



Technically relevant enzymes and proteins produced by LAB suitable for industrial and biological activity

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Abstract

Lactic acid bacteria (LAB) are a unique subset of microorganisms that have co-evolved with humans since the beginning of agricultural practices and animal domestication and throughout our never-ending quest for food preservation, digestibility, and flavor enhancement. LAB have historically played a preponderant role in our foods. In this review, we focus on the enzymatic activities and current or potential applications of LAB in our lives. A description of each of the enzymatic systems in LAB is included. Glycosidases, which hydrolyze the most abundant food molecules and as sources of carbon, sustain the lives of organisms on Earth as well as ensure microbial innocuity by the production of lactic acid from the uniquely mammalian carbohydrate, lactose. Lipases and proteases or proteinases are of fundamental importance in food fermentations and in dairy foods for flavor development. Bacteriocins and peptidoglycan hydrolases are part of the enzymatic system of LAB that has evolved to make these bacteria fierce competitors in various microbiomes, which are highly important for the human gut. In this review, we also present an explanation on how the versatility of the genetics of LAB can adapt to the matrix where they are placed with the advantage of not having any toxicity to humans. The systematic study of LAB enzymes has allowed for some unique applications in foods and biopharmaceutical industries. Here, we summarize how different enzyme systems in LAB are classified, and thus, facilitate much-needed further studies to understand the fundamentals and translate them into applications to improve our lives.

Keywords Lactic acid bacteria · Proteins · Food applications · Biotechnological tools

Introduction

Lactic acid bacteria (LAB) are Gram-positive, nonspore-forming, nonmotile, acid-tolerant bacteria which exist as cocci, coccobacilli, or rods and have a genomic G+C content of 31–49%. Through glucose metabolism, LAB primarily produce lactic acid; however, some LAB are capable of producing other metabolites, including carbon dioxide, ethanol, and acetic acid (Narvhus and Axelsson 2003). Due in part to their metabolic diversity, LAB have several applications in the chemical, pharmaceutical, cosmetic, and food industries. In the food industry, LAB aid in the production of fermented foods such as cheese, milk, meat, vegetable, silage, sourdough

bread, and wine. LAB are commonly used as starter cultures to accelerate ripening, as dietary supplements, to regulate microbiota and as bioconversion agents (Fernández et al. 2015). Fermentation not only extends food shelf life, but also improves the nutritional value of food and confers health benefits. For example, studies have shown that consumption of milk fermented with LAB decreased the percentage of malignant colon tumors in rats compared to milk fermented with non-LAB species (Rao et al. 1983). Additionally, consumption of milk fermented with LAB demonstrated antiallergic effects through reduction of the serum ovalbumin-specific IgE levels in a murine model (Peng et al. 2007). LAB constitute a diverse bacterial group associated with food fermentation and many of them are generally recognized as safe (GRAS) and/or probiotics. Further, LAB contribute to flavor and texture development as a result of various reactions. This occurs through two mechanisms: (i) direct addition of live bacteria during fermentation to produce secondary metabolites with beneficial properties or (ii) direct addition of the secondary metabolites produced by LAB (Giraffa 2014).

