICC19	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*L. plantarum* NFICC163	*L. plantarum* NFICC207	*L. plantarum* NFICC293	*P. pentosaceus* NFICC341
LACTIC ACID	14H	0.00	0.59	12.05 *	6.14	5.50	14.73 *	5.47	4.97	2.88	14.25 *	2.95	13.33 *	7.98 *
	18H		0.86	13.08 *	5.59	6.34	19.78 *	6.16	7.91 *	6.56	16.14 *	3.13	14.77 *	9.18 *
	24H		0.78	5.21	9.38	10.08	19.90 *	16.91 *	12.27 *	20.32 *	20.78 *	4.69	16.11 *	11.43
ETOH	14H	0.00	0.00	0.00	0.00	0.00	0.00	1.32 *	0.00	0.00	0.00	0.00	0.00	0.00
	18H		0.00	0.00	0.00	0.00	0.00	1.58 *	0.00	0.00	0.00	0.00	0.00	0.00
	24H		0.00	0.00	0.00	0.00	0.00	0.37 *	0.00	0.00	0.00	1.74 *	0.00	0.00

Table 4. Sugar fermentation profiles of the tested LAB strains obtained by API 50 CHL after 48 h of incubation at 30 °C.

LAB	D(+)-Glucose	D(−)-Fructose	D(+)-Cellobiose	Maltose	Lactose	D(+)-Melibiose	Saccharose	D(+)-Raffinose	Starch	D(+)-Xylose
F. sanfranciscensis UMCC 2990	+	+	+	+	−	−	−	−	−	−
L. plantarum UMCC 2996	+	+	+	+	+	+	+	+	−	−
F. rossiae UMCC 3002	+	+/−	−	+	−	+/−	−	−	−	+
P. pentosaceus UMCC 3010	+	+	+	+	−	−	−	−	−	−
Lc. citreum UMCC 3011	+	+/−	−	+	−	−	−	−	−	+
P. pentosaceus NFICC10	+	+	+	+	+	+	+	+	−	−
L. plantarum NFICC19	+	+	+	+	+	+	+	+	−	−
L. plantarum NFICC27	+	+	+	+	+	+	+	+	−	−
Lc. citreum NFICC28	+	+	+	+	+	+	+	+	−	+
L. plantarum NFICC58	+	+	+	+	+	+	+	+	−	+
L. plantarum NFICC72	+	+	+	+	+	+	+	+	−	−
P. pentosaceus NFICC87	+	+	+	+	−	−	+	−	−	+
Lc. citreum NFICC94	+	+	+	+	−	−	+	−	−	+
P. pentosaceus NFICC103	+	+	+	+	+	+	+	+/−	−	−
P. pentosaceus NFICC163	+	+	+	+	+	+	+	+	−	+/−
L. plantarum NFICC207	+	+	+	+	+	+	+	+	−	+
L. plantarum NFICC293	+	+	+	+	+	+	+	+	−	−
P. pentosaceus NFICC341	+	+	+	+	+	+	+	+	−	+/−

3.5. Safety Profile of the Selected LAB Strains

3.5.1. Hemolytic Activity Profile

Nonhemolytic activity is considered a safe prerequisite for the selection of new strains for food starter culture [39,40]. Results indicate that all the examined strains did not show signs of β-hemolytic activity when grown in Columbia Blood Agar (Table 5). Eleven LAB strains exhibited green-hued zones around colonies showing α-hemolytic activity (partial hemolysis), while seven strains including *F. rossiae* UMCC 3002, *Lc. citreum* UMCC 3011, NFICC28, NFICC94, and *P. pentosaceus* NFICC58 and NFICC341 were γ- hemolytic (nonhemolysis).

Table 5. Hemolytic capacity * of the tested strains.

Tested Strains	γ-Hemolysis	α-Hemolysis	β-Hemolysis
F. sanfranciscensis UMCC 2990		x	
L. plantarum UMCC2996		x	
F. rossiae UMCC 3002	x		
P. pentosaceus UMCC 3010		x	
Lc. citreum UMCC 3011	x		
P. pentosaceus NFICC10		x	
L. plantarum NFICC19		x	
L. plantarum NFICC27		x	
Lc. citreum NFICC28	x		
L. plantarum NFICC58	x		
L. plantarum NFICC72		x	
P. pentosaceus NFICC87	x		
Lc. citreum NFICC94	x		
P. pentosaceus NFICC103		x	
P. pentosaceus NFICC163		x	
L. plantarum NFICC207		x	
L. plantarum NFICC293		x	
P. pentosaceus NFICC341	x		
S. aureus NFICC1477	x		
Lc. citreum NFICC88		x	
B. cereus DMS 2301			x

* The occurrence of the specific hemolytic activity is marked by "x".

3.5.2. Antimicrobial Susceptibility Profiles (AST)

The sensitivity of LAB strains was determined against various antibiotics (streptomycin, ampicillin, vancomycin, gentamicin, kanamycin, and chloramphenicol), and the obtained results were compared based on the cut-offs, as specified in the guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance from EFSA [32]. Sensitivity profiles are shown in Table 6. Briefly, almost all of the tested LAB show resistance against streptomycin (99%), except *P. pentosaceus* NFICC341 and vancomycin (100%), as already reported by different studies. However, against ampicillin (81%), kanamycin (76%), chloramphenicol (99%), and gentamycin (57%), the majority of them were in accordance with the EFSA cut-off and could be considered susceptible.

3.5.3. In Silico Screening for Resistance Genes

All 18 isolates were analyzed for the presence of acquired resistance genes using ResFinder version 4.2.3 and ResFinder database version 2.0.1 [41] using genome assemblies. Parameters used for a matching identity on the database were required to be at least 80%, whereas the coverage of a matching gene in the database was required to be at least 60%.

3.6. Whole-Genome Sequencing Analysis: Functional Gene and Potential Virulence In Silico Screening

The presence of bacteriocins was initially tested following the protocol by Fugaban et al. [28] with no positive results. In addition, to further confirm the possible absence of bacteriocins,

sequences of the whole genome obtained from all the evaluated LAB were analyzed using the web server BAGEL4. Although phenotypically no bacteriocin-associated inhibitions were observed, in silico analysis showed the presence of bacteriocins belonging to class IIb and Iic (particularly penocin and plantaricins A, E, F, K, J, and N) (Table 7). The structures of the putative operons are shown in Figure S1.

Table 6. Minimum inhibitory concentration (MIC) (μg/mL) of the antibiotic susceptibility of the selected LAB. The values reported in bold and blue color are under or equal to recommended EFSA cut-offs and underline the susceptibility of the strains.

Tested LAB	Tested Antibiotics					
	Streptomycin	Ampicillin	Kanamycin	Vancomycin	Chloramphenicol	Gentamycin
F. sanfranciscensis UMCC 2990	≤128	≤16	**≤32**	512	**≤4**	**≤4**
L. plantarum UMCC 2996	≤128	≤32	**≤64**	512	Resistant	≤256
F. rossiae UMCC 3002	≤128	≤4	**≤32**	512	**≤4**	**≤4**
P. pentosaceus UMCC 3010	≤128	**≤2**	**≤16**	512	**≤4**	**≤4**
Lc. citreum UMCC 3011	≤128	≤4	≤32	512	≤8	≤8
P. pentosaceus NFICC10	≤128	**≤1**	**≤16**	512	**≤4**	**≤4**
L. plantarum NFICC19	≤64	**≤1**	**≤16**	512	**≤4**	≤4
L. plantarum NFICC27	≤64	**≤1**	**≤16**	512	**≤4**	≤4
Lc. citreum NFICC28	≤128	**≤1**	≤32	512	**≤4**	**≤4**
L. plantarum NFICC58	≤128	**≤1**	**≤32**	512	**≤4**	**≤4**
L. plantarum NFICC72	≤64	**≤1**	**≤16**	512	**≤4**	≤4
Lc. citreum NFICC87	≤128	**≤1**	≤32	512	**≤4**	**≤4**
Lc. citreum NFICC94	≤128	**≤1**	≤32	512	**≤4**	**≤8**
P. pentosaceus NFICC103	≤128	**≤2**	**≤32**	512	**≤4**	**≤4**
P. pentosaceus NFICC163	≤64	**≤1**	**≤16**	512	**≤4**	≤4
L. plantarum NFICC207	≤64	**≤1**	**≤16**	512	**≤4**	≤4
L. plantarum NFICC293	≤128	**≤1**	**≤8**	≤8	**≤4**	≤4
P. pentosaceus NFICC341	**≤64**	**≤2**	**≤32**	512	**≤4**	**≤4**

Table 7. Bacteriocins produced by the LAB strains displaying the highest inhibitory activity against ropy agents.

Strain	Species	Bacteriocins Predicted by BAGEL4
NFICC28	*Lc. citreum*	None
NFICC10	*P. pentosaceus*	Penocin A
NFICC58	*P. pentosaceus*	None
NFICC19	*L. plantarum*	Plantaricin E, Plantaricin F Plantaricin K, putative class IIc bacteriocin, putative class IIb bacteriocin
NFICC72	*L. plantarum*	Plantaricin E, Plantaricin F Plantaricin K, putative class IIc bacteriocin, putative class IIb bacteriocin
NFICC163	*L. plantarum*	Plantaricin E, Plantaricin F Plantaricin K, putative class IIc bacteriocin, putative class IIb bacteriocin
NFICC293	*L. plantarum*	Plantaricin A, Plantaricin E, Plantaricin F, Plantaricin J, Plantaricin K, Plantaricin N
UMCC 2996	*L. plantarum*	Plantaricin A, Plantaricin E, Plantaricin F, Plantaricin J, Plantaricin K, Plantaricin N

4. Discussion

Bacillus spp. are well associated with ropy bread and bakery product spoilage [6,7,42]. Clean-label strategies to control microbial spoilage in bakery industries are important, accordingly, some starter cultures, including LAB, are promising alternative biocontrol agents thanks to their ability to produce bacteriocins and organic acids [22,43]. To screen

candidates efficiently, we adapted the high-throughput screening assay developed by Inglin et al. [25], which was identified as a fast, low-cost, and accurate primary screening. Based on the results, most of the LAB strains found to be active against *Bacillus* spp. strains originated from fruits and vegetables (NFICC strains) or sourdoughs (UMCC strains). Their ability to adapt to these highly variable and stressful environments has aided in their capacity to grow into different niches, which can be exploited for their beneficial features, such as acidification ability and competitiveness [11,44,45].

To further assess the ability of the strains, a well diffusion assay in 24-well microtiter plates was conducted using live cells, confirming 18 active strains comprised of representatives from *Lactobacillus*, *Pediococcus*, and *Leuconostoc* genera, from both screening assays. Observations in this study were similar to those of Adesulu-Dahunsi et al. [46] against *Bacillus* spp., *Listeria monocytogenes*, and *Escherichia coli*.

The capacity of the *P. pentosaceus* strain NFICC341 to inhibit the growth of the majority of the spoilage microorganisms can be traced back to its origin, which was Brewers' spent grain. The potential of LAB strains isolated from the vegetable matrix was already demonstrated by Puntillo et al. [47]. In accordance with previous studies, similar behavior can be associated with strains of *Lc. citreum*, *L. plantarum*, and *F. sanfranciscensis*, isolated from plants and sourdoughs, which exhibited promising inhibitory activity [48,49]. Furthermore, it was noted that *L. plantarum* and *P. pentosaceus* are well-known species that are able to produce antimicrobial compounds and organic acids, which can influence the safety of food [44,45,50,51]. Differences between the activities of the strains from the same species might be related to the isolation matrix of the strains and their adaptability to different stress conditions [15,52]. While strains UMCC 2996, UMCC 2990, and NFICC28 were isolated from sourdough and shared a similar antimicrobial activity, sample NFICC19 resulted to be the best acidifier.

A confirmation assay was conducted to further assess the activity of the bioactive metabolites produced by the LAB; however, contrary to the initial observations obtained on the high-throughput screening employed, only bacteriostatic activity was observed, in accordance with the previous literature findings [53]. Thus, identification of the nature of the bioactive compound was performed.

The evaluation of the metabolites in the CFS samples analyzed by using HPLC revealed the presence of organic acids in the supernatant, and therefore suggests they may play a significant role in the inhibition activity of the LAB strains tested [19,54]. Organic acids, in particular lactic acid, reduce the matrix's pH without causing a strong sour taste. A fast acidification of the product is responsible for the inhibition of *B. cereus*, as showed by Yang et al. [45], where a reduction in the initial CFU/mL of the pathogen was detected after LAB fermentation. Treatment of proteolytic enzymes on the CFS showed no significant changes in the inhibitory activity of the bioactive strains—ruling out the possible involvement of protein-based bioactive compounds, including bacteriocins and bacteriocin-like inhibitory substances. Additionally, the elimination of heat-labile antimicrobials was excluded by heat treatment (80 °C) of the CFS.

Even though no bacteriocinogenic inhibitions were detected based on the assays performed, the necessity for further investigation of which other possible metabolites can be further exploited, for future potential application of these studied LAB against different types of Gram-positive pathogens and spoilage bacteria, is needed [55]. Therefore, in silico search for possible antibacterial metabolites was conducted, identifying the presence of Class IIb and Class IIc bacteriocin operons on some of the studied LAB strains.

Class II bacteriocins are nonlantibiotics, heat-stable, and small ($\leq$10 kDa) hydrophobic peptides divided into four subclasses. Albeit these strains are detected on the genome, one of the possibilities for not observing bacteriocins from the CFS might be due to the presence of nonsatisfactory environmental conditions or an incomplete expression of all operons required for bacteriocin synthesis, transport, and regulation [56].

The effective biocontrol activity of the LAB strains evaluated would be associated with their rapid rate of acidification, as this is fundamental in food processing to prevent

and avoid spoilage contaminants. Sourdough's pH varies depending on the state of fermentation; however, it typically ranges between 3.5 and 5. Harmful microorganisms such as botulism bacteria, *E. coli*, and spoilage fungi cannot develop in an environment with a pH below 4.6, as this acidity keeps them away [5,57]. We observed that the majority of the strains were able to lower the pH around 4–4.5 (considered a safe pH to avoid spoilage) after 10 h, exhibiting a high acidification ability and underlining the massive production of acids (lactic and acetic acid). In fact, low pH and high acidity are the major factors for avoiding/delaying the growth of ropy bacterial agents, thus making the rate of pH reduction during the early stage of fermentation crucial for optimum inhibition [45,53,58]. Organic acids were the key metabolites that were identified in this study. The significance of pH reduction in food stability and preservation has been widely researched and recognized by the scientific community [59,60]. Furthermore, pH value also impacts the pKa of the various acids, leading them to a lower or higher dissociation, affecting the safety and taste of the final product [61].

Before the application of any microorganisms in food systems, an assessment of their safety and technological features is important. In this study, we profiled bioactive strains, identifying their sugar fermentations and their ability to be functional for their intended application in simulated ex vivo models. As observed, these strains can use a wide array of carbohydrates primarily found in bakery products, allowing them to be applied efficiently in this system. Consequently, acidification profiles highlight their ability to thrive in bread and produce necessary metabolites for possible bio-protection [13,62]. On the other hand, safety features were assessed as follows: antimicrobial susceptibility and hemolytic activity. Findings indicate that the evaluated strains have a broad range of resistance against streptomycin and vancomycin. Although this might be concerning, it should be noted that resistance to antibiotics in the majority of LAB is intrinsic. Additional assessment, particularly the identification of the location of associated resistance genes, should be performed to identify the occurrence of acquired resistance [63,64]. However, an in silico search for the presence of antibiotic resistance genes showed no matches on all the genomes of the 18 strains evaluated. Evaluation of hemolytic activity is one of the key factors that should be considered when assessing the safety of functional strains [39]. In this study, we identified partial hemolysis (α-hemolysis) on 11 out of 18 strains, whereas the rest showed no hemolysis. Thus, further confirmation, particularly the presence of genes involved in these putative virulence factors, should be investigated to assess potential risk for the application of the strains, especially for food consumption.

5. Conclusions

Cereals and bakery goods are a fundamental part of the human diet, and their higher susceptibility to microbial spoilage could lead to economic losses and health issues. Generally, LAB starter cultures and their metabolites are found to be promising for controlling spoilage agents. In this study, several LAB strains able to contrast the growth of some common rope spoilage agents were selected by using high-throughput and ex vivo screening assays. Specifically, *L. plantarum* NFICC19, NFICC72, NFICC293, *Lc. citreum* NFICC28, and *P. pentosaceus* NFICC58 and NFICC341 showed the best inhibitory activity. The assessment of their technological and safety features supported their suitability for fermentation processes and the production of bakery products. Moreover, the whole-genome sequencing of the selected LAB strains and the in silico analysis showed that some of the strains contain operons for bacteriocins, yet no significant evidence was observed phenotypically, suggesting that additional analysis needs to be performed to better understand the inhibitory mechanisms involved and validate the application of the strains as potential biocontrol agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030290/s1, Figure S1: Gene encoding for bacteriocins detected in the genome of the best candidate strains. Table S1: Results of the high-throughput preliminary screening of LAB-CFS against spoilage bacteria in common media. Table S2: Results of

the dual-plate agar high-throughput screening. Table S3: Results of the complete API test profile after 48 h of incubation at 30 °C.

Author Contributions: Conceptualization, methodology, data curation, investigation, formal analysis, writing—original draft, G.I.; Conceptualization, methodology, data curation, investigation, writing—review and editing, J.I.I.F.; formal analysis, data curation, S.Ö.; data curation, visualization, A.P.W.; investigation, data curation, R.S.K.; data curation, Q.H.; validation, formal analysis, data curation, R.S.; resources, supervision, A.P.; conceptualization, writing—review and editing, supervision, L.D.V.; conceptualization, resources, writing—review and editing, supervision, funding acquisition, C.H.B.-B. All authors have read and agreed to the published version of the manuscript.

Funding: G.I. acknowledges the award of a PhD scholarship from the Department of Life Sciences, University of Modena and Reggio Emilia. R.S. and S.O. were funded by FBCD FFBI grant Apple Pom (project ID 20080). R.S.K. was funded by the European Union's Horizon 2020 research and innovation program (Grant: 874735). C.H.B.-B. has received funding from MycoProtein (Grant Number 34009-20-1682). Lastly, J.I.I.F. was funded by an industrial PhD scholarship (23 IFD LAB DuPont PhD project number 110063) from the Danish innovation foundation. S.Ö. and Q.H. were working as research assistants. Both L.D.V. and A.P. are employees of the Department of Life Sciences, University of Modena and Reggio Emilia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to thank Christina Aaby Svendsen from the Research Group for Genomic Epidemiology, DTU National Food Institute for the technical assistance with performing full-genome Illumina sequencing.

Conflicts of Interest: The authors declare no conflict of interest.

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Table S1. Results of the high-throughput preliminary screening of LAB-CFS against spoilage bacteria in common media.
The OD600 values reported in red marked the inhibition. Bacterial cultures in which the OD_{600} (growth) of both 24h and 48h was inhibited, according to the applied formula, were chosen for further investigations.

PAT/LAB	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*P. pentosaceus* NFICC 10	*L. plantarum* NFICC 19	*L. plantarum* NFICC 27	*Lc. citreum* NFICC 28	*P. pentosaceus* NFICC 58	*L. plantarum* NFICC 72	*Lc. citreum* NFICC 87	*Lc. citreum* NFICC 94	*P. pentosaceus* FICC 103	*L. plantarum* NFICC 163	*L. plantarum* NFICC 207	*L. plantarum* NFICC 293	*P. pentosaceus* NFICC 341
B. cereus **DSM 2301**	0.14	0.14	0.14	0.13	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.13	0.14	0.15	0.14	0.11	0.19	0.15
24h	**0.21**	**0.21**	0.27	0.22	0.27	0.49	**0.21**	0.22	**0.19**	0.50	0.22	0.45	**0.21**	0.57	**0.20**	1.05	**0.23**	0.53
48h	**0.20**	**0.21**	0.23	**0.19**	0.24	0.35	**0.18**	**0.18**	**0.20**	0.43	0.24	0.60	0.27	0.52	0.28	1.11	**0.27**	0.48
B. cereus **DSM 4222**	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.11	0.14	0.14	0.14	0.14	0.14	0.13	0.14	0.15	0.18
24h	0.25	0.26	0.28	0.27	0.30	0.50	0.25	0.28	0.25	0.52	0.25	0.38	0.28	0.56	0.27	0.60	0.32	0.58
48h	0.26	0.25	0.25	0.39	0.29	0.24	0.26	0.26	0.44	0.26	0.37	0.52	0.31	0.52	0.29	0.58	0.33	0.54
B. cereus **DSM 22905**	0.14	0.14	0.14	0.15	0.15	0.15	0.14	0.17	0.14	0.13	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.19
24h	0.27	0.36	0.41	0.47	0.44	0.73	0.54	0.51	0.48	0.77	0.34	0.31	0.27	0.69	0.24	0.54	0.31	0.68
48h	0.30	0.35	0.40	0.48	0.45	1.43	0.50	0.52	0.49	0.77	0.41	1.13	0.37	0.69	0.37	0.79	0.35	0.68
B. cytotoxicus **DSM 4312**	0.14	0.14	0.15	0.14	0.15	0.14	0.14	0.14	0.14	0.13	0.15	0.14	0.14	0.14	0.14	0.14	0.15	0.20
24h	0.31	0.34	0.30	0.34	0.35	0.54	0.35	0.35	0.34	0.41	0.34	0.58	0.31	0.53	0.32	0.68	0.34	0.65
48h	0.30	0.33	0.32	0.32	0.36	0.49	0.32	0.34	0.32	0.45	0.38	1.27	0.31	0.50	0.32	0.70	0.33	0.64
B. mycoides **NFICC503**	0.15	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.11	0.15	0.15	0.15	0.15	0.20
24h	0.31	0.32	0.27	0.31	0.35	0.53	0.31	0.31	0.30	0.50	0.31	0.43	0.33	0.53	0.32	0.61	0.31	0.58
48h	0.29	0.33	0.31	0.30	0.34	0.43	0.30	0.31	0.30	0.48	0.36	1.10	0.32	0.47	0.31	0.60	0.30	0.58
B. altitudinis **NFICC510**	0.17	0.13	0.14	0.14	0.14	0.16	0.17	0.16	0.17	0.16	0.13	0.14	0.13	0.13	0.13	0.13	0.14	0.20
24h	0.32	0.31	0.33	0.31	0.39	0.71	0.39	0.38	0.37	0.95	0.32	0.47	0.21	0.49	**0.20**	0.54	0.26	0.50
48h	0.34	0.36	0.35	0.35	0.41	0.68	0.43	0.42	0.42	1.57	0.38	1.21	0.28	0.46	0.26	0.49	0.31	0.50
B. mycoides **NFICC526**	0.13	0.13	0.14	0.13	0.14	0.13	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.17	0.14	0.14	0.18
24h	0.24	0.23	0.26	0.23	0.28	0.45	0.23	0.24	0.22	0.46	0.24	0.36	0.38	0.51	0.35	0.70	0.41	0.61
48h	0.22	0.22	0.23	0.21	0.27	0.35	0.22	0.22	**0.20**	0.38	**0.21**	0.35	0.29	0.43	0.30	0.55	0.30	0.51
B. subtilis **NFICC528**	0.13	0.13	0.14	0.13	0.14	0.13	0.13	0.11	0.11	0.13	0.11	0.14	0.14	0.14	0.13	0.13	0.13	0.18
24h	0.38	0.26	0.32	0.35	0.41	0.74	0.38	0.30	0.30	0.70	0.38	0.43	0.29	0.69	0.29	0.78	0.30	0.63
48h	0.33	0.26	0.29	0.31	0.36	0.61	0.33	0.31	0.59	0.35	0.57	0.43	0.34	0.60	0.35	0.66	0.33	0.57
B. pumilus **NFICC530**	0.14	0.14	0.14	0.14	0.15	0.14	0.13	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.15	0.15	0.14	0.19
24h	0.42	0.34	0.31	0.40	0.42	0.62	0.44	0.38	0.37	0.59	0.45	0.44	0.40	0.66	0.42	0.80	0.36	0.65
48h	0.32	0.27	0.28	0.33	0.36	0.53	0.37	0.31	0.31	0.55	0.42	1.22	0.31	0.54	0.33	0.73	0.30	0.58
B. simplex **NFICC531**	0.11	0.11	0.12	0.14	0.16	0.15	0.15	0.14	0.15	0.13	0.15	0.15	0.15	0.14	0.14	0.15	0.14	0.18
24h	0.35	0.39	0.33	0.36	0.43	0.61	0.36	0.40	0.35	0.59	0.43	0.95	**0.21**	0.44	0.20	1.02	0.24	0.47
48h	0.31	0.30	0.31	0.31	0.35	0.52	0.31	0.34	0.32	1.05	0.95	0.97	0.24	0.40	0.25	0.95	0.27	0.44
B. cereus **NFICC740**	0.17	0.15	0.18	0.17	0.20	0.19	0.15	0.18	0.16	0.17	0.17	0.19	0.19	0.18	0.17	0.19	0.17	0.20
24h	0.35	0.27	0.37	0.34	0.40	0.54	0.35	0.36	0.32	0.57	0.40	1.07	0.38	0.65	0.38	1.15	0.36	0.52