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LAB display numerous enzymatic activities to enhance nutrient bioavailability with potential synergistic effects on digestion, improved nutritional value, and mitigation of intestinal malabsorption (Naidu et al. 1999). LAB can breakdown carbohydrates, proteins, and lipids, which release more readily digestible sugars, produce vitamin B, and enhance the bioavailability of nutrients such as essential amino acids and others like lysine, tryptophan, and methionine for optimal growth and development (Gilliland 1990). The enzymes involved in these metabolic reactions also play crucial roles in food product characteristics. The most relevant enzymes produced by LAB are proteases, lipases, bacteriocins, glucoamylases, peptidoglycan hydrolases, ureases, and phenoloxidases, among others. LAB have been used in the market food and beverage for many years, producing desirable flavors and sensory characteristics and enhancing safety in fermented products, as they produced diverse compounds that inhibit undesirable bacteria including pathogens (Reddy et al. 2008). Several companies such as Novozymes, DSM, and DuPont lead the industrial enzyme market and sell LAB enzymes that have been modified and selected genetically and chemically for improved properties including as substrate specificity, specific activity, stability to pH, temperature, etc. (Adrio and Demain 2014). LAB enzymes can be used for several applications. For example, amylolytic enzymes have a broad range uses: in the detergent industry to remove starch-based stains, in the paper industry to decrease starch viscosity, in the textile industry to warp the shape of textile fibers, and in the food industry for production of crystalline glucose and diverse glucose and fructose syrups (Raveendran et al. 2018). However, for industrial applications, it is necessary to scale-up expression of these enzymes, and approximately 90% of the industrial enzymes used are heterologously expressed by recombinant DNA technologies. This paper aims to review the different biotechnological proteins and enzymes produced by LAB and their potential applications in the food industry, specifically antibacterial proteins (such as bacteriocins and peptidoglycan hydrolases (PGHs), glycoside hydrolases (GHs), proteases, and lipases). A summary of these proteins is depicted graphically in Fig. 1.

The enzymes we have reviewed have been selected as important enzymes in different biological processes with importance in foods and also those that may be significant in human health via microbiome modification.

Antimicrobial proteins

Bacteriocins from LAB

Antimicrobial peptides have emerged as a family of substances with huge potential as a means of microbiological control due to their broad spectrum of activity and low capacity to develop resistance. These peptides are present in

bacteria, fungi, plants, insects, animals, etc. and are expressed constitutively or inducibly depending on the organism and tissue in which they are expressed (Maria-Neto et al. 2015). In addition, they are produced physiologically as a competition strategy (Cotter et al. 2005; van Heel et al. 2011). Among the antimicrobial peptides used to achieve pathogenic bacteria inhibition, those produced by LAB have significance for applications in food, health, and agriculture. Antimicrobial peptides from bacteria are named bacteriocins and differ from antibiotics in a variety of ways: (1) bacteriocins are ribosomally synthesized, while antibiotics are synthesized by enzymes; (2) bacteriocins have activity in nano- and micromolar concentrations, while antibiotics are used in higher concentrations (μM and mM); and (3) bacteriocins can be used in the clinical and food sector, while antibiotics can only be used clinically (Cotter et al. 2013).

Bacteriocins are synthesized by Gram-positive and Gram-negative bacteria. Among those from Gram-positive, those produced by LAB are from the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (van Heel et al. 2011). In Gram-negative bacteria, colicin was the first bacteriocin reported and is synthesized by *Escherichia coli*; however, it comes from a non-GRAS microorganism, and therefore, its use is limited (Baquero and Moreno 1984). Bacteriocins are defined as ribosomally synthesized peptides with low molecular mass (< 10 kDa) and antimicrobial activity. They are cationic, heat-stable, amphiphilic peptides which have varying spectra of activity with respect to specificity and pH and more. Furthermore, bacteriocin-producing LAB have the ability to protect themselves from the bacteriocin it synthesized by its own metabolism (Perez et al. 2014). Bacteriocins are inactivated by proteolytic enzymes from pancreatic (trypsin and alpha-chymotrypsin) and gastric (pepsin) origin, which is a desirable characteristic for use as biopreservatives in food since they are inactivated by the gastrointestinal conditions (Begley et al. 2009). Bacteriocins produced by Gram-positive bacteria, and especially from LAB, have been extensively studied and described in the last two decades (Cotter et al. 2013).

Due to the heterogeneity of bacteriocins, a series of systems have emerged to try to classify them. Klaenhammer (1993) was a pioneer in this area, classifying bacteriocins into classes I, II, III, and IV. More recently, Alvarez-Sieiro et al. (2016) proposed a new classification of bacteriocins identified in silico, which is based on the mechanism of biosynthesis and biological activity, following the clustering structure previously reported by Cotter et al. (2013) and Amison et al. (2013).

Class I, posttranslationally modified bacteriocins Class I bacteriocins or lantibiotics are small peptides (19 to 38 amino acid residues) containing a leader sequence, which is an enzymatic

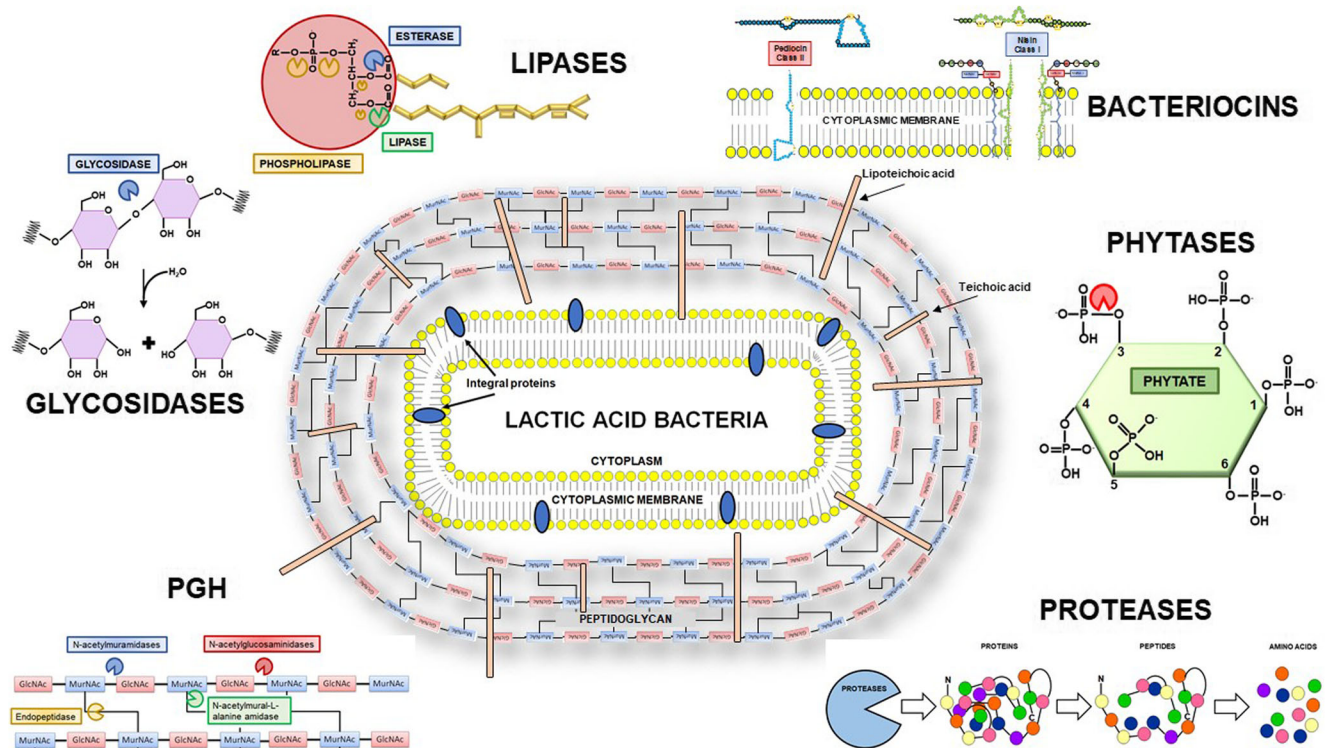


Fig. 1 Graphical depiction of the enzymatic systems in lactic acid bacteria (LAB) covered in this review