48h	0.31	0.31	0.35	0.35	0.37	0.46	0.30	0.30	0.29	0.61	0.88	1.16	0.37	0.57	0.38	1.37	0.36	0.50
B. cereus **NFICC781**	0.18	0.17	0.19	0.19	0.17	0.19	0.14	0.19	0.15	0.17	0.14	0.19	0.13	0.18	0.19	0.19	0.18	0.20
24h	0.40	0.39	0.43	0.39	0.49	0.53	0.35	0.37	0.26	0.54	0.36	1.32	0.32	0.56	0.35	1.43	0.32	0.41
48h	0.39	0.40	0.40	0.39	0.48	0.52	0.36	0.36	0.48	1.15	1.00	1.31	0.40	0.58	0.35	1.31	0.34	0.45
B. thuringiensis **NFICC816**	0.17	0.18	0.17	0.17	0.18	0.16	0.17	0.18	0.17	0.17	0.17	0.17	0.20	0.20	0.20	0.20	0.19	0.21
24h	0.32	0.41	0.44	0.28	0.40	0.39	**0.18**	**0.20**	**0.19**	0.41	0.27	0.68	0.37	0.50	0.30	0.42	**0.10**	**0.13**
48h	0.36	0.45	0.50	0.32	0.44	0.41	**0.21**	**0.23**	**0.22**	0.45	0.37	0.63	0.40	0.53	0.34	0.53	**0.10**	**0.12**
B. weihenstephanensis **NFICC855**	0.22	0.20	0.20	0.19	0.21	0.19	0.22	0.21	0.18	0.21	0.23	0.19	0.22	0.22	0.23	0.21	0.22	0.24
24h	**0.26**	0.34	0.43	**0.18**	0.36	**0.10**	**0.09**	**0.09**	**0.09**	**0.09**	**0.16**	0.53	0.23	0.45	**0.34**	**0.10**	**0.09**	**0.09**
48h	**0.31**	0.35	0.43	**0.21**	0.39	**0.10**	**0.09**	**0.09**	**0.09**	**0.10**	**0.10**	**0.20**	0.31	0.43	**0.32**	**0.14**	**0.09**	**0.09**
B. amyloliquefaciens **NFICC869**	0.18	0.15	0.19	0.17	0.18	0.22	0.16	0.16	0.16	0.18	0.18	0.17	0.16	0.16	0.17	0.16	0.16	0.18
24h	0.33	0.35	0.40	0.34	0.39	0.61	0.29	0.31	0.35	0.58	0.34	1.09	0.35	0.58	0.34	0.71	0.34	0.53
48h	0.31	0.38	0.36	0.33	0.41	1.00	0.28	0.30	0.34	0.76	0.65	1.11	0.41	0.60	0.40	1.16	0.38	0.59
B. simplex **NFICC906**	0.14	0.15	0.16	0.14	0.16	0.15	0.15	0.14	0.14	0.14	0.15	0.14	0.17	0.17	0.15	0.15	0.14	0.16
24h	0.25	0.32	0.39	0.25	0.38	0.52	**0.23**	0.22	0.25	0.54	0.34	0.73	0.32	0.52	**0.20**	0.44	**0.18**	0.39
48h	0.32	0.39	0.36	0.34	0.43	0.52	0.29	0.31	0.33	0.62	0.76	0.95	0.36	0.57	0.29	0.50	0.28	0.45
B. amyloliquefaciens **NFICC1127**	0.17	0.17	0.17	0.16	0.18	0.16	0.17	0.17	0.16	0.16	0.16	0.15	0.17	0.16	0.16	0.17	0.16	0.19
24h	0.27	0.36	0.36	0.24	0.37	0.40	**0.18**	**0.18**	**0.18**	0.40	0.27	0.48	0.33	0.46	**0.21**	0.32	**0.09**	**0.12**
48h	0.33	0.39	0.36	0.30	0.41	0.42	**0.23**	**0.24**	**0.24**	0.45	0.31	0.57	0.38	0.50	0.27	0.33	**0.09**	**0.11**
B. amyloliquefaciens **NFICC1130**	0.17	0.17	0.18	0.15	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.18	0.18	0.18	0.17	0.18	0.19
24h	0.28	0.36	0.37	**0.20**	0.34	**0.18**	**0.09**	**0.08**	**0.08**	**0.08**	**0.12**	0.29	0.34	0.43	**0.18**	**0.09**	**0.08**	**0.09**
48h	0.32	0.42	0.37	**0.20**	0.39	**0.12**	**0.09**	**0.08**	**0.08**	**0.09**	**0.09**	**0.11**	0.34	0.40	**0.15**	**0.09**	**0.09**	**0.09**
B. subtilis **NFICC1525**	0.15	0.14	0.15	0.15	0.17	0.11	0.15	0.15	0.17	0.15	0.14	0.15	0.15	0.16	0.23	0.17	0.14	0.19
24h	0.29	0.32	0.35	0.29	0.38	0.32	0.31	0.31	0.28	0.60	0.31	0.95	0.26	0.64	0.30	1.12	0.25	0.58
48h	0.32	0.32	0.38	0.28	0.41	0.47	0.30	0.32	0.32	1.13	0.85	1.04	0.31	0.64	0.35	1.24	0.32	0.64
B. subtilis **NFICC1534**	0.15	0.14	0.15	0.15	0.14	0.15	0.14	0.15	0.15	0.15	0.15	0.16	0.15	0.16	0.13	0.15	0.15	0.19
24h	0.25	0.26	0.33	0.24	0.33	0.55	0.26	0.26	**0.21**	0.60	0.27	0.35	**0.17**	0.47	0.20	0.39	**0.19**	0.46
48h	0.31	0.34	0.33	0.30	0.34	0.55	0.33	0.35	0.31	0.86	0.83	1.01	0.28	0.47	0.30	0.47	0.33	0.46
B. licheniformis **NFICC1549**	0.14	0.17	0.17	0.19	0.17	0.17	0.14	0.17	0.16	0.16	0.19	0.19	0.17	0.17	0.17	0.16	0.18	0.18
24h	0.27	0.32	0.33	**0.27**	0.34	0.43	0.22	**0.25**	**0.24**	0.45	**0.27**	0.82	**0.21**	**0.26**	**0.14**	0.29	**0.12**	**0.13**
48h	0.23	0.31	0.30	**0.21**	0.32	**0.24**	**0.10**	**0.16**	**0.15**	0.28	**0.20**	1.03	**0.17**	**0.13**	**0.11**	**0.24**	**0.09**	**0.11**
L. fusiformis **NFICC119**	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.17
24h	0.40	0.40	0.49	0.37	0.45	0.65	0.31	0.38	0.31	0.66	0.34	0.70	0.37	0.86	0.49	0.92	0.45	0.64
48h	0.42	0.38	0.53	0.40	0.49	0.90	0.30	0.46	0.30	0.92	0.38	0.84	0.43	1.08	0.61	1.12	0.62	1.04
L. sphaericus **NFICC529**	0.14	0.13	0.14	0.14	0.15	0.15	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.15	0.14	0.14	0.13	0.16
24h	0.39	0.39	0.39	0.42	0.43	0.63	0.37	0.37	0.60	0.39	0.77	0.77	0.36	0.44	0.37	0.92	0.41	0.54

48h	0.43	0.47	0.33	0.43	0.48	0.99	0.42	0.43	0.44	0.99	0.51	0.75	0.40	0.71	0.43	1.15	0.51	0.57
L. fusiformis **NFICC532**	0.14	0.14	0.14	0.13	0.15	0.14	0.14	0.14	0.14	0.13	0.13	0.14	0.14	0.14	0.14	0.15	0.14	0.16
24h	0.36	0.35	0.38	0.38	0.42	0.52	0.39	0.35	0.35	0.48	0.41	0.74	0.35	0.50	0.38	0.67	0.37	0.53
48h	0.43	0.43	0.35	0.44	0.50	0.73	0.45	0.41	0.42	0.91	0.48	0.76	0.44	0.69	0.55	0.95	0.50	0.55
L. sphaericus **NFICC871**	0.14	0.14	0.14	0.14	0.15	0.14	0.13	0.13	0.13	0.14	0.13	0.14	0.14	0.15	0.14	0.14	0.13	0.16
24h	0.39	0.38	0.41	0.42	0.44	0.57	0.39	0.36	0.35	0.68	0.34	0.78	0.35	0.64	0.32	0.76	0.38	0.49
48h	0.47	0.37	0.34	0.47	0.50	0.90	0.43	0.44	0.44	1.03	0.41	0.96	0.26	0.85	0.28	1.01	0.38	0.41
L. boronitolerans **NFICC879**	0.11	0.12	0.12	0.12	0.13	0.12	0.11	0.12	0.12	0.12	0.12	0.12	0.11	0.12	0.11	0.12	0.11	0.14
24h	0.22	0.22	0.24	0.23	0.25	0.39	0.21	0.20	**0.18**	0.35	0.19	0.38	0.24	0.41	0.26	0.70	0.26	0.447
48h	**0.09**	0.19	0.19	**0.09**	0.15	**0.09**	**0.08**	**0.09**	**0.11**	0.27	0.19	0.42	0.22	0.34	0.20	0.53	0.24	0.33
L. fusiformis **NFICC882**	0.13	0.14	0.14	0.14	0.15	0.15	0.14	0.13	0.13	0.14	0.14	0.15	0.15	0.15	0.14	0.15	0.14	0.17
24h	0.39	0.32	0.35	0.37	0.42	0.58	0.33	0.35	0.38	0.61	0.35	0.50	0.35	0.56	0.30	0.67	0.35	0.57
48h	0.33	0.34	0.33	0.32	0.41	0.45	0.32	0.30	0.40	0.56	0.35	0.55	0.45	0.54	0.36	0.60	0.46	0.51
L. boronitolerans **NFICC889**	0.14	0.13	0.15	0.14	0.13	0.14	0.13	0.14	0.13	0.13	0.14	0.15	0.13	0.14	0.14	0.14	0.13	0.17
24h	0.73	0.32	0.33	0.34	0.31	1.11	0.35	0.34	0.35	0.74	0.33	1.12	0.38	0.33	0.30	1.24	0.38	0.59
48h	1.23	0.32	0.42	0.35	0.34	1.27	0.34	0.35	0.36	1.22	0.35	1.05	0.42	0.36	0.34	1.27	0.46	0.65
P. polymyxa **NFICC432**	0.15	0.15	0.19	0.15	0.15	0.15	0.15	0.19	0.15	0.18	0.14	0.19	0.20	0.18	0.19	0.15	0.15	0.17
24h	0.29	0.25	**0.22**	0.25	0.26	0.26	0.27	**0.26**	0.26	0.40	0.25	**0.20**	**0.21**	**0.19**	**0.20**	0.58	**0.20**	0.38
48h	0.60	0.29	0.36	0.30	0.23	0.50	0.29	0.29	0.30	0.72	0.33	**0.24**	**0.27**	**0.21**	**0.20**	0.63	**0.22**	0.40

Table S2. Results of the dual-plate agar high-throughput screening. Complete inhibition of the spoilage bacteria is scored with 3, strong inhibition with 2, partial inhibition with 1, and no inhibition with 0.

PAT/LAB	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*P. pentosaceus* NFICC10	*L. plantarum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC87	*Lc. citreum* NFICC94	*P. pentosaceus* NFICC103	*L. plantarum* NFICC163	*L. plantarum* NFICC207	*L. plantarum* NFICC293	*P. pentosaceus* NFICC341
B. cereus **DSM 2031**	2	1	0	1	1	0	3	0	0	0	1	0	0	1	0	2	1	1
B. cereus **DSM 4222**	0	1	1	0	1	0	0	0	0	0	2	0	1	1	2	0	3	2
B. cereus **DSM 22905**	3	1	1	2	2	3	1	1	1	2	3	2	3	2	3	3	3	3
B. cytotoxicus **DSM 4312**	2	2	2	2	2	1	1	1	1	3	2	1	1	1	1	1	2	2
B. mycoides **NFICC503**	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	2
B. altitudinis **NFICC510**	0	0	0	2	0	0	0	0	0	0	2	0	2	1	1	0	1	2
B. mycoides **NFICC526**	1	0	0	1	2	0	0	0	0	0	0	0	1	2	1	1	0	3
B. subtilis **NFICC528**	2	2	3	2	2	2	2	1	1	1	1	2	1	1	2	2	1	2
B. pumilus **NFICC530**	1	1	2	2	1	1	0	0	1	0	2	0	0	0	3	3	3	3
B. simplex **NFICC531**	3	3	3	3	3	3	3	2	2	3	3	3	2	2	3	3	2	3
B. cereus **NFICC740**	2	2	2	2	2	2	3	0	1	1	2	0	2	1	2	3	2	2
B. cereus **NFICC781**	1	2	3	1	2	2	1	0	0	2	1	2	3	1	2	3	2	2
B. thuringiensis **NFICC816**	2	2	3	3	2	3	3	1	3	3	3	3	2	0	2	2	3	3
B. weihenstephanensis **NFICC855**	2	2	3	2	2	2	0	1	1	1	1	2	2	2	3	3	2	2
B. amyloliquefaciens **NFICC869**	2	2	3	2	2	2	2	2	2	2	3	3	2	2	2	2	2	3
B. simplex **NFICC906**	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
B. amyloliquefaciens **NFICC1127**	2	2	1	2	1	2	0	0	0	0	0	0	3	0	0	0	0	0
B. amyloliquefaciens **NFICC1130**	2	0	0	2	2	0	0	2	0	0	2	2	0	0	0	0	0	0
B. subtilis **NFICC1525**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. subtilis **NFICC1534**	0	0	2	3	0	2	0	0	2	0	0	0	0	0	0	0	0	0
B. licheniformis **NFICC1549**	2	0	3	2	3	0	0	0	0	0	0	0	0	0	0	2	0	0
L. fusiformis **NFICC119**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. sphaericus **NFICC529**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. fusiformis **NFICC532**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. sphaericus **NFICC871**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. boronitolerans **NFICC879**	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
L. fusiformis **NFICC882**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. boronitolerans **NFICC889**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. polymyxa **NFICC432**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S3. Results of the complete API test profile after 48 hours of incubation at 30 °C

48 Hours	*F. sanfranciscensis*	*L. plantarum*	*F. rossiae*	*P. pentosaceus*	*Lc. citreum*	*P. pentosaceus*	*L. plantarum*	*L. plantarum*	*Lc. citreum*	*L. plantarum*	*L. plantarum*	*Lc. citreum*	*Lc. citreum*	*P. pentosaceus*	*P. pentosaceus*	*L. plantarum*	*L. plantarum*	*P. pentosaceus*
30°C	**UMCC 2990**	**UMCC 2996**	**UMCC 3002**	**UMCC 3010**	**UMCC 3011**	**NFICC 10**	**NFICC 19**	**NFICC 27**	**NFICC 28**	**NFICC 58**	**NFICC 72**	**NFICC 87**	**NFICC 94**	**NFICC 103**	**NFICC 163**	**NFICC 207**	**NFICC 293**	**NFICC 341**
Control (negative)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	+/-	-	-	+/-	-	-	-	-	+/-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D(-)-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L(+)-Arabinose	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	+	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
D(+)-Xylose	-	-	+	-	+	-	-	-	+	+	-	+	+	-	+/-	+	-	+/-
L(-)-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-Methyl xyloside	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+/-	+	+/-	+	+	+	+	+	+	-	-	+	+	+	+	+
D(+)-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D(-)-Fructose	+	+	+/-	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)-Mannose	+	+	+/-	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
L(-)-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	+/-	-	-	+/-	-	+/-	-	-	+/-	-	-	-	-	+/-	-	-	+/-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	+	-	-	+/-	+	+	+	+	+	+	+/-	+/-	+	+	+	+	+

Sorbitol	-	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+
alfa-Methyl-p-mannoside	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
alfa-Methyl-D-glucoside	-	-	-	-	-	+/-	-	-	+/-	-	-	+	+	-	-	-	-	-
N-acetyl-glucosamine	+	+	+/-	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+
Arbutine	+	+	-	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+
Esculine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D(+)-cellobiose	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+
D(+)-Melibiose	-	+	+/-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+
Saccharose	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D(-)-Trehalose	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	-	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+
D(+)-Raffinose	-	+	-	-	-	+	+	+	+	+	+	-	-	+/-	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Beta-Gentiobiose	+/-	+	-	+	-	+	+	+	+	+	+	+/-	+	+	+	+	+/-	+

D-Turanose	-	+	-	-	-	+	-	-	+	+	-	+	+	-	-	+	+	-
D-Lyxose	-	-	+	-	+	+	-	-	+/-	+	-	-	-	-	-	-	-	-
D-Tagatose	+	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	+/-	-	-	-	+/-	+	+/-	+/-	+/-	+	-	-	+	+/-	-	+/-	+/-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	+/-	+/-	-	+/-	+	+	+	+	+/-	+	+/-	+	+	+	+/-	+/-	+
+-ceto-gluconate	-	-	-	+/-	-	-	-	-	+/-	-	-	+/-	+/-	+	-	-	-	-
5-ceto-gluconate	-	-	-	-	-	+/-	-	-	+/-	+/-	-	-	-	+/-	-	-	-	-

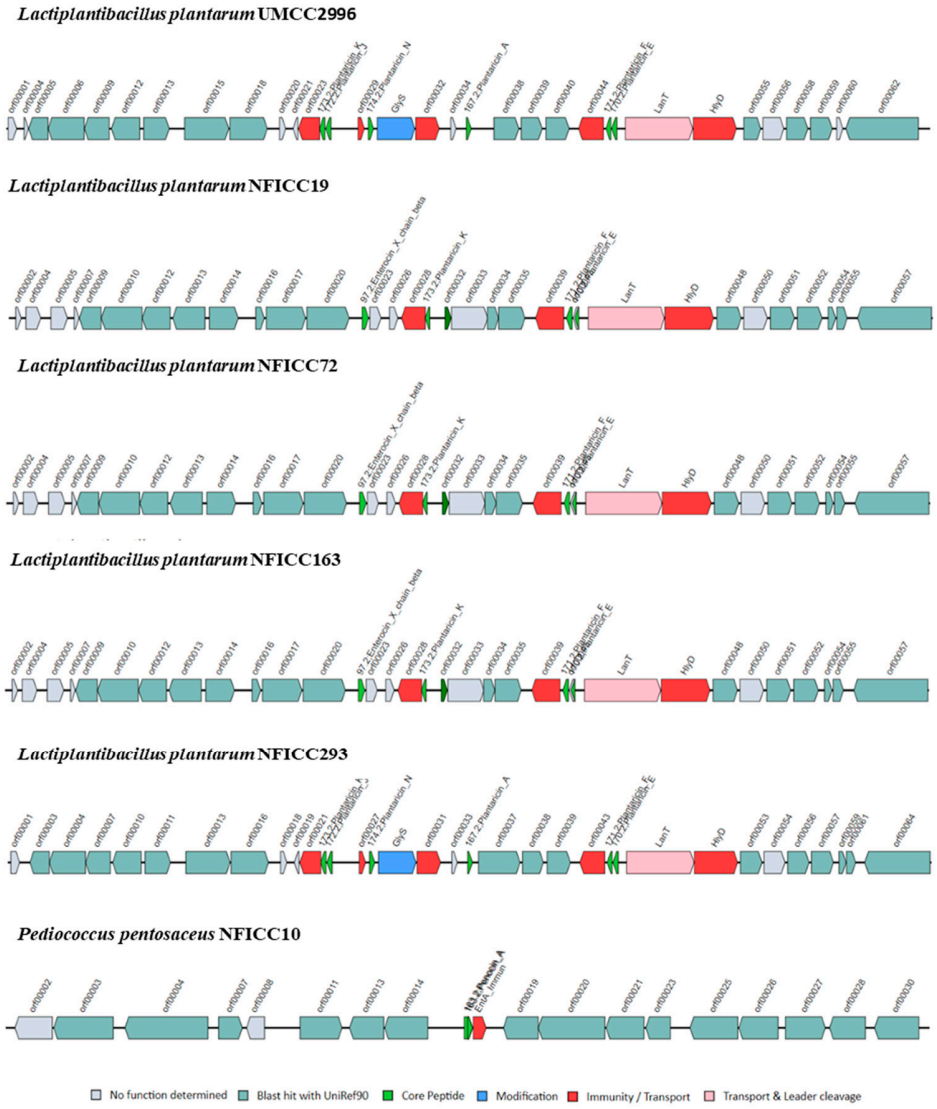


Figure S1. Gene encoding for bacteriocins detected in the genome of the best candidate strains

5. Chapter V

This chapter is represented by the unsubmitted manuscript **"Exploitation of lactic acid bacteria from microbial culture collections against spoilage moulds of bakery products"**.

5.1 Introduction

The most significant and fundamental meal consume worldwide by people is represented by cereal products [1-3]. Cereals are crucial to the daily diet supplying the majority of food calories and roughly half of the protein requirements, thus underlining the importance to identify strategies to enhance the quality and prolong the shelf life of bakery items [1-5]. Bakery items are prone to microbial degradation, particularly fungus infestation, which significantly shortens their shelf life and causes food waste and financial loss [6]. Furthermore, cereal product spoilage raises concerns due to the possibility of producing and consuming poisonous aflatoxins and other mycotoxins. In fact, nearly 300 fungal species are linked to many acute and chronic illnesses, posing a severe threat to the safety of food. Mycotoxins are regarded as the major pollutants in crops including maize, wheat, rice, and several other foods [7]. The World Health Organization (WHO) estimated in 2015 that approximately 600 million foodborne illnesses occur each year globally [8-9]. The genera *Penicillium, Aspergillus, Monilia, Mucor, Endomyces, Cladosporium, Fusarium*, and *Rhizopus* represent the most common spoilage moulds colonizing bread and bakery products [1, 10-11]. During the past decades propionic, sorbic, and acetic acids, as well as their salts, have been employed as chemical preservatives to prevent moulds deterioration of cereal products. Although these substances are generally regarded as safe (GRAS), their prolonged usage is unsatisfactory due to the resistance established by moulds strains, as well as their solubility, toxicity, and potency and their recent linkage with health concerns [1, 12]. Due to the abovementioned issue and the consumer request for clean-label and eco-friendly foods the

application of lactic acid bacteria (LAB) and their metabolites can prevent, avoid and in some cases detoxify moulds and their hazardous metabolites [1, 3, 13-16]. In this study, we highlight how the selection and inclusion of innovative LAB isolates represent the key to answering the request of lowering the use of pesticides, boosting nutrition, and extending the shelf life of bread and cereal products.

5.2 Material and Methods

5.2.1 Strains, growth conditions and preparation of Wheat Bread Media

5.2.1.1 LAB strains and growth conditions

A total of 18 LAB strains, isolated from different food and environmental niches, were selected from the Unimore Microbial Culture Collection (UMCC) and the collection of the Danmark Tekniske Universitat (Table 1) [17-20]. The selected strains were revitalised from the glycerol stock conserved at -80 °C in the respective microbial collections, and cultured on de Man, Rogosa and Sharpe (MRS) at 30 °C for 24 h in an anaerobic environment. Whole genome sequencing was performed to previously characterized and identify the strains species, in addition, API 50 CHL galleries were used to assess their sugar fermentation profile and the safety profile of the strains was evaluated to confirm their suitability in food (Iosca et al., 2023 submitted paper).

Table 1. Lactic acid bacteria strains from Danmarks Tekniske Universitet (DTU) collection and Unimore Microbial Culture Collection (UMCC) tested in the present study.

NFICC CODE	Species	Identification	Isolation source
NFICC10	*Pediococcus pentosaceus*	Whole Genome Sequencing	Sourdough
NFICC19	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Dill
NFICC27	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Sourdough
NFICC28	*Leuconostoc citreum*	Whole Genome Sequencing	Sourdough
NFICC58	*Pediococcus pentosaceus*	Whole Genome Sequencing	Sourdough
NFICC72	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Gooseberry
NFICC87	*Leuconostoc citreum*	Whole Genome Sequencing	Beetroot
NFICC94	*Leuconostoc citreum*	Whole Genome Sequencing	Spinach
NFICC103	*Pediococcus pentosaceus*	Whole Genome Sequencing	Pumpkin
NFICC163	*Lactiplantibacillus platarum*	Whole Genome Sequencing	Field pea
NFICC207	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Glasswort
NFICC289	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Dragsholm plant
NFICC341	*Pediococcus pentosaceus*	Whole Genome Sequencing	Brewer's spent grain
UMCC CODE	**Species**	**Identification**	**Isolation source**
UMCC 2990	*Fructilactobacillus sanfranciscensis*	Whole Genome Sequencing	Sourdough type I
UMCC 2996	*Lactiplantibacillus plantarum*	Whole Genome Sequencing	Dough for Panettone
UMCC 3002	*Furfurilactobacillus rossiae*	Whole Genome Sequencing	Dough for Panettone
UMCC 3010	*Pediococcus pentosaceus*	Whole Genome Sequencing	Gluten-free sourdough
UMCC 3011	*Leuconostoc citreum*	Whole Genome Sequencing	Dough for Panettone

5.2.1.2 Fungal strains and growth conditions

In accordance with the previous literature mould from the genera *Penicillum*, *Aspergillus*, *Mucor*, and *Cladosporium* were selected from the Institut for Bioteknologi, IBT culture collection for fungi from DTU Bioengineering (Table 2). A total of 17 mould strains retained

from various cereals and bakery products were selected and cultured on Yeast Peptone Dextrose Agar (YPDA) at 30 °C with 75% relative humidity (RH) for 72-120 h.

Table 2. Fungi strains selected from the IBT culture collection tested in the present study.

IBT CODE	Species	Identification	Isolation matrix	Isolation's country	Properties of Colony
IBT 7731	*Mucor circinelloides*	C1 Domain	Wheat grain, biological	Denmark	Dark grey
IBT 15367	*Aspergillus glaucus*	Morphological ID	Wheat flour	Denmark	Pale green
IBT 15621	*Aspergillus candidus*	C1 Domain	Wheat flour	Hungary	White
IBT 15711	*Aspergillus flavus*	Unknown	Milled maize, wheat	Bulgaria	Olive green
IBT 17128	*Aspergillus glaucus*	Morphological ID	Rum cake	Denmark	Pale green
IBT 21323	*Aspergillus flavus*	Morphological ID	Easter bread	Denmark	Olive green
IBT 22573	*Aspergillus flavus*	Morphological ID	Bread	Italy	Olive green
IBT 22586	*Aspergillus niger*	Morphological ID	Bread	Italy	Black, fluffy
IBT 22587	*Aspergillus candidus*	Morphological ID	Bread	Italy	White
IBT 23147	*Penicillium sizovae*	Morphological ID	Soft wheat	Italy	Brown/Dark green
IBT 26359	*Penicillium roqueforti*	Morphological ID	Bread	Denmark	Velvety blue, dark greenish grey
IBT 28280	*Aspergillus glaucus*	Morphological ID	Cake	Italy	Pale green
IBT 31230	*Penicillium manginii*	Morphological ID, HPLC-DAD UV	Wheat flour	Denmark	Yellow, green
IBT 34245	*Aspergillus niger*	Morphological ID	Wheat	Denmark	Black, fluffy
IBT 35685	*Penicillium crustosum*	Morphological ID, HPLC-DAD UV	Mouldy bread	Denmark	Heavy dull green
IBT 42662	*Cladosporium carpum*	Morphological ID	Wheat	Turkey	Olivaceous brown
IBT 42663	*Cladosporium herbarum*	Morphological ID	Barley	Netherlands	Brownish

5.2.1.3 Preparation of Fungal Spore Suspension

The concentrations of fungal spore suspensions were adjusted to approximately 10^5 CFU/mL with saline (9 g/L NaCl solution) and were counted by microscopic counting using a haemocytometer (Fisher Scientific, Denmark). The prepared spore suspensions of fungi were stored at 4 °C for a period of 14 d and were then employed in the following experimental procedure.

5.2.1.4 Preparation of LAB Cell-Free Supernatant (CFS)

After incubation at 30 °C for 48 h in MRS broth or Wheat (WBM), the LAB-CFS was obtained from the various strains by centrifugation at 8000× g for 10 min at 4 °C and following sterile filtration (0,20 µm) in new Eppendorf (ThermoFisher, Waltham, MA USA).

5.2.1.5 Preparation of Wheat Bread Media (WBM)

To assess the *ex vivo* antifungal activity of the various selected LAB, a culture medium from plain wheat bread has been simulated following the protocol designed by Verni et al. [21] and Iosca et al., 2023 (In print) [22]. Briefly, wheat bread was homogenized utilizing a blender to ease the subsequent enzyme treatment, and water was added in a 1:4 ratio (w/w). In order to facilitate nutrient compound availability, proteolytic and amylolytic enzymes were adjunct to the mixture. Specifically, Neutrase, an endo-protease (1.5 AU/g) from *Bacillus amyloliquefaciens* (Novozymes, Denmark) and Amylase (Novozymes, Denmark) were used at 1,5 mL/L and 0.5 g/L, respectively. Following, the wheat bread mixture was incubated at the enzyme's optimal working conditions, centrifuged (6000 rpm for 20 min) and the obtained supernatant was finally collected. The medium was then filtered, sterilized at 121 °C for 15 min and the pH was adjusted.

5.2.2 Screening of LAB strains for Antifungal Activity

5.2.2.1 High-throughput Microtiter Plates (96 wells) Preliminary Screening in ex vivo condition

A preliminary screening following the protocol of Zhao et al. [23] was conducted with slight modifications. Sterile 96-well microtiter plates were employed for preliminary screening, plates were filled with an undiluted suspension (100 µL) of each selected moulds (Table 2) and LAB-CFS (100 µL) or BM as a control (100 µL), and then incubated at 30 °C for 48 h. Using. using a plate reader infinite M200PRO (Tecan, Männedorf, Switzerland), the absorbance value (OD_{600}) at 600 nm was determined, and the following formula was used to compute the antifungal rate:

$$(\%)\ \text{Antifungal rate} = \left(1 - \frac{OD_{600}LAB}{OD_{600}CONTROL}\right)$$

OD_{600}LAB identifies the absorbance value of the moulds spores' growth with the LAB-CFS for 48 h, while OD_{600}CONTROL represents the absorbance value of mould spores cultured in WBM liquid for 48 h.

5.2.2.2 Dual Plate Agar Screening Assay in Common Media

A high-throughput dual plate agar assay in 24 wells-plates (ThermoFisher, Waltham, MA USA) was performed according to Iosca et al. [18] with minor modifications. Briefly LAB strains were grown overnight in MRS broth at 30 °C and 10 µL of each culture at 10^8 CFU/mL was mixed with 1 mL of MRS agar poured in each well of the Petri dishes. After incubation at 30 °C for 24 h, each well was overlaid with 500 µL of YPDA. Spores suspension at 10^5 CFU/mL for each moulds was obtained as aforementioned and 5 µL of each spoilage agent was placed in the

middle of YPDA after solidification. All plates were incubated in aerobic conditions at 30 °C with 75% oh RH for 7 days. Results were observed and taken after 2, 4 and 7 days.

5.2.3 Confirmatory antifungal assay on common media

Confirmatory assays to validate the results obtained with the high-throughput screening were performed both against spores and fungal cells.

5.2.3.1 Dual plate agar assay against spore

A dual plate assay agar against fungal spores was conducted following the protocol of Fugaban et al. 2022 [24]. Briefly, LAB strains were grown overnight in liquid MRS at 30°C, and 10 µL of each culture at 10^8 CFU/mL was spotted at the center of Petri dishes with MRS agar. After incubation at 30 °C for 24 h, plates were overlaid with 10 mL YPDA containing moulds spore in a concentration of 10^5 CFU/mL. All plates were incubated in aerobic conditions at 30 °C with 75% RH and inhibition of fungal spores growth was detected. Results were taken after 2, 4 and 7 days, the experiment was conducted in triplicate.

5.2.3.2 Dual plate agar assay against fungal growth

The LAB strains were tested for their inhibitory antifungal activity using a dual agar plate assay, according to Iosca et al. [18]. Briefly, LAB strains were grown overnight in MRS broth at 30 °C and 100 µL of each culture at 10^8 CFU/mL was mixed with 10 mL of MRS agar. Plates were incubated at 30 °C for 24 h and then overlaid with 10 mL of YPDA. After solidification, 10 µL of spores suspension at 10^5 CFU/mL was obtained and placed in the middle of the Petri dishes. All plates were incubated in aerobic conditions at 30 °C for 9 days and the radial inhibition of

fungal growth was measured and calculated following the formula from Chen et al. [25] in order to obtain the percentage of inhibition. The experiment was repeated three times. Statistical analysis of data was determined by one-way analysis of variance (ANOVA) followed by Tuckey's test using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). P-values ≤ 0.05 were considered statistically significant. Results were expressed as mean ± standard deviation.

5.2.4 In vivo fungal inhibition assay

Based on the previously obtained results the best inhibitory LAB were selected to challenge the various moulds directly in wheat bread. Wheat bread was sliced to obtain uniform slices of 6.5 cm length, 4 cm width and 1 cm depth. LAB cultures were grown in liquid MRS for 18 h at 30 °C, set at 10^7 CFU/mL and washed with saline solution three times (5000 rpm x 10 min) in order to avoid any trace of MRS broth. Before inoculation, bread slices were put in Petri dishes and sterilised under UV light for 30 minutes, to avoid the presence of any other microorganisms except the desired one. After 1 mL of saline solution containing each LAB was inoculated in the various bread slices and following incubated at 30°C for 24 h. To insurance enough humidity on the surface of the bread slices 1 mL of saline solution was added before inoculating the bread with 100 µL of spores at 10^5 CFU/mL of each moulds at the center of the slice. Petri dishes containing the bread sliced were then aerobically incubated at 30°C with 75% RH for 9 days. Results were measured after 2, 4, 7, and 9 days and triplicates were made. Statistical analysis of data was determined by one-way analysis of variance (ANOVA) followed by Tuckey's test using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) and t-test were ANOVA was not applicable. P-values ≤ 0.05 were considered statistically significant. Results were expressed as mean ± standard deviation.

5.2.5 Identification of LAB antifungal compounds

5.2.5.1 Untargeted dynamic headspace Gas Chromatography-Mass Spectrometry (GC-MS) analysis

To identify LAB's volatile metabolites with putative antifungal compounds an untargeted dynamic headspace " purge and trap" analysis was conducted. LAB cultures were grown in liquid MRS for 24 h at 30 ℃ and the extraction of metabolites was carried out with the following protocol. Briefly, 4 mL of fermented MRS broth for each LAB strain was placed in a 100 mL purge bottle using an automatic pipette and 1 mL of antifoam (Synperonic) was added. A thermoregulated bath was used to heat all the samples at 37 ℃, and volatile compounds were then collected for 30 min into a Texan-tube using an N_2 flow of 150mL/min. Subsequently, Texan-tube was dried for 20 min with an N_2 flow of 50mL/min. Volatile compounds were then desorbed in the injection port of the GC (HP 6890 Series, Hewlett Packard, Palo Alto, CA, USA). The program was set as follows: an initial temperature of 45°C for 5 min was used followed by increments of 1.5 ℃/min until 55 ℃, increments of 2 ℃/min until 90°C, and increments of 8 °C/min to a final temperature of 230 ℃ held for 8 min. The obtained compounds were then analysed by MS (HP 5973 inert mass-selective detector, Aligent Technologies, USA; Electron ionisation mode, 70 eV, mass to charge ratio scan between 30 and 250. All the retrieved compounds were identified with the use of MS-library Wiley 138 K (John Wiley and Sons, Hewlett-Packard). The presence or the absence of the various metabolites found were asses for each LAB strain along with their antifungal activity. Samples were made in double and an internal standard and plain MRS were run as a control.

5.3 Results and Discussion

5.3.1 High-throughput screening for the detection of LAB antifungal activity

5.3.1.1 High-throughput screening in ex vivo conditions

The antifungal activity of LAB-CFS was initially asses using high-throughput screening following the recent protocol by Zhao et al. [23]. As reported in table 1, the obtained results demonstrated a different range of sensitivity between the various moulds and a different range of inhibitory ability of the various LAB-CFS strains employed, underlining the specific range of activity of the various employed LAB strains [18]. In particular, the growth of *A. candidus* IBT 22587 was strongly inhibited by all the tested LAB-CFS. This fungi behaviour might be explicable with a higher sensitivity to LAB's organic acid. In fact, as reported by Liu et al. [3] and previously by Russo et al. [26] higher concentrations of lactic acid in the CFS have positively correlated with fungal inhibitions due to this acid synergic role with other metabolites such as fatty acid, bacteriocins and antifungal compounds, enhancing the final antifungal activity. On the other hand, against the mould strain *A. candidus* IBT 15621, no inhibition was detected for any of the tested LAB-CFS strains. The observed opposite behaviour might be ascribable to the different isolation matrices and isolations country (Table 2) of the abovementioned moulds strains. The lower a_w present in wheat flour alongside less disposable nutrient respect bread might have contributed to heightening the resistance and competitiveness of strain IBT 15621. Among LAB-CFS strains *L. plantarum* UMCC 2996 and *P. pentosaceus* UMCC 3010 display larger activity showing strong inhibition against different strains of *A. glaucus* (IBT 17128 and 28280) and *A. flavus* (IBT 15711; 21323 and 22573) commonly regarded as some of the major cereals and bakery spoilage agents [1-2, 27-30]. It is extremely interesting to notice the high inhibition of 90% against *A. niger* IBT 22586 by *P. pentosaceus*

NFICC103 and the inhibition by *Lc. citreum* NFICC28 (82%) and *L. plantarum* NFICC19 (80%) against *P. roqueforti* IBT 26359. The particular activity of these LAB strains is probably due to their isolated environment, sourdough and plant, regarded as stressful habitats and helping the strains develop the strongest competitiveness [4, 31-33]. Furthermore, the antifungal ability of LAB-CFS might be due to its richness in compounds such as primary and secondary LAB metabolites affecting the growth of the moulds [33]. Nevertheless, the production of these compounds is strictly related to LAB species, strains, growth medium, sugar content, and temperature explaining the different ranges of activity observed in this experiment [33].

Table 3. High-throughput screening results using LAB-CFS against spore's moulds on WBM. The data were analysed following the formula from Zhao et al. (2022) and are expressed in percentages. Data ≥ 80% were considered inhibited agents and are highlighted in green.

PAT/LAB-CFS	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*P. pentosaceus* NFICC10	*L. planatrum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC 28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC 87	*Lc. citreum* NFICC 94	*P. pentosaceus* NFICC 103	*L. plantarum* NFICC163	*L. planatrum* NFICC207	*L. plantarum* NFICC289	*P. pentosaceus* NFICC341	Control
M. circinelloides IBT 7731	12	19	29	37	51	20	41	41	37	27	41	36	47	29	41	34	27	20	0,59
A. glaucus IBT 15367	-75	-32	-19	-55	-61	-32	-41	-44	-54	-14	-48	-41	-36	-32	-44	-53	-49	-46	1,36
A. candidus IBT 15621	-226	13	-45	6	23	-29	32	35	13	-123	-52	-6	39	0	16	23	16	-19	0,31
A. flavus IBT 15711	32	60	87	88	55	78	55	53	55	64	42	41	47	83	38	82	51	83	2,87
A. glaucus IBT 17128	-5	80	75	78	45	65	83	78	52	70	73	75	77	75	75	75	76	72	1,66
A. flavus IBT 21323	61	82	78	83	57	26	37	69	57	35	79	67	62	38	74	81	53	77	2,43
A. flavus IBT 22573	66	86	84	84	79	73	75	73	68	83	72	88	72	79	72	83	74	75	2,53
A. niger IBT 22586	-18	14	20	0	-3	11	6	-3	-2	14	4	-6	4	90	-5	7	7	20	2,51
A. candidus IBT 22587	91	89	85	86	90	82	90	89	80	84	90	86	88	86	87	88	89	86	2,2
P. sizovae IBT 23147	-105	-45	-75	-45	-15	-55	-35	-35	-35	-98	-35	-65	-45	-55	-35	-55	-30	-180	0,2
P. roqueforti IBT 26359	-50	72	75	62	70	69	80	63	82	79	88	76	80	73	74	78	75	72	1,38
A. glaucus IBT 28280	30	89	81	83	47	79	74	84	82	82	86	76	69	78	68	57	70	78	2,51
P. manginii IBT 31230	28	52	48	51	68	42	63	58	60	43	66	46	57	62	63	51	62	51	0,65
A. niger IBT 34245	15	14	81	24	21	22	19	4	5	33	68	7	19	39	55	17	63	44	3,23
P. crustosum IBT 35685	-261	-35	-35	-12	23	-73	19	-15	19	-38	-8	-19	27	-12	-4	-8	-4	-31	0,26
C. carpum IBT 42662	69	55	60	58	68	46	71	76	68	58	73	63	65	71	68	69	64	69	0,8
C. herbarum IBT 42663	48	56	53	62	73	36	71	60	68	49	66	59	66	53	64	64	62	45	0,73

5.3.1.2 High-throughput dual plate agar screening

To screen the inhibitory ability in optimal nutrient growth conditions for both spoilage agents and LAB, a dual plate screening in 24-wells using common media was performed. The results were collected after 2, 4 and 7 days. The latter are reported the following table 4. Fungal growth was evaluated visually and results were expressed as (-) complete inhibition (no mould growth) ; (-/+) partial inhibition (small moulds spots); (+) no inhibition (moulds growth). As observed from the results, in this cell-to-cell screening, the majority of the LAB were able to inhibit the thriving of *A. candidus* 22587. In particular, *L. plantarum* UMCC 2996, NFICC72 and 289 alongside *Lc. citreum* NFICC28 and 87, and *P. pentosaceus* NFICC58 were able to completely inhibit the spreading of the moulds for all 7 days of testing. Positive results, in contrast with the previous screening, with the same LAB, were obtained also against *A. candidus* 15261, where *P. pentosaceus* NFICC58 and *L. plantarum* UMCC 2996 display a complete inhibitory ability. This behaviour might be explained by the difference in the media and nutritional sources and the direct challenge against the moulds, which has potentially triggered a higher production of antifungal compounds [33]. Prominent results with partial inhibition of both *A. niger* strains IBT 22586 and 34245 have been observed. *Lc. citreum* NFICC28 affect the spreading of both moulds strains with complete inhibition of the spoilage agents until the 4^{th} day of incubation. This LAB activity might be related to the production of phenyllactic acid (PLA) or various antifungal compounds such as fatty acids or benzoic acid [3, 32-35]. In addition, its isolation environment (sourdough) has probably influenced the LAB metabolic ability and its metabolite production [18]. A good inhibitory activity was showed also against the moulds of the *Penicillium* genus, especially challenging *P. manginii* and *P. crustosum* while almost no sign of antifungal activity was detected against the two *A. flavus* strains. Regarding the selected LAB strains in general,

after the dual plate preliminary screening, the broader anti-moulds strains resulted belonged to the *L. plantarum*, *F. sanfranciscensis* and *Lc. citreum* species. Specifically, strains *L. plantarum* NFICC19, 163, 207 and 289, *F. sanfranciscensis* UMCC 2990 and *Lc. citreum* 28 and 87. On the contrary, a complete anti-moulds inability was detected for *F. rossiae* UMCC 3002 and *P. pentosaceus* NFICC103. The absence of any antifungal activity regarding these two LAB strains could be related to their slow acidification ability and the consequent presence/absence of weak organic acid in their undissociated form.