recognition signal for transportation and activation of the peptide. This class includes all polycyclic peptides that contain an unusual amino acid such as β -methylanthionine, lanthionine, dehydrated residues, and thioether cross-links. These are formed as a posttranslational modification by dehydration of Ser/Thr residues and subsequent additions of Cys residues to the resulting dehydro-amino acids. These rare amino acids have the ability to form covalent bonds between them, giving unique structural characteristics to class I bacteriocins (Ongley and Neubauer 2016; Rea et al. 2011). Lantibiotics can be further classified by structure and mode of action. Two mechanisms of action have been reported for this class: (a) they can bind to type II lipid, which is a transporter of peptidoglycan subunits from the cytoplasm to the cell wall, preventing the synthesis of the wall and consequent cell death; and (b) they can bind lipid II and insert themselves into the membrane to create pores, which lead to rapid death. Structurally, the C-terminal region is important for specificity of target cells, since this is the region where the formation of disulfide bridges takes place, providing the final conformation (Cotter et al. 2005). Total cysteine content is correlated to antimicrobial activity, since bacteriocins with two or more cysteine residues can form disulfide bridges, increasing the inhibition spectrum. Therefore, those without Cys residues have a reduced activity spectrum (Tominaga and Hatakeyama 2006). An example of class I is nisin, which is produced by *Lactococcus lactis* subsp. *lactis*. Nisin is 3.5 kDa in molecular weight, considered a

GRAS additive, and has been used in the food industry as a preservative in approximately 50 countries with authorization by the FDA (Food and Drug Administration; Barboza-Corona et al. 2007; Campion et al. 2013).

Class II, bacteriocins without posttranslational modifications

Class II, or bacteriocins that do not contain lanthionine residues, are small peptides (less than 10 kDa) that are thermostable, cationic, and hydrophobic. Class II bacteriocins are not posttranslationally modified, except for the removal of the pre-bacteriocin leader peptide. The mechanism of action of these bacteriocins is through membrane permeabilization and pore formation, resulting in loss of the proton-motive force and the depletion of ATP, consequently leading to cell death. These bacteriocins are attracted to bacterial cells by electrostatic interaction, where the N-terminal interacts with membrane phospholipids due their hydrophilic characteristics, while the more hydrophobic C-terminal interacts with the fatty acid component of the cell membrane. It has been suggested that these antimicrobial peptides are active only against a limited number of closely related bacteria (Cotter et al. 2005; Song et al. 2014). The most studied class II bacteriocin is the PA-1 pediocin which has disulfide bridges, strong anti-listerial activity, and a consensus N-terminal sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (Kjos et al. 2011; Tiwari et al. 2015). This pediocin has been used in cheese, where bacteriocin-producing strains can reduce up to 50 CFU/g of

Listeria innocua SA1, compared to the cheese control without the pediocin-producing strain (3.7 million CFU/g) (Reviriego et al. 2007).

Bacteriolysins or peptidoglycan hydrolases

Bacteriolysins or PGHs are thermolabile proteins, greater than 10 kDa. Unlike bacteriocins, the mechanism of action of bacteriolysis targets the bacterial cell wall, through hydrolysis of the glycosidic or peptide bond of peptidoglycan (Briers et al. 2014). These enzymes can be used as antimicrobial compounds in both clinical and food applications and have been reported to control pathogens in biofilms and on surfaces (Fenton et al. 2010; Pastagia et al. 2013; Oliveira et al. 2014; Rodríguez-Rubio et al. 2016). For example, in the clinical setting of two PGHs, PlyG and PlyPH have been used in mice to prevent death after infection by *Bacillus anthracis* and *B. anthracis*-like *Bacillus cereus* cells (Yoong et al. 2006). Others indicate that PGHs have potential therapeutic use against antibiotic-resistant Gram-positive pathogens, as medical material disinfectants to prevent colonization, and for prevention of postsurgical infections (Fischetti 2010; Briers et al. 2014). Additionally, PGHs have potential use in food preservation due to high specificity over pathogenic bacteria. Hen egg white lysozyme (HEWL) is a PGH which has been approved as a biopreservative in the USA and in other countries. The proposed use of this enzyme is to inhibit *Clostridium tyrobutyricum* in ripened cheeses and control growth of undesirable bacteria in wines (Callewaert et al. 2011). Another biopreservative role of PGHs in foods is lysostaphin (Szweda et al. 2007); however, this PGH has not been approved by any regulatory institution because it is from a genus (*Staphylococcus* spp.) that is not recognized as GRAS. The target of lysostaphin is the pentaglycine peptide bond of peptidoglycan and its activity is highly specific against *Staphylococcus aureus* and related species. It is a zinc-dependent, 27-kDa protein produced by *Staphylococcus simulans* var. *staphylolyticus* (Turner et al. 2007).