Table 4. Results were obtained after 7 days with the high-throughput dual plate agar screening. The date reported in green highlights the inhibition of mould growth. -: complete inhibition; -/+: partial inhibition (small moulds spot); + : no inhibition

PAT/LAB	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*.F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*P. pentosaceus* NFICC10	*L. planatrum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC 87	*Lc. citreum* NFICC 94	*P. pentosaceus* NFICC 103	*L. plantarum* NFICC163	*L. planatrum* NFICC207	*L. plantarum* NFICC289	*P. pentosaceus* NFICC341	C
	Days 7																		
M. circinelloides IBT 7731	+	+	+	-/+	-	+	+	+	+	+	+	-/+	+	+	+	+	+	+	+
A. glaucus IBT 15367	-/+	+	+	+	+	+	-	-	+	-/+	+	-/+	+	+	-/+	-	+	-	+
A. candidus IBT 15621	+	-	+	+	+	+	-	+	-/+	-	-/+	-/+	-/+	+	-/+	-/+	+	+	+
A. flavus IBT 15711	+	+	+	+	+	+	-	+	+	+	-/+	-/+	+	+	-/+	-/+	+	+	+
A. glaucus IBT 17128	+	+	+	+	+	+	-/+	-/+	-/+	-/+	-	-/+	+	+	-/+	-/+	+	+	+
A. flavus IBT 21323	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. flavus IBT 22573	-/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. niger IBT 22586	+	+	+	+	+	-	-/+	-/+	-/+	+	+	-/+	+	+	-/+	+	+	+	+
A. candidus IBT 22587	-/+	-	+	+	+	+	-/+	+	-	-	-	-	+	+	-/+	-/+	-	-/+	+
P. sizovae IBT 23147	+	+	+	+	+	+	+	+	+	+	+	-/+	+	+	+	+	+	+	+
P. roqueforti IBT 26359	+	+	+	+	+	+	+	+	+	-/+	+	+	+	+	+	+	+	+	+
A. glaucus IBT 28280	-/+	+	+	+	+	-	+	+	+	-/+	+	-/+	+	+	+	+	+	+	+
P. manginii IBT 31230	-/+	-	+	-	-/+	-	-	-	-/+	-	-	-	-/+	+	-	-/+	+	-	+
A. niger IBT 34245	+	+	+	+	+	+	+	+	-/+	+	+	-/+	+	+	+	+	+	+	+
P. crustosum IBT 35685	-/+	-	+	+	+	+	-	-	-	-	-/+	-	+	+	-	+	-	-	+
C. carpum IBT 42662	+	+	+	+	+	+	+	-/+	+	+	-/+	+	+	+	+	+	+	-	+
C. herbarum IBT 42663	+	-	+	+	+	+	-	-	-/+	-	-/+	-	-	+	+	-	+	-	+

5.3.2 Confirmatory Screening

To validate and confirm the positive results obtained from the high-throughput screenings, two different types of confirmatory dual plate screening were performed guaranteeing an antifungal LAB strain selection.

5.3.2.1 Spore antifungal assay

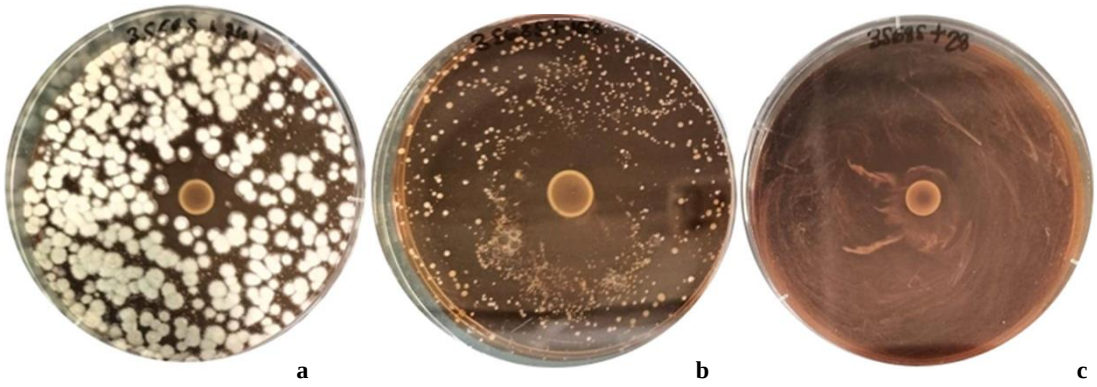
The results obtained from the validation of the high-throughput screening are reported in table 5 and confirm the majority of the detected LAB's antifungal ability. In fact, species belonging to *L. plantarum*, *P. pentosaceus* and *Lc. citreum* confirm their strong antifungal potential, showing accordance with the findings in the previous literature [35-38]. Particularly prominent results with complete inhibition of the moulds' growth, stated in table 5 as (--), were obtained against different moulds genera and species revealing a different sensitivity of the fungal agents toward the various LAB's antifungal compounds. In fact, as reported by Shi et al. [39] different moulds strains and/or species respond differently to various antifungal substances and competition mechanisms. *A. flavus* IBT 21323 showed resistance against the tested strains and completely colonized the plates after only four days of incubation. This specie is well-known to have a resistance against acetic acid considered one of the most effective organic acids against fungal contaminations in food [40]. A high resistance against LAB was also displayed by the *Penicillium* species tested in this study, in particular *P. roqueforti*. In the spoilage microbiota, *P. roqueforti* represents one of the dominant species. In fact, due to the highly developed physiology of *Penicillium* species, very little nutritional support is needed for the strain development aiding in adaptation to a wide variety of living environments and resistance to different types of preservatives [39]. However, major inhibitory results were obtained against *P. manginii* IBT 31230, *P. crustosum* IBT 35685 (Figure 1), and *P. sizovae* IBT 23147 by various

strains belonging to *L. plantarum*, *P. pentosaceus* and *Lc. citreum* species. Even if resistance against some LAB strains was detected for the two *A. niger* IBT 22586 and IBT 34245 strains, promising results were obtained when challenged with *L. plantarum* NFICC19 and *Lc. citreum* NFICC28, respectively. This LAB strains behaviour is probably related to their higher production of lactic and acetic acid bacteria in MRS and a speed acidification rate shown in the previous experiment by Iosca et al. [41]. A strong inhibition was assets also for *M. circinelloides*, considered an opportunistic pathogen due to its ability to produce the mycotoxin 3-nitropropionic acid. In particular, *Lc. citreum* UMCC 3011 and NFICC87 completely inhibited the thriving of these moulds for the all incubation period. As already reported by various studies, in fact, the thriving of this mould specie could be controlled and avoid by the use of LAB as bio-preservatives due to their ability in pH changes and environmental modifications [13, 42]. A strong sensitivity against LAB was also reported for *A. glaucus* (Figure 2), *A. candidus* and *C. herbarum*.

Table 5. Results of the dual plate spore antifungal assay after 7 days of incubation. --: complete inhibition; -: partial inhibition; +: no inhibition. The experiment was repeated three times.

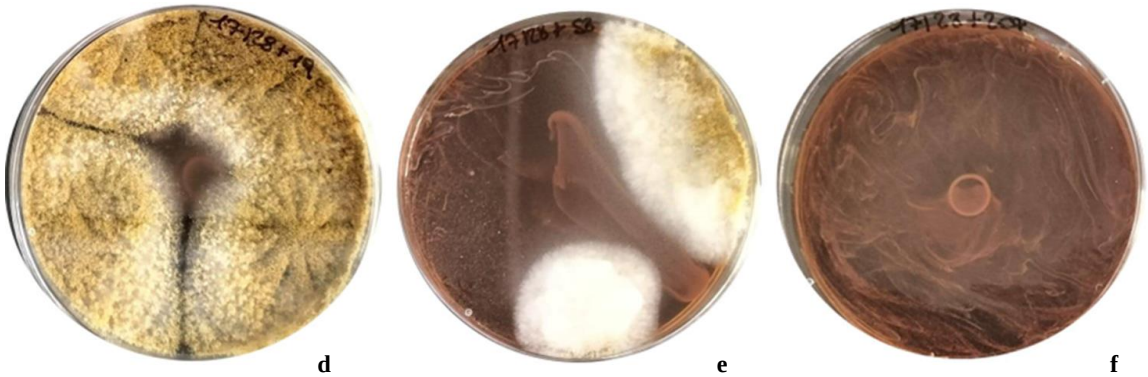
LAB/ PAT	*M. circinelloides* IBT 7731	*A. glaucus* IBT 15367	*A. candidus* IBT 15621	*A. flavus* IBT 15711	*A. glaucus* IBT 17128	*A. flavus* IBT 21323	*A. flavus* IBT 22573	*A. niger* IBT 22586	*A. candidus* IBT 22587	*P. sizovae* IBT 23147	*P. roqueforti* IBT 26359	*A. glaucus* IBT 28280	*P. manginii* IBT 31230	*A. niger* IBT 34245	*P. crustosum* IBT 35685	*C. carpum* IBT 42662	*C. herbarum* IBT 42663	Control
F. sanfranciscensis UMCC 2990		+					+		-			-	+		+			+
L. plantarum UMCC 2996			--			+			--				-		--		-	+
P. pentosaceus UMCC 3010	-					+							--					+
Lc. citreum UMCC 3011	--												-					+
P. pentosaceus NFICC10								+				+	-					+
L. planatrum NFICC19		+	--	-	+			-	+				+		--		--	+
L. plantarum NFICC27		+			-			+					-		--	+	-	+
Lc. citreum NFICC28			+		--			+	-				+	-	--		-	+

P. pentosaceus NFICC58		--	--		-				--		+	-	--		+		+	+
L. plantarum NFICC72			--	+	-				+				+		--	+	+	+
Lc. citreum NFICC87	--	+	-	-	-			+	+	-		+	-	+	--		--	+
Lc. citreum NFICC94			-										-				--	+
L. plantarum NFICC163		+	+	+	-			+	+				+		+			+
L. planatrum NFICC207		+	--	+	--	+			+				+				+	+
L. plantarum NFICC289									-						-			+
P. pentosaceus NFICC341		+							+				-		+	--	-	+



a **b** **c**

Figure 1. Plates showed different inhibitory ranges. A: no inhibition *P. crustosum* IBT 35685 + *P. pentosaceus* NFICC341; b: partial inhibition *P. crustosum* IBT 35685 + *L. plantarum* NFICC289; c: complete inhibition *P. crustosum* IBT 35685 + *Lc. citreum* NFICC28



d **e** **f**

Figure 2. Plates showed different inhibitory ranges. D: no inhibition *A. glaucus* IBT 17128 + *L. plantarum* NFICC19; e: partial inhibition *A. glaucus* IBT 17128 + *P. pentosaceus* NFICC58; f: complete inhibition *A. glaucus* IBT 17128 + *L. plantarum* NFICC207

5.3.2.2 Dual plate antifungal assay

The anti-mould activity of the LAB strains was assessed in a dual plate confirmatory assay after 9 days of incubation with optimal conditions for the spoilage agents (Table 6). A different strain behaviour was observed between the various moulds and the inhibiting action of the various LAB. The most sensitive moulds resulted to be *M. circinelloides* IBT 7731 with a complete absence of growth when challenged with the various LAB, followed by *A. candidus* IBT 15621 and IBT 22587, and *A. flavus* IBT 15711. Concerning the specific moulds belonging to the *Penicillium* genus, in general, positive results were also obtained. Only the strains *P. manginii* IBT 31230 and *P. crustosum* IBT 35685 display less sensitivity with inhibitions value <50% when challenged with some LAB such as *L. plantarum* NFICC163 and NFICC72. In fact, *Penicillium* species can develop in environments with low pH (down to pH 3 or pH 2), low temperature (below 5 °C, and some may even survive at 0 °C), low water activity (about 0.8), and low oxygen levels (usually 2%, sluggish growth at 0.5% O_2) results in high resistance against adverse environments [39]. Regarding the two different *A. niger* strains (IBT 22586 and IBT 34245), promising results were obtained. In particular, against the latter strain mould, inhibitions ≥50% were reached using *Lc. citreum* NFICC28 and NFICC87. In general, as reported from the following table, strains belonging to *P. pentosaceus*, *Lc. citreum*, and *L. plantarum* confirmed again their strongest activity against spoilage moulds showing accordance with the previous results. Their activity could be correlated with the different origins of the strains (sourdough and plants), which could affect their behaviour causing different stress responses and enhancing their metabolic production of antifungal compounds [36-37, 39, 41].

Table 6. Results of the radial growth (in mm) of the various moulds after 9 days of incubation. Superscript capital letters represent the statistical differences within a row, while small superscript letters represent differences in the same column. Different letters are significantly different ($P < 0.05$). Data are presented as mean ± SD, n = 3. The percentage of inhibition obtained with the formula by Chen et al. [23] was also reported.

LAB/ PAT	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*P. pentosaceus* NFICC10	*L. planatrum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC 87	*Lc. citreum* NFICC 94	*L. plantarum* NFICC163	*L. planatrum* NFICC207	*L. plantarum* NFICC289	*P. pentosaceus* NFICC341	C
M. circinelloides **IBT 7731**			0 ± 0^{Bb} 100%	0 ± 0^{Bb} 100%							0 ± 0^{Be} 100%						32 ± 0^{A}
A. glaucus **IBT 15367**	20 ± 0^{BCc} 56%					18 ± 2.16^{BCcd} 60%	22 ± 4.24^{Bcab} 51%		21 ± 4.32^{Bcab} 53%		25 ± 0^{Bbc} 44%		20 ± 4.08^{BCbc} 56%			15 ± 0^{Cbc} 67%	45 ± 0^{A}
A. candidus **IBT 15621**		0 ± 0^{Dd} 100%				0 ± 0^{De} 100%		10 ± 0^{Cc} 77%	0 ± 0^{Db} 100%	10 ± 0^{Cb} 77%	30 ± 0^{Bb} 33%	10 ± 0^{Ca} 77%	13.33 ± 4.71^{Cc} 70%	11.67 ± 2.36^{cd} 74%			42 ± 0^{A}
A. flavus **IBT 15711**						5 ± 0^{Be} 88%				0 ± 0^{Cc} 100%	0 ± 0^{Ce} 100%		0 ± 0^{Cd} 100%	0 ± 0^{Cd} 100%			45 ± 0^{A}
A. glaucus **IBT 17128**						35 ± 7.07^{Ba} 22%	31.67 ± 2.36^{B} a 29%	40 ± 0^{Aa} 11%	33.33 ± 2.36^{Ba} 26%	40 ± 0^{Aa} 11%	28.33 ± 2.36^{B} Cb 37%		20 ± 0^{Cbc} 56%	33.33 ± 2.36^{B} ab 26%			45 ± 0^{A}
A. flavus **IBT 22573**	45 ± 0^{Aa} 0%																45 ± 0^{A}
A. niger **IBT 22586**					23 ± 0.37^{Cda} 48%	21.67 ± 2.36^{CDbc} 52%	30 ± 0^{BCDa} 33%	31.67 ± 2.36^{Bcab} 30%			36.67 ± 2.36^{Ba} 19%		31.67 ± 2.36^{Bcab} 30%			30 ± 4.08^{BCDa} 33%	45 ± 0^{A}
A. candidus **IBT 22587**	0 ± 0^{Be} 100%	10 ± 0^{Bc} 75%				20 ± 0^{Bbc} 50%		5 ± 0^{Bc} 88%	20 ± 7.07^{Bab} 50%	15 ± 7.07^{Bb} 63%	20 ± 0^{Bc} 50%		20 ± 7.07^{Bbc} 50%	20 ± 7.07^{Bbc} 50%	20 ± 0.75^{Ba} 50%	20 ± 0^{Bb} 50%	40 ± 0^{A}
P. sizovae **IBT 23147**											20 ± 0^{Bc} 55%						43 ± 0^{A}
P. roqueforti **IBT 26359**									22 ± 2.16^{Aab} 51%								45 ± 0^{A}
A. glaucus **IBT 28280**	15 ± 4.08^{Cd} 67%			0 ± 0^{Cb} 100%	0 ± 0^{Cb} 100%				31.67 ± 2.36^{Ba} 30%		0 ± 0^{Ce} 100%						45 ± 0^{A}
P. manginii **IBT 31230**	31.67 ± 2.36^{BCb} 30%	15 ± 0^{DEFb} 67%	10 ± 0^{Fa} 78%	10 ± 0.41^{Fa} 78%	25 ± 0.41^{CDEa} 44%	30 ± 4.08^{BCDab} 33.33%	9 ± 2.94^{Fb} 80%	35 ± 0^{ABCab} 22%	18.33 ± 2.36^{DEFab} 60%	40 ± 0^{Aba} 5%	20 ± 0^{Fc} 55%	10 ± 0.71^{Fa} 78%	41.67 ± 2.36^{Aba} 0%	38 ± 5.89^{Aba} 15%		15 ± 4.08^{DEFbc} 67%	45 ± 0^{A}
A. niger **IBT 34245**								21.67 ± 2.36^{Babcd} 52%			19.67 ± 3.40^{Bc} 56%						45 ± 0^{A}
P. crustosum **IBT 35685**	20 ± 0^{BCc} 56%	20 ± 0^{Bca} 56%				20 ± 4.08^{BCbc} 56%	20 ± 7.07^{Bcab} 56%	20 ± 12.25^{BCbc} 56%	30 ± 10.80^{Aba} 33.33%	15 ± 0^{BCb} 67%			40 ± 0^{Aa} 11%		10 ± 0.25^{Ca} 78%	15 ± 0^{BCbc} 67%	45 ± 0^{A}
C. carpum **IBT 42662**							15 ± 0^{Bb} 50%			15 ± 0^{Bb} 50%						10 ± 0^{Cc} 67%	30 ± 0^{A}
C. herbarum **IBT 42663**		8 ± 1.63^{Bc} 77%				10 ± 0^{Bde} 71%	15 ± 7.07^{Bb} 57%	10 ± 7.07^{Bc}	10 ± 0^{Bb} 71%		10 ± 3.60^{Bd} 71%	10 ± 0^{Ba} 71%		10 ± 4.08^{Bcd} 71%		10 ± 0^{Bc} 71%	35 ± 0^{A}

5.3.3 In vivo antifungal activity

After the confirmatory screening, an *in vivo* experiment using wheat bread was performed to assess the actual inhibitory ability, in non-laboratory growth conditions, of the LAB strains previously showing the most promising and consistent results. The obtained results are reported in table 7 and display a particular efficiency of some LAB strains to inhibit the bread colonization by the moulds. *A. candidus* IBT 15621 confirmed its strength regarding the matrix colonisations and display a statistical sensitivity to the various LAB, in particular when challenged with *L. plantarum* NFICC19 in complete accordance with the data reported in the previous paragraphs (Figure 3). The strong antifungal action of this specific *L. plantarum* strain could be explained by its habitat of isolation (plant) and its high production of organic acids in particular lactic and acetic acids [41]. Furthermore as stated from the untargeted results obtained from the GC-MS numerous metabolites known to display an antifungal activity have been found in the MRS broth fermented by this LAB (Table 8).

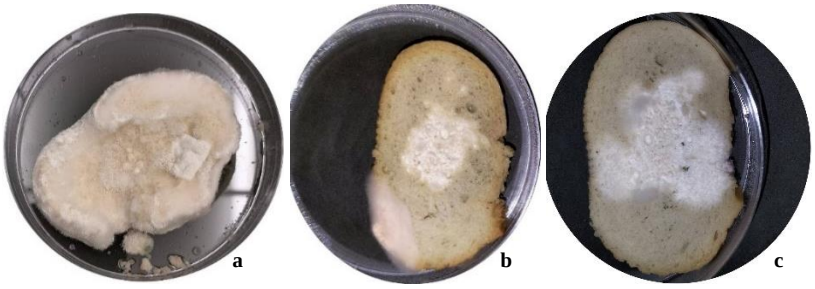


Figure 3. *A. candidus* IBT 15621 on wheat bread slice after 9 days of growth. Alone (a), challenged with *L. plantarum* NFICC19 (b) and with *L. plantarum* NFICC72 (c),

On the contrary, *A. candidus* IBT 22587, resulted in being more resistant to the LAB antifungal action in a non-optimal lactic acid bacteria habitat such as bread, showing statistical evidence of inhibition only with *L. plantarum* UMCC 2996, *P. pentosaceus* NFICC58 and *L. plantarum* NFICC207 (Figure 4). *A. candidus* alongside with *P. roqueforti*, represent one of the common

moulds present in raw material such as wheat flour and rye, explaining its particular adaptation to bakery products such as bread, in which this mould strain was also isolated [43].

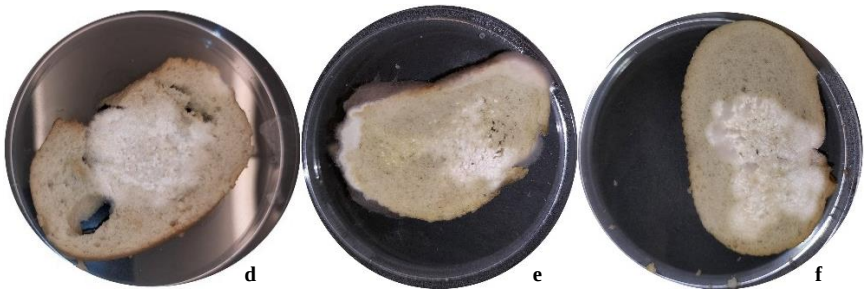


Figure 4. *A. candidus* IBT 22587 on wheat bread slice after 9 days of growth. Alone (d), challenged with *L. plantarum* UMCC 2996 I and *F. sanfranciscensis* UMCC 2990 (f)

P. manginii IBT 31230 and *P. crustosum* IBT 35685, are reported to be common spoilage agents not only in bread but also in food products containing cereal in general as reported by Wigmann et al. [44]. Although *Penicillum* species are considered one of the most adaptable moulds genera to unfavourable growing conditions, statistically relevant inhibitions were detected particularly when in co-culture with *P. pentosaceus* NFICC341, *L. plantarum* NFICC27 (Figure 5) and NFICC289, respectively. As reported in the subsequent paragraph (*3.4.1*) these LAB strains were able to produce different compounds, such as 2-methylbutanoic acid, generally produced under fermentative stress and reported to have an antagonistic effect against moulds related to vegetable and cereal spoilage [45-47].

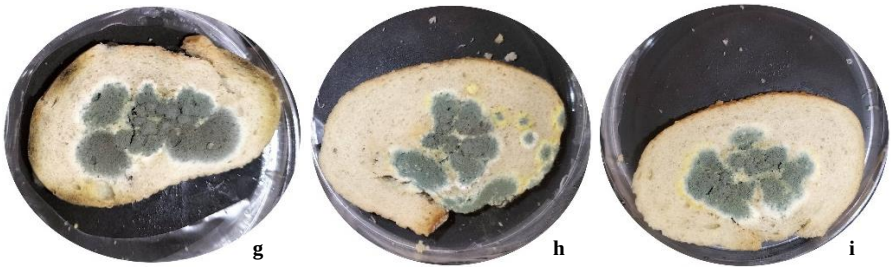


Figure 5. *P. manginii* IBT 31230 on wheat bread slice after 9 days od growth. Alone (g), challenged with *L. plantarum* NFICC27 (h) and *P. pentosaceus* NFICC341 (i).

Corroborating the activity already detected in the previous confirmatory screenings, a strong inhibition against *A. niger* IBT 34245 was detected again when tested with *Lc. citreum* NFICC28. As visible in figure 6, the presence of the LAB caused also a change in the morphology of the mould strain, suggesting the production and presence of particularly antagonistic compounds negatively affecting the mould thriving such as 1-Pentanol and propanoic acid, and a putative detoxification activity [48-49].

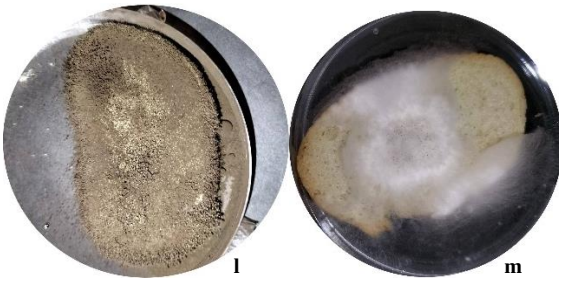


Figure 6. *A. niger* IBT 34245 on wheat bread slice after 9 days of growth. Alone (l), in challenged with *Lc. citreum* NFICC28 (m)

In contrast, no statistical significance was observed for *A. niger* IBT 22586 and complete colonization of the bread slices was reported already after four days of incubation (Figure 7).

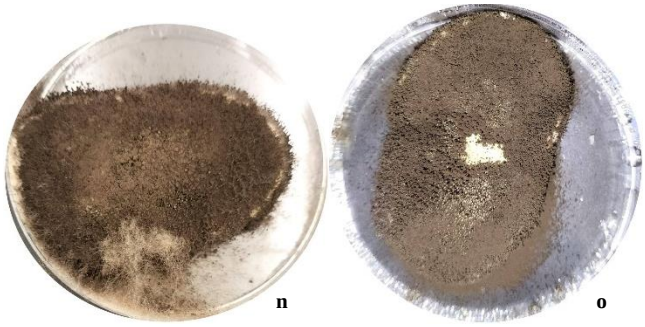


Figure 7. *A. niger* IBT 22586 on wheat bread slice after 4 days of growth. Alone (n), in challenged with *L. plantarum* NFICC19 (o)

The origin of the raw material from which the strain has been isolated wheat from Italy) possibly explain the deepest mould's resistance to antifungal compounds and nutritional competition. In fact, as recently reported by Mateo et al. [50], toxigenic fungi, such as *Aspergillus* spp., colonizing cereals of the South-European area exhibited adaptative abilities leading to massive economic loss and health concerns. Positive results were validated again also in the inhibitory capacity of *P. pentosaceus* UMCC 3010, *Lc. citreum* UMCC 3011 and *Lc. citreum* NFICC87 against the thrive of *M. circinelloides* IBT 7731, indicating the presence of different antifungal metabolites working synergically as previously proposed by Shi et al. [51]. Besides, statistical significance, with a complete absence of growth, was obtained regarding *A. glaucus* IBT 17128 and IBT 28280 (Figure 8) when tested with *L. plantarum* NFICC207 and *F. sanfranciscensis* UMCC 2990, respectively, connotating possible implication of a conspicuous change in the fungi metabolic profiles.

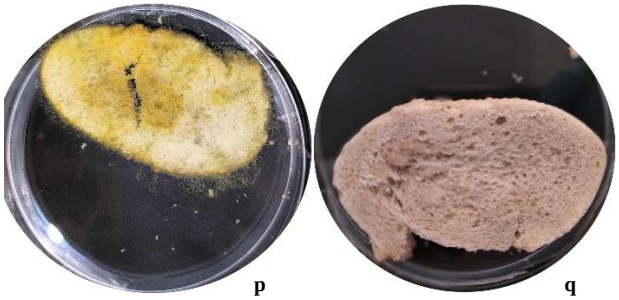


Figure 8. *A. glaucus* IBT 28280 on wheat bread slice after 9 days of growth. Alone (p), challenged with *F. sanfranciscensis* UMCC 2990 (q).

Table 7. *In vivo* antifungal screening results on wheat bread slice expressed in mm. Data were analysed using one-way ANOVA. Superscript letters represent the statistical differences within a row. Different letters are significantly different ($P < 0.05$). Data are presented as mean ± SD, n = 3. In the cases where ANOVA was not applicable, data were analysed using one-tailed t-tests, and asterisks marked samples significantly different from the control.

Moulds/LAB		*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*P. pentosaceus* UMCC 3010	*Lc. citreum* UMCC 3011	*L. plantarum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC28	*P. pentosaceus* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC 87	*Lc. citreum* NFICC 94	*L. plantarum* NFICC163	*L. plantarum* NFICC207	*L. plantarum* NFICC289	*P. pentosaceus* NFICC341	Control
***M. circinelloides* IBT 7731**	Length			30 ± 0.16^{b}	30 ± 0^{b}						30 ± 0^{b}						$50 \pm 0,16^{a}$
	Widht			10 ± 0^{c}	10 ± 0^{c}						25 ± 0.41^{b}						40 ± 0^{a}
***A. candidus* IBT 15621**	Length		30 ± 0.20^{b}			14.3 ± 0.06^{e}			33 ± 0.17^{cd}	35 ± 0.10^{c}				40 ± 0^{b}			65 ± 0.20^{a}
	Widht		30 ± 0^{d}			21.7 ± 0.29^{c}			31.3 ± 0.32^{b}	30 ± 0^{b}				23.3 ± 0.31^{c}			40 ± 0^{a}
***A. glaucus* IBT 17128**	Length												0± 0*				50.2± 0.01
	Widht												0± 0*				39.6± 0
***A. niger* IBT 22586**	Length					65± 0											65± 0
	Widht					40± 0											40± 0
***A. candidus* IBT 22587**	Length	35 ± 0.44^{b}	20 ± 0.50^{d}			35 ± 0.50^{b}		36.6 ± 0.28^{b}	25 ± 0.50^{cd}	35 ± 0.20^{b}	65 ± 0.00^{a}		35 ± 0.50^{b}	25 ± 0.30^{cd}	30 ± 0^{bc}	35 ± 0^{b}	35 ± 0^{b}
	Widht	25 ± 0.44^{d}	15 ± 0^{f}			35 ± 0.20^{b}		25 ± 0^{d}	25 ± 0.10^{d}	30 ± 0.20^{c}	40 ± 0.10^{a}		35 ± 0^{b}	23.3 ± 0.15^{de}	30 ± 0^{c}	30 ± 0^{c}	35 ± 0^{b}
***P. sizovae* IBT 23147**	Length										36.6 ± 0.02*						45± 0,04
	Widht										24.7 ± 0*						30.6±0
***A. glaucus* IBT 28280**	Length	0± 0*															64.7 ± 0
	Widht	0± 0*															39.5± 0
***P. manginii* IBT 31230**	Length		40± 0^{a}	30± 0^{c}	40± 0.30^{a}		23.3 ±0.15^{d}		36 ± 0.26^{ab}		30±0^{c}	32.3 ± 0.25^{bc}				20 ± 0^{d}	40± 0^{a}
	Widht		30± 0^{ab}	20± 0^{c}	30± 0.26^{ab}		21.7 ±0.15^{c}		30± 0^{ab}		20± 0^{c}	25 ± 0.20^{bc}				10± 0.50^{d}	35 ± 0^{a}
***A. niger* IBT 34245**	Length							25 ± $0,41^{b}$			65 ± 0.07^{a}						65 ± 0.02^{a}
	Widht							40 ± $0,03^{a}$			40± 0.03^{a}						40± 0.03^{a}
***P. crustosum* IBT 35685**	Length		50 ± 0.20^{bc}			45 ± 0.46^{cd}	40 ± 0^{d}	40 ± 0^{d}		55 ± 0.10^{b}	40 ± 0.30^{d}				33.3 ± 0.31^{e}		65 ± 0^{a}
	Widht		30 ± 0^{bc}			25 ± 0.17^{c}	30 ± 0^{bc}	26 ± 0.10^{c}		33.3 ± 0.29^{b}	35 ± 0^{ab}				26.7 ± 0.15^{c}		40 ± 0^{a}
***C. carpum* IBT 42662**	Length															0± 0*	65± 0
	Widht															0± 0*	40.6± 0

5.3.4 Detection and identification of antifungal compounds

5.3.4.1 Untargeted dynamic headspace GC-MS analysis

In order to identify the putative antifungal compounds production of the promising tested LAB when growing solo, untargeted analysis on fermented MRS broth was conducted. The obtained results are shown in table 8 and plain MRS was also reported and used as a standard in order to detect the media compounds already present. However, due to the qualitative outline of the conducted analysis, no data on the exact amount of the produced metabolites are available. LAB bio-control activity and the various beneficial outcomes derived from their employment in food are strictly related to their metabolisms. Even though no fungi were present in co-colture with the LAB, the production of various compounds recognized to have antifungal activity has been detected. As displayed in the subsequent table, majority of the LAB were able to produce alcohols, generally affecting mould development, such as isoamyl alcohols, 1- Butanol and 2-ethylhexanol [52-57]. Various acids like acetic acid, 2-methylbutanoic acid, benzoic acid and propanoic acid were also observed highlighting the potential inhibitory activity of the various LAB. In fact, as aforementioned these compounds are regarded as some of the main players affecting and controlling mould spoilage in cereals [45-47, 58-59]. As the pH of the medium decreases due to acid production, fungus growth is inhibited and the cell membrane becomes damaged due to acidic substances. Low pH is responsible for the fragmentation and recombination of acids in their active undissociated forms which may pass through the cytoplasmic membrane and disrupt the membrane proton motive force. Luz et al. [60] also reported several ketones, aldehydes, pyrazine, and alcohols observed in this study confirming their effect against mould thriving, and the particular anti-spoilage capacity of LAB isolates from sourdough. Substantiating this evidence, various tested LAB isolated from sourdough demonstrated a strong anti-mould ability, *F. sanfranciscensis* UMCC 2990 completely block the

growth of *A. glaucus* IBT 28280 when tested *in vivo* experiment, while other LAB strains statistically reduced the spoilage agents growth both in the *in vivo* and common media experiments. Promising results were achieved as well with LAB belonging to *P. pentosaceus*, *L. plantarum* and *Lc. leuconstoc* species isolated from vegetable matrices. These species are generally regarded as the main active against spoilage agents and reported in various studies underlining their crucial importance in food safety [61-62]. Regardless of the isolation matrices, the main key of the potent antifungal and antibacterial activity of LAB is represented by the production of different types of metabolites working in synergy between them and the consequent dropping pH [61].

Table 9. Results of the untargeted dynamic headspace GC-MS on MRS broth fermented by the various LAB strains selected for this study. The table listed all the different compounds that have been detected and x marks the strains in which they were present.

Compounds	MRS	*F. sanfranciscensis* UMCC 2990	*L. plantarum* UMCC 2996	*P. pentosaceus* UMCC 3010	*L. citreum* UMCC 3011	*P. pentosaceus* NFICC10	*L. plantarum* NFICC19	*L. plantarum* NFICC27	*Lc. citreum* NFICC28	*P. pentosacues* NFICC58	*L. plantarum* NFICC72	*Lc. citreum* NFICC87	*Lc. citreum* NFICC94	*L. plantarum* NFICC163	*L. plantarum* NFICC207	*L. plantarum* NFICC289	*P. pentosaceus* NFICC 341
1-Butanol			x		x	x	x	x		x	x	x	x	x	x	x	x
1-Butanol, 3-methyl- acetate				x													
1-Hexanol		x		x			x				x						
1-Hexenal	x																
1-Methylnonane														x			
1-Nonanol											x						
1-Octen-3-ol	x																
1-Pentanol	x		x						x	x	x	x	x		x	x	x
1-Penten-1-ol	x																
1-Penten-3-ol	x																
1-Penten-3-one	x																
1-Phenylethanol			x		x		x			x					x	x	x
1-Tetradecanol									x	x	x		x	x		x	
1-Undecanone										x							
2,3,4-Trimethyloxetane		x		x													
2,3,5-Trimetylhexane														x			
2,3-Butanedione	x							x	x			x	x	x	x	x	x
2,3-Hexanedione	x																
2,4(1H,3H)-Pyrimidinedione	x																
2,4-Decadienal	x																
2,4-Dimethylheptane									x		x		x		x	x	x
2,4-Heptadienal	x																
2,4-Hexadienal	x																
2,4-Methylheptane									x								

2,5-Dimethyl-2-hexanol					x												
2,5-Dimethyl-3-hexanol						x							x	x	x	x	x
2,5-Dimethylfuran		x		x													
2,5-Dimethylpyrazine		x	x	x	x	x	x			x	x	x	x	x	x	x	x
2,5-Methyl-3-hexanol								x	x			x					
2,5-Methylfuran			x														
2,5-Methylpyrazine								x	x								
2,6,10-Trimethyltetradecane													x				
2-Butanol		x		x							x		x				
2-Butanone			x				x				x						
2-Butyl-1-octanol									x			x					x
2-Decenal	x																
2-Dimethyl-2-butenal															x		
2-Ethyl-5-methylpyrazine		x	x	x					x	x		x	x	x	x		
2-Ethyl-6-methylpyrazine												x				x	
2-ethylhexanol		x	x	x	x		x	x	x	x	x	x	x	x		x	x
2-Ethylpyrazine						x		x									
2-Heptanol		x															
2-Heptanone	x		x		x	x		x	x	x	x	x	x	x	x	x	x
2-Heptenal	x																
2-Hydroxy-2-methylpropanenitrile			x		x							x	x				x
2-Methyl-1-butanol		x		x			x		x	x				x		x	
2-Methyl-1-propanol			x		x		x	x		x	x	x	x				
2-Methyl-2-butenal			x	x	x	x	x	x		x	x	x	x	x		x	x
2-Methyl-5-ethylpyrazine					x			x			x					x	x
2-Methyl-6-ethylpyrazine											x						
2-Methylbutan									x								
2-Methylbutanoic acid		x	x	x						x				x	x	x	x
2-Methylbutenal		x				x			x								
2-Methylfuran	x																
2-Methylhexanoic acid											x	x	x				

2-Methylhexanol															x		
2-Methylpropanoic acid		x		x													
2-Methylpyrazine									x	x	x						
2-Nonanol		x															
2-Nonanone						x			x	x		x	x	x	x	x	x
2-Octenal	x																
2-Pentanol		x		x													
2-Pentanone					x												
2-Pentenal	x	x															
2-Penthylfuran	x																
2-Propanol										x	x						
2-Propanone					x					x							
2-Tridecanone					x				x	x		x	x	x	x	x	
2-Undecanol		x															
2-Undecanone			x		x				x		x	x	x	x		x	x
3,4-Dimethylbenzaldehyde					x				x	x		x	x	x	x	x	x
3,4-Trifluoracetic										x							
3-Ethyl-2,5-dimethylpyrazine		x										x					
3-Ethyl-2,5-methylpyrazine												x					
3-Hydroxy-2-butanone			x			x		x				x	x		x		x
3-Methyl-1-butanol		x						x									
3-Methyl-2-butanone									x	x	x			x			x
3-Methyl-2-butenal					x	x			x	x	x	x	x	x	x	x	x
3-Methylbutanoic acid		x		x													
3-Trifluoroacetoxypentadecane												x				x	
4,5-Dimethylnonane		x	x	x									x				
4,6-Dimethyldodecane									x								
4-Heptenal	x																
4-Methyl-1-pentanol	x																
4-Methyl-2-pentanol		x															

4-Methyldecane									x		x	x					
5-Ethyl-2-methyloctane						x			x	x		x		x			x
5-Ethyl-2-nonanol													x				
5-Ethyl-5-methyldecane		x															
5-Methyl-2-hexanol				x													
6-Methyl-5-hepten-2-one					x				x	x	x	x	x	x	x	x	x
9-Octadecenoic acid					x						x						
Acetic acid		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Acetoin					x												
Acetone							x	x			x			x	x	x	
Acetophenone									x		x	x	x	x			
Benzaldehyde	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x
Benzene					x												
Benzene 1,3-bis(1,1-dimethyl)													x	x	x	x	
Benzene 1,3-bis(1,1-dimethylethyl)		x	x	x		x		x	x	x	x	x					x
Butanal	x																
Butanoic acid		x	x	x	x	x	x	x	x	x	x		x	x	x	x	x
Carbone dioxide	x	x															
Decane					x						x	x	x				
Dimethyl propanedioic acid																	x
Dodecane		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Dymethil ether		x		x													
Ehtylpyrazine		x		x	x	x	x		x	x	x	x	x	x	x	x	x
Ethylacetato																	
Heptanal	x																
Heptane	x										x				x		
Hexadecane											x	x					
Hexanal	x																
Hexanoic acid									x								
Hydrazoic acid													x				

Hydrogen azide		x															
Isoamyl alcohol					x	x					x			x			
Isobutane		x															
Isobutyl alcohol														x	x	x	x
Isopropyl alcohol		x		x													
Isovaleric acid			x		x	x		x	x	x	x	x	x	x	x	x	x
Malonic acid					x												
Methane nitrous	x		x				x	x	x	x	x	x	x	x	x		x
Methylpyrazine		x	x	x	x	x	x	x		x	x	x	x	x	x	x	x
Nonanal	x									x	x	x		x	x	x	x
Nonenal	x																
Octanal	x																
Octane	x				x												
Pentadecane								x	x	x	x	x		x	x	x	x
Pentanal	x																
Phenol, 2,4-bis-(1,1-dimethyl)			x							x					x		
Phenol, 2,4-bis-(1,1-dimethylethyl)												x		x			x
Phenol, 3,5-bis-(1,1-dimethyl)								x	x							x	
Phenol, 3,5-bis-(1,1-dimethylethyl)											x		x				
Propanedioic acid			x			x	x	x	x	x	x	x	x	x	x	x	
Propanenitrile									x								
Propanoic acid		x	x						x	x	x	x	x		x	x	x
Pyrazine		x	x	x		x	x	x		x	x	x	x	x	x	x	x
Styrene									x		x	x					
Undecanal		x															

5.4 Conclusion

LAB represent a powerful source of antifungal compounds and numerous studies have outlined their crucial activity in the preservation of meals and feeds. As stated in this study the scouting of anti-fungal LAB in different environmental sources such as fermented food and vegetable matrices and their preservation in microbial culture collections are fundamental for the exploitation and creation of innovative starter cultures able to answer the preservation needs of bread and cereal products in general. Among the different LAB species tested in this experiment, *L. plantarum* and *Lc. citreum* display the broadest and strongest antifungal activity even in a non-optimal environment such as wheat bread slices. Furthermore, the investigation of LAB metabolites may help to understand the particular interaction between the various compounds and the different spoilage agents revealing innovative strategies to overrun spoilage resistance mechanisms. Nevertheless, further metabolomics and proteomics analysis are required to properly understand the interaction mechanisms and putative detoxification activity of the LAB.

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6. Final remarks

Fermented foods have an enormous potential to improve nutrition, promote health, and lower the risk of many diseases all around the world. In addition to the numerous positive outcomes on health, they also help extend the product shelf-life and are a source of different species of LAB and yeast. Contemplating the numerous studies highlighting the importance of LAB and their metabolic outcomes, this Ph.D. thesis aims to contribute to the exploitation of the LAB collected in the UMCC and the isolation of innovative LAB with enhance anti-spoilage ability in order to answer the new consumer and eco-green necessity. In fact, food security is crucial, especially with the high globalization of food and the rapid increase in the world population. LAB due to their ability to suppress pathogens and spoilage agents, both bacterial and fungi, have emerged as excellent alternatives to chemical preservatives. Their QPS status, their bacteriocins, organic acid, fatty acid, and antifungal metabolites, found in this study, have proved their competitiveness and efficiency as bio-preservatives with possible employment in innovative technologies such as food biofilm and circular economy. The corpus of knowledge on the industrial uses of LAB and their metabolites, the different roles they play as part of several food products to stop/avoid the spread of spoilage bacteria and foodborne pathogens, and the consequent reduction of viral diseases linked to food, is represented in the outcomes of this Ph.D. thesis. In conclusion, the use of different species of LAB characterised by different metabolic ability represent the key for the production of safer and healthier functional foods.

7. Other publications and Conference Posters

fermentation

MDPI

Article

Selection of Wine *Saccharomyces cerevisiae* Strains and Their Screening for the Adsorption Activity of Pigments, Phenolics and Ochratoxin A

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Received: 22 July 2020; Accepted: 1 August 2020; Published: 5 August 2020



Abstract: Ochratoxin A is a dangerous mycotoxin present in wines and is considered the principal safety hazard in the winemaking process. Several authors have investigated the ochratoxin A adsorption ability of *Saccharomyces cerevisiae* yeasts, and specifically selected strains for this desired trait. In the present work, a huge selection of wine yeasts was done starting from Portuguese, Spanish and Italian fermenting musts of different cultivars. Firstly, 150 isolates were collected, and 99 non-redundant *S. cerevisiae* strains were identified. Then, the strains were screened following a multi-step approach in order to select those having primary oenological traits, mainly (a) good fermentation performance, (b) low production of H_2S and (c) low production of acetic acid. The preselected strains were further investigated for their adsorption activity of pigments, phenolic compounds and ochratoxin A. Finally, 10 strains showed the desired features. The goal of this work was to select the strains capable of absorbing ochratoxin A but not pigments and phenolic compounds in order to improve and valorise both the quality and safety of red wines. The selected strains are considered good candidates for wine starters, moreover, they can be exploited to obtain a further enhancement of the specific adsorption/non-adsorption activity by applying a yeast breeding approach.

Keywords: adsorption; ochratoxin A; phenolics; pigments; *Saccharomyces*; winemaking; yeasts

1. Introduction

Ochratoxin A (OTA) is a mycotoxin largely detected in several foods such as cereals, bread, coffee, dried wine fruits, as well as in beer, grape juice and wine [1–3].

OTA is composed of a 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-R-methylisocoumarin (OTA α) moiety and an L-β-phenylalanine molecule linked through the 7-carboxy group by an amide bond [4] (Figure 1).

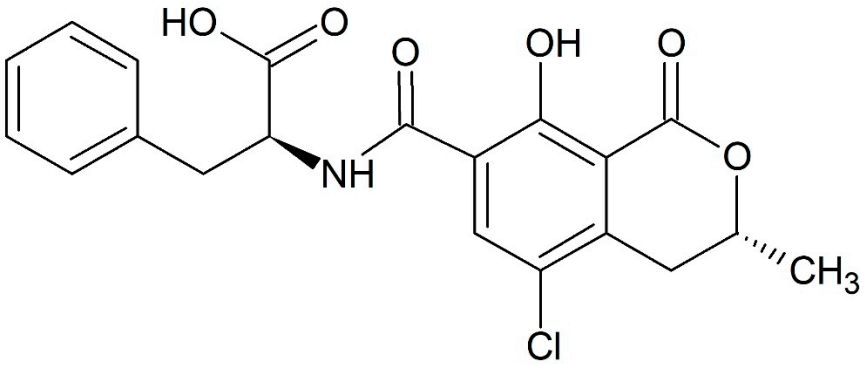


Figure 1. Chemical structure of Ochratoxin A.

The presence of OTA in wines and juices occurs due to the growth of a fungal contamination in grapes at pre- and post-harvest [5]. Moulds belonging to *Aspergillus* and *Penicillium* genera are mainly responsible for the contamination [6,7].

During the last few years, OTA has received a special focus as it is considered the principal safety hazard in the winemaking process [8,9]. OTA contamination is, in fact, very dangerous due to its carcinogenic, nephrotoxic and teratogenic effects [6]. The contamination of grapes seems to be mainly influenced by the different geographical and climatic zones. In Europe OTA is prevalent in wines originating from southern areas of the Mediterranean basin with typically warmer climates [10,11]. In fact, in these wine-growing regions, red wines are frequently more contaminated than white wines [12]. In order to protect consumers, the EU committee has established the maximum OTA levels for wines and musts at 2 μg/L [2]. Common strategies used for the containment of the toxin include good agricultural practices, selection of fungal resistant crop varieties, correct application of fungicides as well as proper storage of commodities. However, these individual measures could result in unsuccessful or be inadequate with the results of still having OTA in feed and products which require additional decontamination or detoxification procedures [13]. Inorganic adsorbents such as zeolites, bentonites and activated carbon can be used to control OTA but, in many cases, they decrease the nutritive value and organoleptic properties as well as affect the production cost. An alternative approach, which has received a growing interest, is the mycotoxin detoxification by microorganisms. Numerous studies reveal that some bacteria and yeasts species, such as *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Saccharomyces cerevisiae*, and *Kloeckera apiculata* are able to detoxify mycotoxins [2]. In particular, several authors have investigated the OTA adsorption ability of wine *S. cerevisiae* strains [4,14–16] and have proposed polyphasic approaches to select the strains with this desired trait in order to use them as a wine starter [17,18]. Although the exact mechanism of OTA-removal from contaminated grape must by yeasts is still not fully understood, it has been supposed a fundamental role of the cell wall and its primary components such as β-glucans and mannoproteins [4,19]. In particular, mannoproteins could be implicated in the OTA adsorption because of their common ability to bind mycotoxins, attributed to modified mannanoligosaccharide [14].

Unfortunately, yeast cell wall can adsorb not only OTA, but, at different levels of adsorption, also other components, such as phenolic compounds and pigments, causing colour loss in wines [14,19,20].

Therefore, the aim of this work was to select wine *S. cerevisiae* strains that are able to show an opposite adsorption activity of OTA, pigments and phenolic compounds. The selection was performed with a multi-step screening starting from the sampling of a large number of wine yeasts, which were preliminary selected for primary oenological traits. Only the best strains were then screened for the following traits of interest: (a) wine colour protection, (b) preservation of phenolics, and (c) removal of OTA. The final goal was to find strains with all the desired traits, to be used as wine starters able to improve and valorise both quality and safety of red wines.

2. Materials and Methods

2.1. Samples and Yeast Isolation

In this study, spontaneously fermenting musts were collected in three different Mediterranean areas (Italy, Spain and Portugal).

Each sample was diluted in physiological solution (9 g/L NaCl) by a tenfold dilution series. A volume of 0.1 mL of the highest dilution (10^{-7}) was spread onto plates of Yeast Peptone Dextrose Agar (YPDA) medium (1% *w/v* yeast extract, 1% *w/v* peptone, 2% *w/v* dextrose and 2% *w/v* agar). The plates were incubated at 27 °C for two days and the isolated colonies were purified by streaking on fresh YPDA medium. A total of 150 isolates were initially characterised according to the standard procedures of the Microbial Resource Research Infrastructure-Italian Joint Research Unit (MIRRI-IT) [21]. rDNA ITS-RFLP restriction analysis and interdelta regions typing [22] allowed us to identify the 99 non-redundant *S. cerevisiae* described in Table 1. Periodical transplants of the yeast cultures in a fresh YPDA were performed to ensure growth and viability of the microbial cells used in the different screenings. Strain sporulation activity was tested on acetate medium (1% *w/v* anhydrous sodium acetate, 2% *w/v* agar). The plates were incubated at 28 °C and asci formation was microscopically checked after 14 days.

Table 1. Origin of the 99 *S. cerevisiae* strains used in the present work.

Wine Sample Cultivar.	Region (Country)	Number of Non-Redundant *S. cerevisiae* Strains (Codes Range)
Nerello mascalese	Sicily (Italy)	21 (RE001–RE021)
Carricante		3 (RE022–RE024)
Grecanico		8 (RE025–RE032)
Grecanico	Penedès and La Rioja (Spain)	2 (RE033–RE034)
Tempranillo		33 (RE035–RE067)
Touriga		3 (RE068–RE070)
Touriga national	Porto (Portugal)	7 (RE071–RE077)
Touriga franca		12 (RE078–RE089)
Tinta rorizi		10 (RE090–RE099)

Culture copies cultivated on YPDA were preserved at −80 °C in cryovials supplemented with glycerol (Merck, Darmstadt, Germany) at 25% (*v/v*) final concentration and safe deposited at Unimore Microbial Culture Collection (UMCC).

2.2. Preliminary Screening

The strains were first screened by evaluating: (a) growth modality and foam production during grape must fermentation. Assays were performed in test tubes containing 10 mL of pasteurised (110 °C × 10 min) and filtered (through sterile gauze) must from white grape of the cultivar *Greco bianco*. The must was inoculated with each culture strain and tested for acetic acid production on chalk agar at 30 °C for 3 days according to Lemaresquier et al. [23]; H_2S production on BiGGY agar (Oxoid, Milan, Italy) at 25 °C for 48 h, according to Nickerson [24].

2.3. Fermentation Trials and Adsorption Activity of Pigments and Phenolics

Based on the results obtained with the preliminary screening, the resulting strains were further studied for their aptitude for adsorbing grape pigments. To this purpose, the strains were grown in Petri dishes with a chromogenic grape-skin agar medium following the procedure described by Caridi [20]. The dishes were put into plastic bags and hermetically closed after the insufflation of nitrogen gas for 1 min. After 10 days of anaerobic incubation at 28 °C, yeast biomass was subjected to the computer-assisted evaluation of the red, green, and blue component. The image was processed for colour using Adobe Photoshop CS for Windows XP. The region of interest was set to 5 × 5 pixels

taking four replicates for each strain. Photoshop's red-green-blue colour mode assigned an intensity value to each region. The same strains were tested in micro-winemaking trials to assess their aptitude for adsorbing grape pigments and phenolics during fermentation. The must was prepared starting from red grapes subjected to a pre-fermentative maceration to extract phenolic compounds from skins and seeds. They were destemmed, crushed and cold soaked at 0 °C for 3 days, performing a punch down twice per day. The must obtained after pressing (pH 3.50, °Brix 23) was divided in aliquots of 20 mL, suddenly inoculated at 5% in triplicate with the *S. cerevisiae* strains and incubated at 20 °C. After three days, the fermentation vigour was assessed as the weight loss caused by CO_2 production (g CO_2/100 mL). At the end of fermentation, wine samples were centrifuged at 2300× *g* for 5 min and diluted 1:5 (*v*/*v*) with a pH 3.5 buffer (citric acid monohydrate 0.1 M and Na_2HPO_4 0.2 M). The wine absorbance was read at 420, 520, and 620 nm; the intensity (I) was calculated with the following formula: I = A420 + A520 + A620 [25]. The total phenolic content was determined using the Folin-Ciocalteu index (FC index) according to Singleton and Rossi [26]. The strains which showed a high adsorption activity of grape pigments and phenolics were excluded from the final trial.

2.4. OTA Adsorption Activity

The remaining strains were studied for their aptitude for removing OTA from synthetic must (6.7 g/L yeast nitrogen base, 5.0 g/L tartaric acid, 5.0 g/L malic acid, 0.2 g/L citric acid, 110 g/L dextrose, 100 g/L fructose, and 7 g/L saccharose, pH 3.3) contaminated with the addition of 5 µg/L OTA (Sigma-Aldrich, St. Luis, MO, USA). Yeast precultures were prepared in YPD broth and cultivated at 28 °C for 48 h. Tests were performed inoculating in triplicate 10 mL of the synthetic must with 0.2 mL of the precultures (~10^6 cells/mL final concentration). The fermentations were carried out at 25 °C and after 28 days the OTA content of the samples was detected by HPLC applying the protocol described by Meca et al. [4].

2.5. Statistical Analysis

All experiments were carried out at least in triplicate. The results are expressed as mean value ± standard deviation (sd). One-way ANOVA followed by a Tukey HSD post hoc test was applied to the OTA adsorption activity and fermentative vigour data, showed by the selected strains, to establish significant differences between means ($p < 0.05$). For the statistical analysis, the software GraphPad Prism version 8 was used (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Preliminary Trials on the S. cerevisiae Strains

Firstly, the 99 *S. cerevisiae* strains were screened in order to choose those with desired primary oenological traits related to growth modality, foam production, acetic acid and H_2S production. In particular, the strains which exhibited a non-flocculant growth and null or low fleeting foam production during grape must fermentation overtook the trial. Concerning the acetic acid production on chalk agar, the strains producing null or little halos (<3 mm) in the used medium passed the screening. Regarding the H_2S production, only the strains showing white or light brown colony colours on BiGGY agar medium were chosen as low hydrogen sulphide-producing yeasts. On the basis of their different behaviours, 14 strains were excluded for one or more parameters. The remaining 85 strains shown in Table 2 were further screened.

Table 2. Fermentation vigour and assessment of pigments and phenolics adsorption activity showed by the 85 preselected *S. cerevisiae* strains.

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days (g CO_2/100 mL)	Wine Colour Intensity	Phenolics Content
	Red Component	Green Component	Blue Component		Absorbance (420 + 520 + 620) nm	Folin–Ciocalteu's Index (FC)
RE001	81 ± 4	40 ± 2	45 ± 2	8.58 ± 0.11	3.908 ± 0.181	11.08 ± 5.68
RE002	73 ± 3	37 ± 1	40 ± 2	8.45 ± 0.14	4.933 ± 0.392	11.98 ± 6.48
RE004	95 ± 2	52 ± 2	50 ± 1	8.65 ± 0.22	4.580 ± 1.052	10.98 ± 5.87
RE005	78 ± 3	39 ± 2	39 ± 2	9.14 ± 0.08	3.145 ± 0.818	8.83 ± 3.30
RE006	73 ± 3	37 ± 4	40 ± 4	8.56 ± 0.07	3.897 ± 0.161	11.33 ± 5.68
RE007	60 ± 5	28 ± 2	34 ± 2	8.58 ± 0.11	4.108 ± 0.590	10.22 ± 2.55
RE008	82 ± 3	38 ± 2	42 ± 2	8.12 ± 0.14	1.855 ± 0.398	9.18 ± 2.55
RE010	71 ± 2	36 ± 2	39 ± 2	8.14 ± 0.22	3.528 ± 0.763	12.08 ± 7.07
RE011	65 ± 1	32 ± 2	38 ± 2	9.15 ± 0.08	5.418 ± 0.460	22.85 ± 3.50
RE012	66 ± 2	31 ± 2	37 ± 1	8.41 ± 0.07	5.557 ± 0.333	10.25 ± 8.46
RE013	67 ± 4	33 ± 1	41 ± 1	8.25 ± 0.11	2.833 ± 0.855	9.12 ± 5.74
RE014	80 ± 2	43 ± 1	46 ± 2	7.45 ± 0.14	4.715 ± 0.654	12.82 ± 2.33
RE015	86 ± 2	48 ± 2	49 ± 3	7.12 ± 0.18	3.532 ± 0.488	12.25 ± 0.73
RE017	108 ± 5	65 ± 4	61 ± 4	8.51 ± 0.08	6.417 ± 0.336	21.55 ± 0.41
RE018	91 ± 3	49 ± 3	50 ± 1	9.56 ± 0.07	1.588 ± 0.172	9.65 ± 3.34
RE019	69 ± 3	34 ± 1	39 ± 2	7.75 ± 0.11	4.498 ± 0.595	11.00 ± 2.35
RE020	69 ± 2	36 ± 3	41 ± 3	9.12 ± 0.14	4.870 ± 0.441	12.85 ± 6.29
RE022	72 ± 4	39 ± 2	42 ± 2	8.90 ± 0.22	4.940 ± 0.440	18.42 ± 2.68
RE023	84 ± 2	45 ± 3	45 ± 2	7.52 ± 0.08	3.128 ± 0.418	8.38 ± 6.13
RE024	70 ± 5	35 ± 1	38 ± 2	7.75 ± 0.07	3.147 ± 0.511	12.97 ± 1.88
RE025	70 ± 4	34 ± 2	39 ± 4	8.42 ± 0.11	3.317 ± 0.587	8.62 ± 5.15
RE026	68 ± 4	34 ± 2	38 ± 3	8.21 ± 0.14	2.358 ± 0.660	9.62 ± 5.40
RE027	114 ± 3	69 ± 2	62 ± 3	7.21 ± 0.18	3.957 ± 0.305	12.07 ± 1.83
RE028	62 ± 1	30 ± 2	36 ± 1	8.05 ± 0.22	4.958 ± 0.398	12.77 ± 2.48
RE029	57 ± 3	33 ± 2	37 ± 3	8.74 ± 0.08	1.530 ± 0.411	9.43 ± 5.34
RE030	70 ± 2	36 ± 2	40 ± 3	7.78 ± 0.07	3.483 ± 0.700	10.17 ± 3.60
RE031	79 ± 2	38 ± 1	40 ± 2	7.89 ± 0.11	2.888 ± 0.935	12.17 ± 3.12
RE032	91 ± 3	50 ± 2	51 ± 2	7.56 ± 0.14	3.522 ± 0.713	15.08 ± 0.53
RE033	76 ± 4	36 ± 2	40 ± 3	9.78 ± 0.18	4.990 ± 0.156	15.45 ± 2.99
RE034	61 ± 3	28 ± 4	33 ± 2	8.95 ± 0.22	3.903 ± 0.582	9.72 ± 3.77
RE036	83 ± 3	44 ± 1	47 ± 3	8.45 ± 0.07	4.817 ± 0.189	16.43 ± 1.43

Table 2. *Cont.*

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days (g CO_2/100 mL)	Wine Colour Intensity	Phenolics Content
	Red Component	Green Component	Blue Component		Absorbance (420 + 520 + 620) nm	Folin–Ciocalteu's Index (FC)
RE037	76 ± 2	40 ± 2	40 ± 2	9.67 ± 0.11	2.580 ± 0.420	7.55 ± 6.37
RE038	76 ± 3	40 ± 4	41 ± 3	9.56 ± 0.14	4.462 ± 1.220	11.37 ± 2.02
RE039	74 ± 1	37 ± 2	42 ± 4	9.63 ± 0.18	4.235 ± 0.075	13.78 ± 0.88
RE040	62 ± 2	31 ± 3	35 ± 3	8.67 ± 0.22	4.922 ± 0.473	12.32 ± 3.07
RE041	85 ± 3	45 ± 3	48 ± 3	8.36 ± 0.08	2.383 ± 0.626	6.15 ± 4.90
RE042	76 ± 2	36 ± 2	41 ± 2	9.67 ± 0.07	3.532 ± 0.647	12.53 ± 1.46
RE043	76 ± 3	37 ± 2	42 ± 1	7.89 ± 0.11	2.517 ± 0.756	7.52 ± 5.95
RE044	112 ± 6	69 ± 4	64 ± 2	6.89 ± 0.14	4.397 ± 0.305	19.90 ± 0.97
RE045	81 ± 3	43 ± 1	43 ± 1	9.89 ± 0.18	4.912 ± 0.903	17.5 2 ± 11.23
RE046	94 ± 4	54 ± 1	54 ± 2	7.45 ± 0.22	4.435 ± 0.371	12.40 ± 2.35
RE048	112 ± 4	69 ± 2	64 ± 3	8.92 ± 0.07	5.008 ± 0.375	23.67 ± 0.13
RE049	102 ± 2	56 ± 1	57 ± 1	7.12 ± 0.11	5.845 ± 0.153	25.18 ± 0.65
RE050	93 ± 3	52 ± 3	53 ± 2	8.76 ± 0.14	4.653 ± 0.888	12.72 ± 2.15
RE051	64 ± 5	36 ± 3	41 ± 3	9.82 ± 0.18	4.372 ± 0.518	12.97 ± 0.95
RE052	86 ± 5	45 ± 2	49 ± 4	9.75 ± 0.22	3.150 ± 1.197	9.92 ± 3.03
RE053	93 ± 4	47 ± 3	47 ± 1	7.56 ± 0.08	5.072 ± 0.719	12.20 ± 2.68
RE054	97 ± 4	50 ± 1	53 ± 1	7.50 ± 0.07	2.010 ± 0.752	9.45 ± 2.84
RE055	106 ± 3	62 ± 2	59 ± 3	8.72 ± 0.11	5.284 ± 0.367	22.90 ± 0.80
RE056	75 ± 4	37 ± 1	39 ± 2	7.69 ± 0.14	1.968 ± 0.450	14.58 ± 4.82
RE057	102 ± 3	56 ± 2	59 ± 2	7.98 ± 0.18	2.873 ± 0.475	19.82 ± 0.60
RE058	67 ± 1	31 ± 1	34 ± 2	8.23 ± 0.22	5.375 ± 0.679	19.98 ± 5.57
RE059	73 ± 2	37 ± 3	43 ± 2	7.54 ± 0.08	1.900 ± 0.566	9.05 ± 4.93
RE060	96 ± 2	53 ± 1	52 ± 2	8.88 ± 0.07	3.485 ± 0.805	13.03 ± 2.99
RE061	110 ± 2	68 ± 3	63 ± 2	6.56 ± 0.11	3.343 ± 0.296	18.82 ± 0.60
RE062	82 ± 2	43 ± 2	46 ± 1	9.55 ± 0.14	2.197 ± 0.955	10.87 ± 2.62
RE063	73 ± 2	37 ± 3	41 ± 1	8.21 ± 0.18	6.210 ± 0.874	22.83 ± 7.61
RE064	107 ± 2	64 ± 1	60 ± 2	8.84 ± 0.22	4.056 ± 0.318	19.17 ± 0.23
RE065	64 ± 1	32 ± 2	36 ± 2	7.71 ± 0.08	4.640 ± 1.043	14.30 ± 3.36
RE066	100 ± 3	56 ± 2	54 ± 1	7.34 ± 0.07	5.620 ± 0.231	20.48 ± 0.21
RE068	107 ± 2	65 ± 1	57 ± 2	7.65 ± 0.14	3.220 ± 0.304	14.37 ± 0.25

Table 2. *Cont.*

Strain	Yeast Biomass Colour on Grape-Skin Agar			Fermentation Vigour after 3 Days (g CO_2/100 mL)	Wine Colour Intensity	Phenolics Content
	Red Component	Green Component	Blue Component		Absorbance (420 + 520 + 620) nm	Folin–Ciocalteu's Index (FC)
RE069	127 ± 2	87 ± 1	78 ± 2	7.87 ± 0.18	3.473 ± 0.476	16.15 ± 0.52
RE070	107 ± 1	65 ± 1	59 ± 1	7.45 ± 0.22	2.943 ± 0.927	16.42 ± 0.40
RE071	111 ± 3	68 ± 3	66 ± 2	7.76 ± 0.08	5.093 ± 0.213	25.48 ± 1.52
RE072	96 ± 3	53 ± 2	51 ± 2	7.68 ± 0.07	5.803 ± 0.363	12.87 ± 3.91
RE073	68 ± 2	34 ± 2	36 ± 2	7.38 ± 0.11	1.670 ± 0.527	7.70 ± 4.45
RE074	89 ± 2	48 ± 2	48 ± 1	7.98 ± 0.14	1.452 ± 0.414	7.57 ± 1.76
RE075	87 ± 3	45 ± 2	46 ± 2	7.89 ± 0.18	6.125 ± 0.423	10.55 ± 8.71
RE076	129 ± 5	88 ± 1	82 ± 1	8.93 ± 0.22	4.543 ± 0.508	22.77 ± 1.27
RE077	112 ± 3	71 ± 2	67 ± 1	9.78 ± 0.08	2.628 ± 0.340	15.80 ± 0.18
RE079	51 ± 2	28 ± 1	31 ± 3	6.98 ± 0.11	3.823 ± 0.078	13.32 ± 1.70
RE080	82 ± 2	41 ± 1	43 ± 1	7.78 ± 0.14	1.715 ± 0.373	8.48 ± 1.04
RE081	68 ± 4	33 ± 2	36 ± 3	7.12 ± 0.18	6.253 ± 0.437	19.13 ± 1.01
RE083	87 ± 4	47 ± 4	49 ± 3	7.54 ± 0.08	2.453 ± 1.164	8.08 ± 1.81
RE084	68 ± 3	33 ± 2	39 ± 1	7.66 ± 0.07	2.108 ± 0.798	7.47 ± 3.18
RE085	69 ± 3	35 ± 1	39 ± 2	8.12 ± 0.11	2.013 ± 0.894	7.00 ± 3.43
RE086	65 ± 2	35 ± 1	39 ± 2	9.31 ± 0.14	2.038 ± 0.736	7.33 ± 2.38
RE087	64 ± 5	29 ± 1	35 ± 4	7.23 ± 0.18	2.365 ± 0.573	9.17 ± 1.37
RE088	96 ± 3	54 ± 2	54 ± 2	6.99 ± 0.22	2.198 ± 0.778	8.42 ± 1.52
RE089	85 ± 3	44 ± 4	45 ± 1	7.23 ± 0.08	2.132 ± 0.878	7.53 ± 3.96
RE090	106 ± 3	65 ± 1	62 ± 1	7.54 ± 0.07	5.588 ± 0.316	23.57 ± 1.25
RE092	80 ± 3	40 ± 2	43 ± 2	7.98 ± 0.14	2.295 ± 0.898	10,42 ± 2.93
RE093	82 ± 4	42 ± 3	48 ± 3	7.54 ± 0.18	5.325 ± 0.158	15.07 ± 3.66
RE097	59 ± 3	27 ± 2	33 ± 2	6.87 ± 0.18	4.222 ± 0.762	10.50 ± 3.50
RE098	82 ± 1	41 ± 1	42 ± 1	7.78 ± 0.22	5.570 ± 0.413	17.17 ± 3.27

Data highlighted in grey have caused the exclusion of the strains as they indicate a higher adsorption activity of pigments and phenolics. Bold font indicates the strain selected for further tests. The data are expressed as mean value ± standard deviation.

3.2. Assessment of the Fermentation Vigour and Adsorption Activity

The screening of the pigment's adsorption activity, assessed as biomass colour on grape-skin agar, underlined a different aptitude of the preselected strains (Table 2). According to Caridi [20], in a colour image, the intensity values ranged from zero (black) to 255 (white) for each of the red, green, and blue components, therefore, high grape pigment adsorption matched low values of these components.

In our screening, the lowest value of the red component was observed for the strain RE079 (51 ± 2) while the strain RE076 showed the highest value (129 ± 5) with a mean value of 83.

The green component of the strain biomass colour ranged from a minimum value of 27 ± 2 (strain RE097) to a maximum value of 88 ± 1 (strain RE076) with a mean value of 45. Regarding the blue component, data ranged from a minimum value of 31 ± 3 (strain RE079) to a maximum value of 82 ± 1 (strain RE076) with a mean value of 46.

The strains which exhibited values less or equal to the mean value (arbitrarily chosen as threshold) of a colour component were considered unsuitable for the final selection due to their greater adsorption activity.

Regarding the micro-winemaking trials, the fermentation vigour, detected for all the strains after 3 days, was generally considered satisfying and no exclusions were made for this parameter in the screening. At the end of the fermentation process, the wine colour and the phenolics content were also assessed. In particular, the colour intensity, obtained by adding the wine absorbance read at 420, 520, and 620 nm, ranged from a minimum value of 1.452 ± 0.414 (strain RE074) to a maximum value of 6.417 ± 0.336 (strain RE017) with a mean value of 3.798. Regarding the adsorption activity of phenolics, assessed on the basis of the FC index, it ranged from a minimum value of 6.15 ± 4.90 (strain RE041) to a maximum value of 25.48 ± 1.52 (strain RE071) with a mean value of 13.35.

Similar to what is described above, the mean values were chosen as threshold, therefore, the strains which exhibited values less or equal to the mean values were excluded due to an adsorption activity that was considered too high. In total, 75 strains were discarded at the end of the screening for one or more data highlighted in grey in Table 2.

3.3. Assessment of the OTA Adsorption Activity and Final Strain Selection

The strains, which passed the previous screening, were tested for the OTA adsorption activity (Figure 2). The percentage of OTA removed from the preselected strains ranged from a minimum value of 34.52 ± 1.80 (strain RE064) to a maximum value of 48.96 ± 4.93 (strain RE049). The ANOVA analysis of the OTA data revealed no significant difference among the strains with the only exception being that between RE049 and RE064 ($p < 0.05$). Interestingly, significant differences were observed among the fermentative vigour data of the 10 strains (Figure 3). In particular, the strains RE048 (8.92 ± 0.07) and RE076 (8.93 ± 0.22) showed the highest values. The latter is definitively the best strain as it combines all the desired traits, although the other nine strains can also be considered good candidates for winemaking.

The values (%) of OTA adsorption activity, showed by the selected strains, are summarised in Table 3. The strains have been safe deposited in UMCC and recorded in the database with a UMCC code.

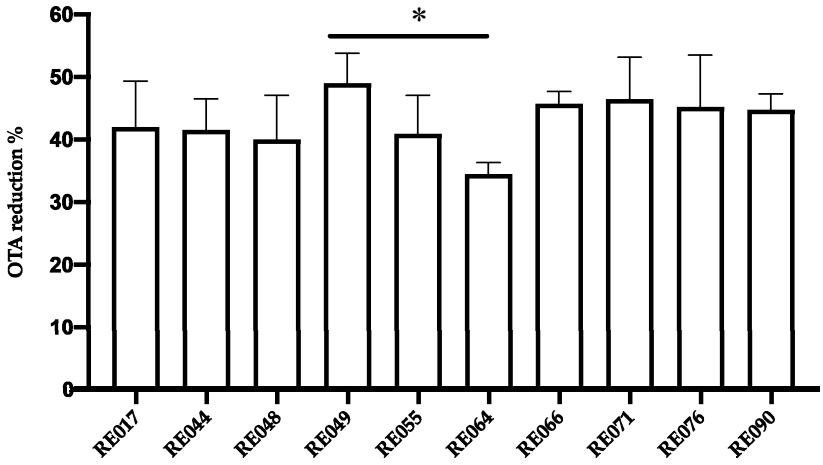


Figure 2. Ochratoxin A (OTA) reduction (%) obtained with the *S. cerevisiae* strains which passed the previous trials. The asterisk indicates significant differences at 95% of confidence level ($p < 0.05$) between strains RE049 and RE064. Bars indicate ± standard deviation.

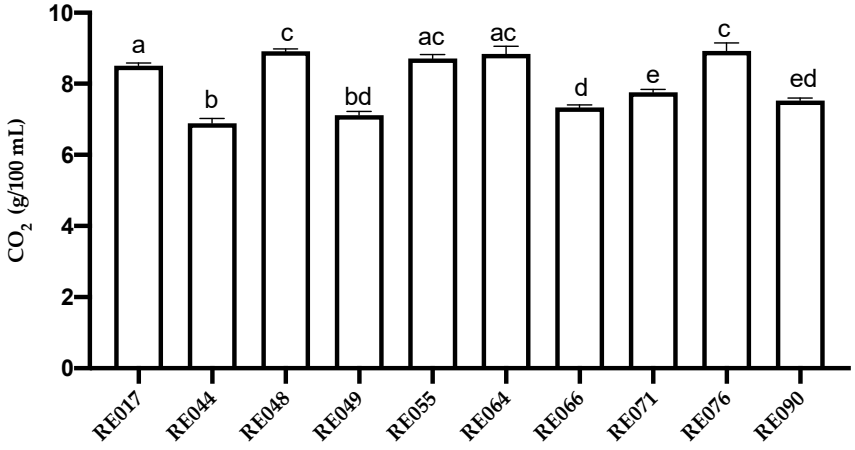


Figure 3. Fermentative vigour of the *S. cerevisiae* strains for which OTA reduction activity was detected. Different superscript letters within the column indicate significant differences in data set according to one-way ANOVA and Tukey's HSD post hoc test tests at $p < 0.05$. Bars indicate ± standard deviation.

Table 3. *S. cerevisiae* strains deposited in Unimore Microbial Culture (UMCC) and their OTA absorption activity.

UMCC Code	Original Code	OTA Absorption Activity (%)
UMCC 2954	RE017	42.03 ± 7.33
UMCC 2955	RE044	41.54 ± 4.98
UMCC 2956	RE048	40.01 ± 7.08
UMCC 2957	RE049	48.96 ± 4.83
UMCC 2958	RE055	40.90 ± 6.18
UMCC 2959	RE064	34.52 ± 1.80
UMCC 2960	RE066	45.72 ± 1.95
UMCC 2961	RE071	46.42 ± 6.75
UMCC 2962	RE076	45.23 ± 8.28
UMCC 2963	RE090	44.75 ± 2.55

4. Discussion

Nowadays, winemakers demand starter cultures with a whole range of specific properties that largely differ according to the type and style of wine to be made, as well as to the technical requirements

of the winery [18]. A new frontier goal for yeasts selection intended as a starter for wines is represented by the ability to remove OTA.

During the past years, significant correlations between yeast strains used for winemaking and phenolic content of wines have been reported, proving that yeast behaviour can somewhat modify chromatic properties, phenolic profile, antioxidant power [27,28] as well as the aroma profile of wine [29,30]. In fact, greater or lesser phenolic adsorption on a yeast cell wall influences the concentration and the composition of phenolics in wine [31–33]. Moreover, the parietal adsorption of pigments during alcoholic fermentation has important consequences because of the loss of wine colour [34,35].

Several authors [2,18,36,37] have proposed a specific selection of wine yeasts that are able to get a selective removal of OTA. However, strains which remove more OTA, also adsorb more colour and phenolics from wine. For this reason, in our selection, only the strains with pigments and phenolics adsorption activity below the mean observed values were chosen for the OTA screening. This was carried out after 28 days of fermentation, considering that OTA is mainly adsorbed during the yeast exponential growth phase and, in some cases, again released in wine, probably due to the premature autolysis of yeast cells [16].

The percentages of OTA adsorption assessed are considered good in accordance with the data reported by Petruzzi et al. [17] and Aponte and Blaiotta [16].

In the present work, the extensive yeasts selection, starting with the collection of oenological samples from three Mediterranean areas particularly interested in OTA contamination [10,11], allowed us to isolate a high number of strains that were potentially able to possess the ability to remove the toxin. Certainly, good wine yeasts must always feature both good primary traits, which are related to the overall fermentative fitness of the strain, and secondary traits, which provide accessory features of technological value [38,39]. Nevertheless, even if the naturally isolated strains do not meet all the desired traits for winemaking, they generate a biodiversity background, which is very useful for successive improvements [40]. In particular, the genetic improvement of yeast strains can be achieved in several ways by exploiting the suitable method according to the complexity of the targeted character and the knowledge of molecular and regulatory interactions, which lie behind a specific desired trait [41,42].

In the context of our work, the inheritable nature of the adsorption of wine colour and OTA was recently analysed on descendants derived from wine strains of *S. cerevisiae* [33,43,44]. Investigation on the progeny demonstrated that adsorption of wine colour and OTA are polygenic inheritable quantitative traits loci, partially and interdependently correlated to colour and phenolic content of wines. This may justify the further improvement of the 10 strains obtained from our selection. In fact, as all the selected strains were able to sporulate, a yeast breeding approach to obtain new strains with traits of interest could be performed through sexual recombination and hybridisation strategies.

Moreover, since indigenous strains are believed to be able to maintain the typical sensory properties and to enhance the peculiarities of a wine [45], the influence of the strains on the aroma profile could also be considered in a further study.

5. Conclusions

The sequential screening implemented in this study allowed a selective reduction in a high number of isolates by excluding those definitely unable to be candidates as wine starters.

The strains reported in Table 3 were specifically selected for the desired features and, among them, the strain RE076 stands out for its high fermentative vigour.

To our knowledge, this is the first work in which the selection of wine strains, exhibiting good fermentative performance and OTA removal activity, was achieved, taking into account the low adsorption activity of pigments and phenolics.

The natural variability of yeasts for the studied traits may be exploited to obtain a further enhancement of the definite adsorption activity of wine strains through specific genetic improvement

strategies in order to develop new strains that possess the desired features for winemakers above the high fermentative fitness.

Author Contributions: All authors participated in the design and discussion of the research. Specific contributions were conceptualisation and supervision, A.P. and A.C.; methodology and investigation, A.P., G.B., R.S., A.C.; data curation, writing—review and editing, L.D.V.; visualisation and editing, G.I.; review and editing, M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Italian Ministry of Education, University and Research, PRIN (Projects of Relevant National Interest), title of the project: "Wine strain improvement strategies to enhance red wine safety based on parietal adsorption activity".

Conflicts of Interest: The authors declare no conflict of interest.

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Exploring the Microbial Community of Traditional Sourdoughs to Select Yeasts and Lactic Acid Bacteria†

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† Presented at the 1st International Electronic Conference on Microbiology, 2–30 November 2020; Available online: https://ecm2020.sciforum.net/

Published: 2 November 2020

Abstract: Sourdoughs represent an awesome example of ecosystem in which yeasts and lactic acid bacteria (LAB) interact with each other, defining the characteristics of the final product in terms of composition, texture, taste and flavor. Therefore, the identification of dominant yeasts and LAB involved in the fermentation process can lead to the selection of starters with suitable fermentation aptitude and capable of producing desired aromas and/or aromatic precursors. In this work, two sourdoughs samples (A and B) for Panettone production were collected from an artisan bakery. Yeasts and bacteria were isolated at different fermentation steps on selective agar media. A total of 120 isolates were obtained and firstly characterized by conventional microbiology methods. Afterward, genomic DNA was extracted from the cultures, and $(GTG)_5$-MSP-PCR fingerprinting analysis was carried out to reduce the redundance among the isolates. Representative yeasts and LAB strains, having a unique profile, were identified by sequencing the D1/D2 domain of the 26S rRNA and the 16S rRNA genes, respectively. The results highlighted the occurrence of *Kazachstania humilis* and *Fructilactobacillus sanfranciscensis* in both sourdoughs. Among LAB also some other strains belonging to *Lactobacillus* genus were found. Moreover, *Saccharomyces cerevisiae* and *Staphylococcus spp.* strains were detected in sample B. In this study, a pool of yeasts and LAB strains for producing starter cultures with specific technological traits for sourdough productions was obtained.

Keywords: sourdoughs; starter culture; sequencing

1. Introduction

Sourdough is a microbial ecosystem of lactic acid bacteria (LAB) and yeasts in a matrix of mainly cereal flour and water [1]. Although yeasts and LAB mainly occur, other microorganisms may be present, such as acetic acid bacteria and those coming from the raw material and/or from the process [2]. The microbial community of sourdough is continuously kept active due to the back-slopping procedure that characterize the manufacturing process. Since millennia, humankind has exploited microbial diversity associated to different food matrices and used their microbial community as natural starter culture. The research of new starter cultures is of great interest in the scientific community because they can be exploited as driver of innovation in the food and beverage field [3].

In this preliminary work we investigated the microbial community of two sourdoughs samples used for producing traditional Panettone. Yeasts and LAB were isolated and identified with the final aim to select strains capable of producing desired aromas and/or aromatic precursors, in order to exploit them as new starter cultures.

2. Methods

2.1. Collection of samples

Two sourdoughs, used to produce artisanal Panettone, were collected from a traditional bakery of the Emilia Romagna region (Italy). Above them, other samples were collected during two different back-slopping methods started from the sourdoughs, respectively (the former was refreshed three times, the latter only two). In particular, from the two sourdoughs MA and MB, the samples IMPA, IMPB and FINA, FINB were obtained. They were taken respectively from the final dough before the leavening and at the end of leavening before cooking. Except for the sourdough back-slopping procedure, the same ingredients were used, and all the samples were prepared and collected at the same day.

2.2. Yeasts and LAB isolation

To obtain microbial isolates, 10 g of each sample was collected and mixed with 90 ml of physiological solution (9 g/l of NaCl) in a Stomacher bag and homogenized for 2 minutes. From the homogenate samples serial dilutions from 10^{-2} to 10^{-5} were made and appropriated dilutions were plated in duplicate on selective agar media. Yeasts were isolated onto WL nutrient medium (OXOID) and Yeast peptone dextrose agar (YPDA; 10 g/L yeasts extract, 10 g/L peptone, 20 g/L dextrose and 20 g/L agar) containing 0.125 g/l of chloramphenicol. LAB were isolated onto de Man Rogosa Sharpe (MRS, OXOID) and Sourdough Bacteria Agar (SDB; 6 g/L trypticase, 3 g/L yeast extract, 20 g/L maltose, 3 ml of 10% TWEEN 80, 15 g/L fresh yeast extract, 12 g/L agar). These media were supplemented with 0.125 g/l cycloheximide. Totally 120 isolates were chosen and purified by streaking on the correspondent isolation medium. Basic phenotypic tests, such as the observation of the morphology of the colonies for yeasts and gram staining and catalases for bacteria, respectively, were done. After the molecular characterizations yeasts and bacteria strains have been preserved in the Unimore Microbial Culture Collection (UMCC) in accordance with standard procedures [4].

2.3. DNA extraction and preliminary characterization

Genomic DNA from yeast and bacterial cultures was extracted followed the protocols of Hoffman e Winston [5] and Ausubel et al. [6], respectively. PCR-RFLP of the internal transcribed spacer (ITS) region and 5.8S gene of rDNA was performed on yeast by using the oligonucleotide primers ITS1 and ITS4 and *Hae* III (Thermofisher) restriction endonucleases [7]. To typing all the isolates, $(GTG)_5$-MSC-PCR was conducted according to Lattanzi et al. [8].

2.4. Species assignment

To obtain a species assignment, the D1/D2 domain of 26S rDNA of the yeast showing different $(GTG)_5$ clusters was amplified with the primers NL1 and NL4, following the protocol of Kurtzman et al. [9]. PCR products were cleaned before sequencing by MWG Biotech Company (Ebersberg, Germany). Regarding the bacteria, amplification of 16S rRNA gene of isolates with different $(GTG)_5$ profiles was performed using primers 27f and 1490r [8] and the PCR products were sequencing as

describe before. All the sequences obtained were processed with Chromas and Bioedit software and then aligned in GeneBank with the Blast program [10] to determine the closest known relatives.

3. Results and discussion

The microbial density found in in MA and MB samples showed values around 7-7,5 ufc/ml. In the other samples the population was slightly higher with values of about 8 ufc/ml. After a preliminary microbiological screening, 60 yeast isolates and 25 bacterial isolates (Gram +) were selected and furtherly investigated (Table 1,2).

3.1. Yeasts molecular characterization

$(GTG)_5$-MSP-PCR fingerprinting technique allowed to group isolates in 15 different clusters. The results of the typing and D1/D2 domain sequencing are reported in Table 1. Sourdough A shows a strong dominance of *Kazachstania humilis* species, with only one sample identified as a group-species *K. humilis*/*K. parahumilis*. This homogeneous result was already observed in traditional sourdoughs [2,11–13]. In sourdough B, four strains belonged to the *S. cerevisiae* species while the other samples belonged to *K. humilis* species (Table 1). These two species represent typical yeasts interactions found in other traditional sourdoughs [12,14]. Furthermore, as demonstrated by Fujimoto et al. [15], the reduced fermentation of sourdough could favored the dominance of *S. cerevisiae,* as happened in sourdough B.

Table 1. PCR-RFLP, $(GTG)_5$-PCR and sequencing of D1/D2 domain of the 26S rDNA gene.

Sample	Strain	RFLP-PCR ITS-5.8S	(GTG)5--PCR	D1/D2 domain of 26S rRNA gene sequence comparison
		*Hae*III restriction enzyme	Cluster	Species, accession N. (% identity)
MA	LMA2, **LMA3***	400-230	A	*K. humilis* NG_055707(99%)
	LMA1, LMA4, **LMA5**,	400-230	-	*K. humilis* NG_055707(98%)/*K.*
	LMA6, LMA7, LMA8	400-230	-	*pseudohumilis* NG_058281(98%)
IMPA	**LMA9**, LMA10	400-230	B	*K. humilis* NG_055707(99%)
	LIA1, LIA9, LIA10	400-230	C	*K. humilis* NG_055707(99%)
	LIA2, LIA3, LIA4,	400-230	D	
	LIA5, LIA7	400-230	D	*K. humilis* NG_055707(99%)
	LIA6, LIA8	400-230	E	*K. humilis* NG_055707(99%)
FINA	**LFA1**	400-230	F	*K. humilis* NG_055707(100%)
	LFA2, **LFA3**, LFA4,	400-230	G	*K. humilis* NG_055707(97%)
	LFA5	400-230	G	
	LFA6	400-230	H	*K. humilis* NG_055707(99%)
MB	LFA7, LFA8	400-230	A	
	LFA9, LFA10	400-230	C	
	LMB1	400-230	-	*K. humilis* NG_055707(100%)
	LMB2, LMB3, LMB6,	320-230-180-150	I	
	LMB7, LMB10	300-230-180-150	I	
	LMB4, LMB5	400-230	L	*K. humilis* NG_055707(100%)

	LMB8	400-230	M	
	LMB9	320-230-180–150	N	*S. cerevisiae* NG_042623 (100%)
IMPB	LIB1, LIB4, **LIB**7,	320-230-180-150	I	*S. cerevisiae* NG_042623(100%)
	LIB10	320-230-180-150	I	
	LIB2, LIB3, **LIB5**,	400-230	O	*K. humilis* NG_055707(100%)
	LIB6, LIB8, LIB9	400-230	O	
FINB	**LFB1**, LFB2, LFB3,	320-230-180-150	P	*S. cerevisiae* NG_042623(100%)
	LFB4, LFB6	320-230-180 -150	P	
	LFB5, LFB7, LFB8,	400-230	O	
	LFB9	320-230-180-150	O	
	LFB 10	320-230-180 -150	Q	*S. cerevisiae* NG_042623(100%)

*: the sequenced strains are reported in bold

3.2. Bacteria molecular characterization

The bacteria isolates belonged to the LAB group with the exception of four strains, identified as *Staphylococcus* spp. species (Table 2). The 60 strains subjected to (GTG)$_5$-MSP-PCR fingerprinting highlighted 14 different clusters. The results showed the presence of heterofermentative obliged bacteria except for *Lactobacillus plantarum*, a facultative heterofermentative bacterium, normally colonizing sourdough continuously back-slopped and maintained at low temperature [1] (Table 2). *Fructilactobacillus sanfranciscensis* (formerly *Lactobacillus sanfranciscensis*) was found in both sourdoughs with a major presence in the A samples. This species is typical of traditional sourdough environment and is selected precisely by its process condition [16], allowing its presence both in dominant and non-dominant conditions [15,17–19]. The presence of *Furfurilactobacillus rossiae* (formely *Lactobacillus rossiae)* in both sourdough samples is interesting, this species was recently described by Corsetti et al. [20] and subsequently studied for its fermentative properties by Di Cagno et al. [21]. *F. rossiae* can be found both individually and in association, as a dominant species, with other LAB species, as shown in sample IMPA (Table 2). The species *Leuconostoc citreum* and *Leuconostoc mesenteroides*, found in both sourdoughs, are typical of the last stages of Panettone process [19]. Moreover, their presence, along with *L. plantarum*, *F. rossiae* and *Fr. sanfranciscensis* can be explained with the low temperature of the process [1]. The presence of *Lactobacillus plantarum* in sourdough is also widely described both as a sub-dominant and dominant species [19,22]. Less common but explainable [22] is the presence of *Lentilactobacillus parabuchneri (*formerly *Lactobacillus parabuchneri*), commonly found in beer or dairy product. In contrast to sample A, sourdough B shows a contamination of *Staphylococci,* probably of human origin (Table 2) and explainable by the manipulation and the back-slopping of the dough.

Table 2. (GTG)5-PCR cluster and sequencing of the 16S rRNA gene.

Sample	**Strain**	**(GTG)5--PCR**	**16S rRNA gene sequence comparison**
		Cluster	Species, accession N. (% identity)
MA	**BMA2, BMA8, BMA10***	A	*L. sanfranciscensis* X76327 (99%)
	BIA2, BIA3	B	*L. parabuchneri* LC383822 (100%)
	BIA5, BIA8, **BIA10**	C	*L. rossiae* NR_112758 (99%)
IMPA	**BIA6**	D	*L. citreum* NR_041727 (99%)
	BIA7	E	*L. mesenteroides* NR_074957 (100%)

	BIA9	F	
FIN A	**BFA1**	G	*L. plantarum* CP039121 (100%)
	BFA2	H	*L. citreum* NR_041727 (99%)
	BMB5	I	*L. sanfranciscensis* X76327 (99%)
MB	**BMB7**	L	*L. sanfranciscensis* X76327 (99%)
	BMB10	-	*S. epidermidis* LN681574 (99%)
	BIB6	M	*S. epidermidis* LN681574 (100%)
IMPB	**BIB7,** BIB8	N	*L. rossiae* NR_112758 (99%)
	BIB9	-	
	BFB1	M	*S. epidermidis* LN681574 (99%)
FINB	BFB3**, BFB8**	O	*L. citreum* NR_041727 (99%)
	BFB6	P	*L. mesenteroides* NR_074957 (100%)
	BFB7	-	*S. hominis* subsp. *novobiosepticus* MF678884 (100%)

*: the sequenced strains are reported in bold

4. Conclusion

The constant search for new starter cultures with optimal and increasingly precise performance is leading to a rediscovery of the great microbial diversity present in food processes such as sourdoughs. In addition, the new food trends are increasingly asking industries for natural, eco-friendly products with beneficial properties. The molecular and characterization of these two sourdoughs (composed of samples MA, IMPA, FINA and MB, IMPB, FINB) microbiota showed the presence of the main microorganisms normally colonizing this environment and can allow the selection of yeasts and LAB to be used as new starters.

Acknowledgments: We thank Panificio Fantuzzi for providing us the two sourdoughs samples in the different stages of the process.

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Abstract

Selection of Promising Exopolysaccharide-Producing Starter Cultures for Gluten-Free Sourdough †

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Abstract: Celiac disease is an autoimmune enteropathy that affects the small intestine and is caused by the gliadin fraction of wheat gluten and other alcohol-soluble proteins (prolamines) of barley and rye in genetically predisposed subjects. Nowadays, the only recognized cure for this pathology is the use of gluten-free (GF) products. Although the market for GF products has grown in the last few years, their improvement is still a challenge for the food industry. With regard to GF bakery products, some of them can result in an unappetizing taste with a high fat content. These inconveniences can be minimized or solved by using sourdough fermentation. Sourdough is a stable culture of lactic acid bacteria (LAB) and yeast in a mixture of flour and water. In particular, GF sourdough represents a rich source of naturally occurring LAB and wild yeasts, which play important roles in food fermentation. This is mainly due to their potential in improving functional, technological, and probiotics properties, contributing to safe food with a better taste. Moreover, exopolysaccharide (EPS)-producing strains can also contribute to improving the sensory and rheological quality of the different GF products. This study aims to isolate and characterize the microbial pool from a GF sourdough made with rice flour and to evaluate the ability of selected LAB strains to produce exopolysaccharides (EPS). The yeast and LAB were isolated in selective agar media. A total of 220 isolates (110 yeasts and 110 bacteria) were obtained. They were taxonomically identified and characterized using both traditional and molecular approaches. Sequencing analysis of the D1/D2 domain of the 26S rRNA and the 16S rRNA genes, for yeasts and bacteria, respectively, revealed the occurrence of *Saccharomyces cerevisiae* as predominant yeasts and *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* as dominant LAB. Regarding the EPS production, 25 strains were able to produce dextran-like EPS with a maximum yield of 2 g/L. This study allowed the isolation and selection of promising EPS-producing starter cultures for the different types of flour sourdough, in particular, GF sourdough.

Keywords: sourdough; EPS; lactic acid bacteria

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Citation: Iosca, G.; Febo, C.; Haghighi, H.; De Vero, L.; Gullo, M.; Pulvirenti, A. Selection of Promising Exopolysaccharide-Producing Starter Cultures for Gluten-Free Sourdough. *Biol. Life Sci. Forum* **2021**, *6*, 38. https://doi.org/10.3390/Foods2021-10952

Academic Editor: Christopher J. Smith

Published: 13 October 2021

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Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/Foods2021-10952/s1, Poster: Selection of Promising Exopolysaccharide-Producing Starter Cultures for Gluten-Free Sourdough.

Author Contributions: Conceptualization and original draft preparation G.I., L.D.V.; investigation and visualization: G.I., C.F., H.H.; writing-review and editing L.D.V., M.G.; supervision, project administration and funding acquisition A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Selection of Promising Exopolysaccharides-Producing Starter Cultures for Gluten-Free Sourdough

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Foods 2021 — The 2nd International Electronic Conference on Foods — Future Foods and Food Technologies for a Sustainable World — 15–30 OCTOBER 2021 | ONLINE — foods MDPI

Introduction

Celiac disease is an autoimmune enteropathy that affects the small intestine and is caused by the gliadin fraction of wheat gluten and other alcohol-soluble proteins (prolamines) of barley and rye in genetically predisposed subjects. Although the market of gluten-free (GF) products has been increased in the last few years, their improvement is still a challenge for the food industry. Considering the GF bakery products, some of them can result in unappetizing taste with high-fat content. These inconveniences can be minimized or solved by using sourdough fermentation.

Sourdough is a stable culture of lactic acid bacteria (LAB) and yeast in a mixture of flour and water. In particular, GF sourdough represents a rich source of naturally occurring LAB strains and wild yeasts which play important roles in food fermentation. This is mainly due to their potential in improving functional, technological, and probiotics properties contributing to safe and more tasty food. Moreover, exopolysaccharide (EPS)-producing strains can contribute to improving the sensory and rheological quality of the different GF products as well [1].

This study aims to isolate and characterize the microbial pool from a GF sourdough made with rice flour and to evaluate the ability of selected LAB strains to produce EPS for producing an innovative starter culture.

Methodology

GF sourdough samples

↓

Homogenization in Stomacher x 2 min
(90 mL 0.9% saline solution with 10 g of samples)

↓

Serial dilutions

↓ Growth on selective agar media (MRS and YPDA)

Purification and isolation of 110 strains

Phenotypical characterization
- Gram staining
- Catalase test
- Fermentative test
- Morphology observation

Molecular characterization [2]
- Extraction of gDNA
- Species-specific PCR
- $(GTG)_5$-PCR fingerprinting
- Sanger sequencing of 16S rRNA gene
- Identification of 25 LAB

EPS characterization
- Preliminary screening of ropiness/slimy colonies for EPS production
- Extraction of EPS following the protocol by [3]
- Analysis of the high yield producing strains (PP_34, LP_42, and LP_50) by:
  - Fourier-transform infrared spectroscopy (FT-IR)
  - Scanning electron microscopy (SEM)
  - X-ray diffraction (XRD)

Results

Molecular and phenotypical characterization

Among the 110 isolated strains tested for the production of EPS only three LAB with the highest production yield were selected and characterized. The results are shown in table 1.

Table 1. Results of the molecular characterization of the three strains and their EPS production yield.

Code	Species	EPS yield (g/L)	Morphology	Gram staining	Catalase test	Fermentative test
PP_34	*Pediococcus pentosaceus*	1.64	Cocci	Positive	Negative	Positive
LP_42	*Lactiplantibacillus plantarum*	1.14	Rod	Positive	Negative	Positive
LP_50	*Lactiplantibacillus plantarum*	4.88	Rod	Positive	Negative	Positive

XRD

XRD analyses of EPS-like compounds produced by *P. pentosaceus* PP_34 and *L. plantarum* LP_42 are presented in the figures 1a and 1b, respectively. The EPS produced by both strains showed a single large diffraction peak at 21°.

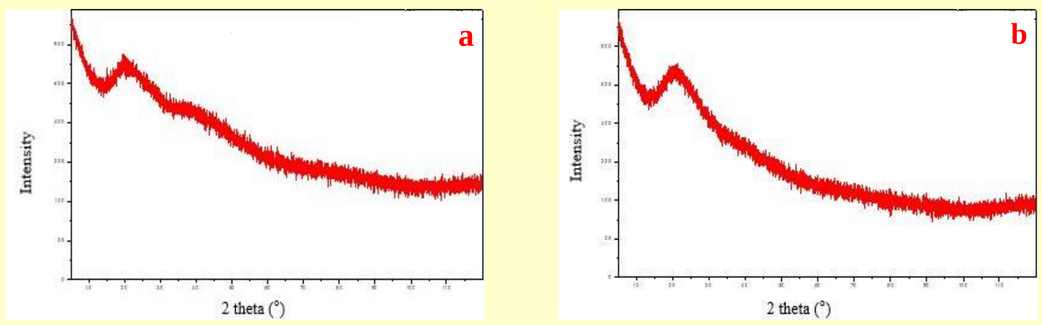


Figure 1. XRD analysis of EPS produced by (a) *P. pentosaceus* PP_34 and (b) *L. plantarum* LP_42.

SEM

SEM showed cracks and irregular microstructures and in particular EPS produced by *L. plantarum* LP_42 showed rough and a more inhomogeneous surface morphology (Figure 2b).

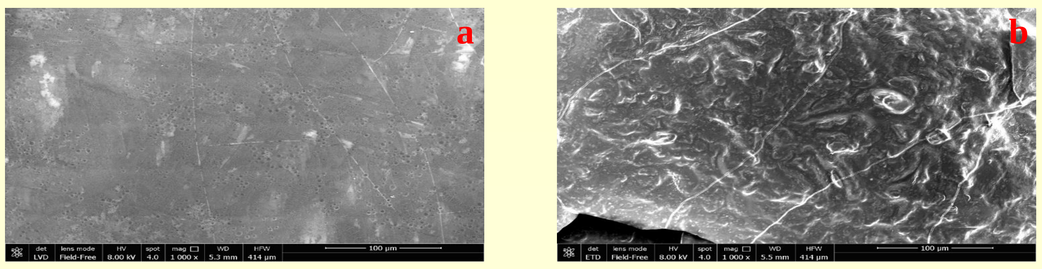


Figure 2. Surface SEM images of EPS produced by (a) *P. pentosaceus* PP_34 and (b) *L. plantarum* LP_42.

FT-IR

The FT-IR spectra of EPS produced by *L. plantarum* LP_50 and *P. pentosaceus* PP_34 showed characteristic peaks at 900-1100 cm^{-1} and 3000-3500 cm^{-1} (Figure 3) [4].

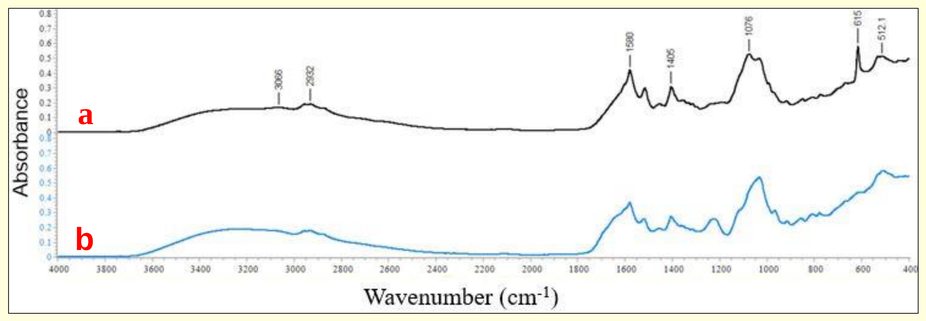


Figure 3. FT-IR spectra of (a) *P. pentosaceus* PP_34 and (b) *L. plantarum* LP_50.

Conclusion

The natural microbial biodiversity of sourdoughs and the ability of these microorganisms for producing EPS or EPS-like compounds can be successfully exploited for the production of both traditional and gluten-free baked products. In this preliminary study, a total of 110 LAB isolated from GF sourdough. Among them, high EPS yield was only obtained by three strains which were identified as *Pediococcus pentosaceus* and *Lactiplantibacillus plantarum*. The preliminary analysis performed on these two strains are in accordance with the previous literature and confirmed the presence of EPS with irregular surface microstructure and the typical EPS peaks obtained by the FTIR and XRD analysis. However, further studies are required in order to improve the extraction protocol and to define the composition of these EPS-like compounds.

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Detection of the microbial community in gluten-free sourdoughs and selection of exopolysaccharides-producing strains



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Introduction

Fermented food and its microbial diversity represent a starting resource for the characterization and selection of new starter cultures, able to satisfy the growing demand of clean label products and sustainability in the food market. In particular, sourdough is a matrix of flour and water including yeasts and lactic acid bacteria (LAB), which can greatly impact the structure and sensory features of final products, included the gluten-free (GF) ones. Celiac disease is an autoimmune enteropathy that affects the small intestine and is caused by the gliadin fraction of wheat gluten and other alcohol-soluble proteins (prolamines) of barley and rye in genetically predisposed subjects. Common problems of GF products include low fibre contents, reduced shelf life, crumbly and dry texture (bread), and low palatability. Exopolysaccharides (EPS)-producing strains can contribute to improve the sensory and rheological features due to EPS properties as hydrocolloids enhancing the textural stability, viscosity and sensory acceptability of different GF products [1]. The EPS produced by LAB are divided into heteropolysaccharides (HePS), synthesised intracellularly, and homopolysaccharides (HoPS) produced using extracellular enzymes [2] (Figure 1). The aim of this study was to exploit the microbial community of GF sourdoughs used for bread production, as well as to investigate LAB strains ability to produce EPS. Selective agar media were used to isolate yeasts and lactic acid bacteria , which were characterized with a polyphasic approach. Non-redundant strains were identified and collected in the Unimore Microbial Culture Collection (UMCC), Italy.

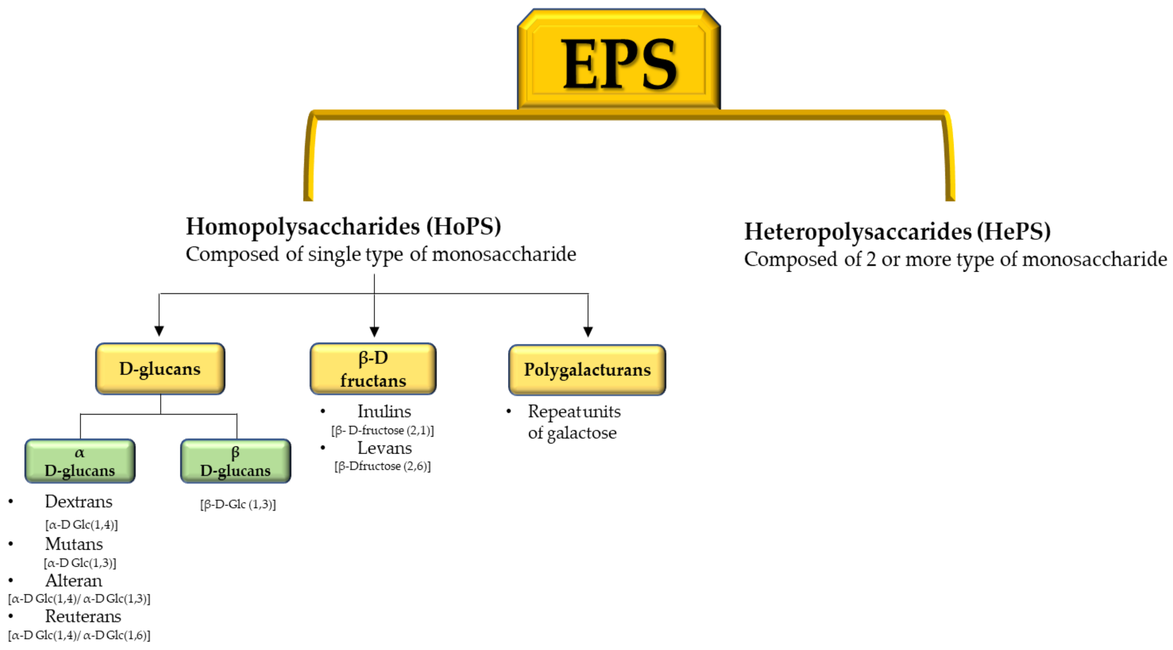
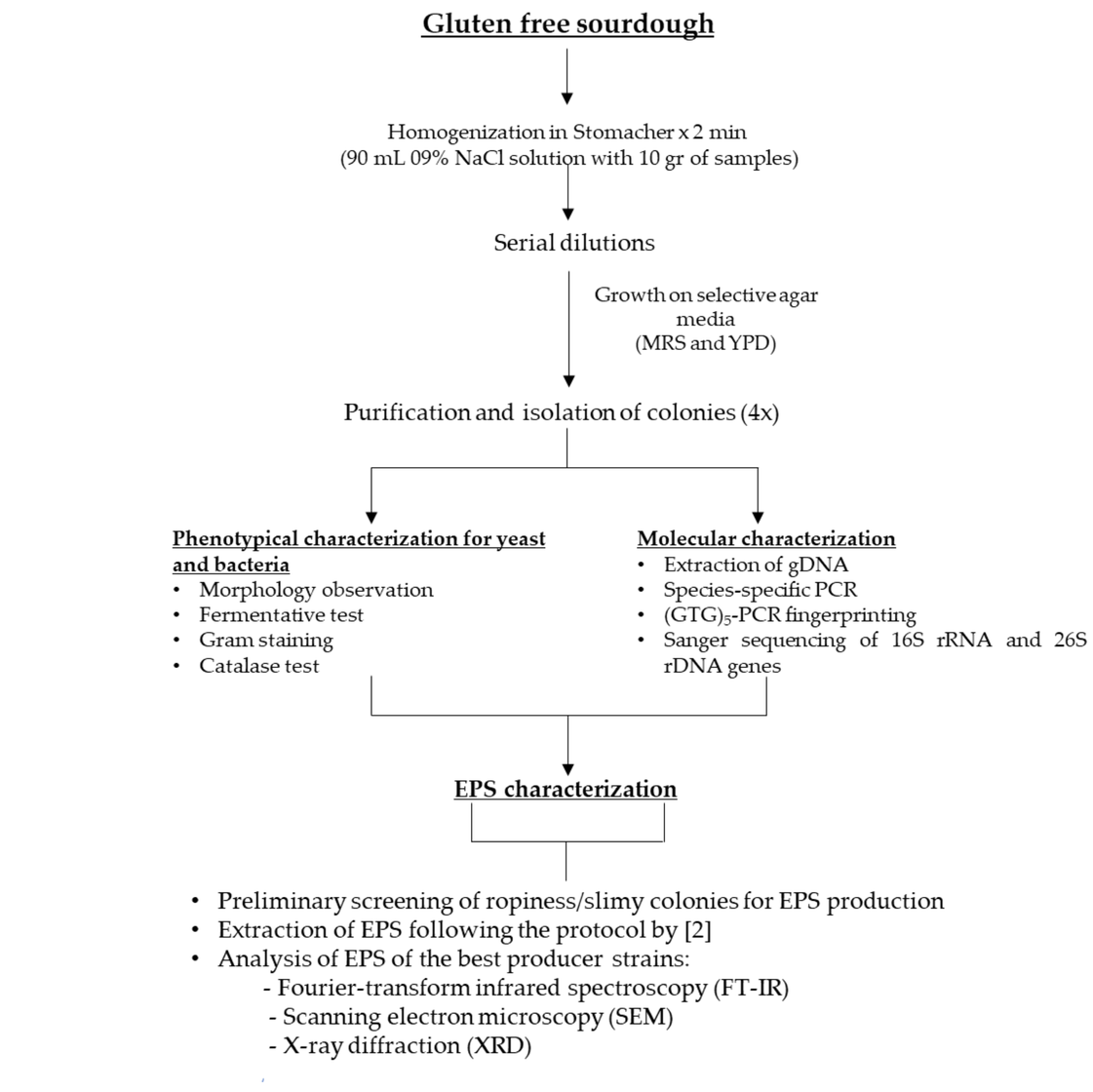


Figure 1. Classification of EPS

Methodology



Results

Among the yeasts detected *Saccharomyces cerevisiae* was the dominant species, while *Pediococcus pentosaceus* and *Lactiplantibacillus plantarum* were the dominant LAB. All the isolated strains was phenotypical, molecular characterized and tested for the production of EPS. Among them only three LAB were selected and characterized due to their highest production yield. The results are shown in table 1.

Table 1. Description of the three strains with the highest EPS production yield

Code	Strain	Morphology	EPS yield (g/L)
PP_34	*Pediococcus pentosaceus*	Cocci	1,64
LP_42	*Lactiplantibacillus plantarum*	Rod	1,14
LP_50	*Lactiplantibacillus plantarum*	Rod	4,88

Scanning electron microscopy

The SEM analysis exhibit irregular surfaces for both EPS showing cracks and irregular microstructures (Figure 2). In particular, the EPS produced by LP_42 reveal a rough and more inhomogeneous surface morphology (Figure 2b).

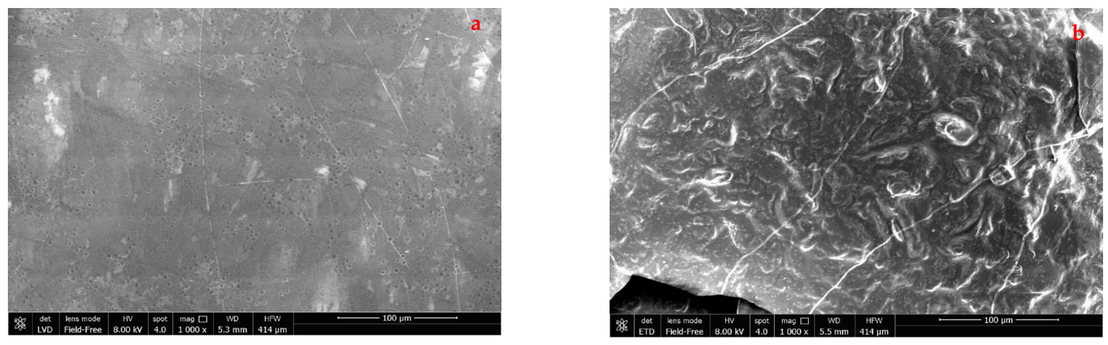


Figure 2. SEM images showing the surface of EPS produced by (a) *P. pentosaceus* PP_34 and (b) *L. plantarum* LP_42.

X-ray diffraction

XRD analyses of EPS-like compounds produced by PP_34 and LP_42 are shown in the figures 1a and 1b, respectively. A single large large diffraction peak at 21° is evident at both strains.

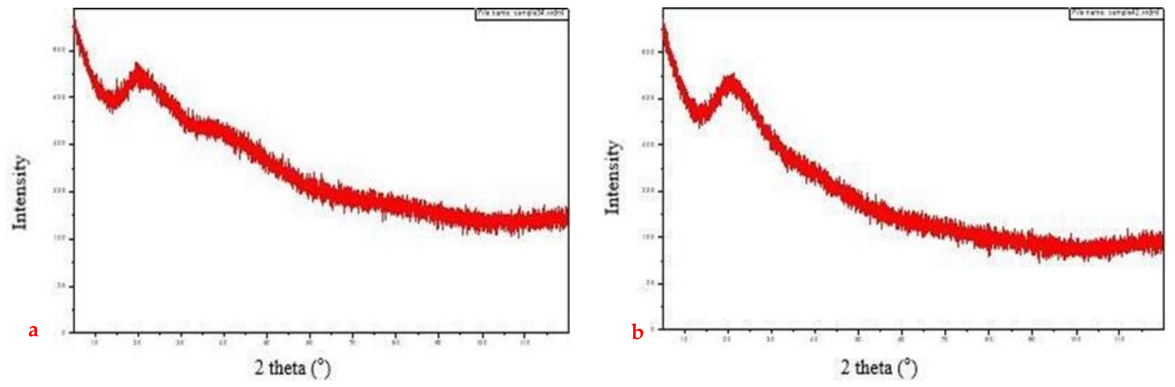


Figure 1. XRD analysis of EPS produced by (a) PP_34 *P. pentosaceus* and (b) LP_42 *L. plantarum*

FT-IR

The FT-IR spectra of EPS produced by *L. plantarum* LP_50 and *P. pentosaceus* PP_34 showed characteristic peaks at 900-1100 cm^{-1} and 3000-3500 cm^{-1} (Figure 3) [4].

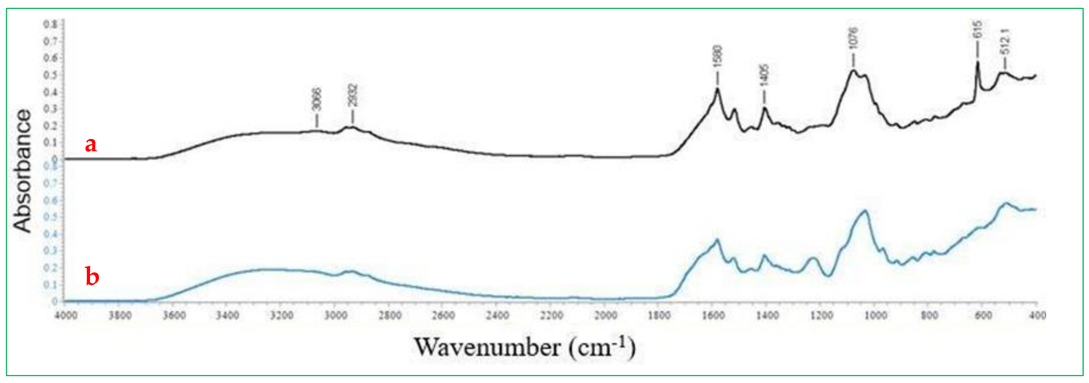


Figure 3. FT-IR spectra of the EPS produced by (a) *P. pentosaceus* PP_34 and (b) *L. plantarum* LP_50.

Conclusion

The natural ability of lactic acid bacteria for producing EPS or EPS-like compounds can be successfully exploited for the production of more tasty gluten-free baked products. In this preliminary study a high EPS production was detected in three strains identified with the species *Pediococcus pentosaceus* and *Lactiplantibacillus plantarum*. The preliminary analysis performed on these strains are in accordance with the previous literature and confirmed the presence of the characteristic EPS peaks. However, further studies are required in order to improve the extraction protocol and to define the composition of these EPS-like compounds.

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