Physiologically, PGHs are involved in different cellular functions, such as growth, division, and autolysis (Vollmer et al. 2008). PGHs are classified into four types: N-acetylmuramidases (muramidases), N-acetylglucosaminidases (glucosaminidases), N-acetylmural-L-alanine amidase (amidases), and endopeptidases (García-Cano et al. 2011).

Glycoside hydrolases

Glycoside hydrolases (glycosidases or carbohydrases, EC 3.2.1) are a widely distributed group of enzymes, which cleave O-glycosidic bonds in glycosides, glycans, and glycoconjugates (Vuong and Wilson 2010). GHs are found in almost all living organisms. The GHs found in microorganisms depend on their ecological niche. Small changes in the

primary structure of GHs drastically change their substrate specificity (Naumoff 2011). Starch, a target of GHs, is a polymer of α -D-glucose residues linked through the C1 oxygen and is one of the most abundant polymers on Earth. Starch is composed for two heterologous polymers: amylose (15–25%) and amylopectin (75–85%). Amylose is essentially a linear polymer of glucose linked by α -1,4 bonds. Amylopectin is a branched polysaccharide with linear chains of 10–60 glucose residues joined by α -1,4 bonds and α -1,6 linked side chains of 15–45 glucose residues. The number of glucose residues depends on the amylose and amylopectin source (Tester et al. 2004; Nalin et al. 2015). The hydrolysis of the starch requires the coordinated action of different enzymes like α -amylases, glucoamylases, glucose isomerases, and pullulanases, each having a specific role as shown in graphic form in Fig. 2 (Hii et al. 2012).

Amylases

The starch-degrading enzymes include endo- and exoamylases. Endoamylases cleave internal α -1,4 glycosidic bonds present in the inner part of amylose and amylopectin, releasing oligosaccharides and dextrin as α -anomeric products. Exoamylases can cleave α -1,4 and α -1,6 bonds of the external glucose residues like glucoamylase (EC 3.2.1.3) or α -glucosidase (EC 3.2.1.20), releasing glucose as a final product. The best substrates for glucoamylase are long-chain polysaccharides, while α -glucosidase acts best over short malto-oligosaccharides. β -Amylases release maltose and β -limit dextrins converting the anomeric configuration or the released maltose from α to β (van Der Maarel et al. 2002; Sarian et al. 2017). Another group that converts starch corresponds to the debranching enzymes that exclusively hydrolyze α -1,6 bonds and isoamylases (EC 3.2.1.68) and type I pullulanases (EC 3.2.1.41). Pullulanases hydrolyze the α -1,6 bonds in pullulan (polymer of maltotriose, linked by α -1,6 bonds) and amylopectin, while isoamylase can hydrolyze only amylopectin (Kim et al. 2008).

The α -amylases (EC 3.2.1.1) as endoamylases catalyze the hydrolysis of starch, cleaving the α -1,4-glycosidic bonds. These enzymes are metalloenzymes and require Ca^{2+} ions for enzymatic activity and protein stability (De Souza and de Oliveira 2010). In addition, amylases are produced by a wide variety of microorganisms. Fungal and bacterial amylases are used by the industry due to low cost, low time, and space required for production and ease of process modification and optimization. For thermostable α -amylases production, *Bacillus* sp. is widely used for commercial production for different applications. An important alternative source of amylases comes from filamentous fungi, most commonly the genus *Aspergillus* (Sivaramakrishnan et al. 2006). Amylases are one of the most frequently used enzymes by the industry and consist of around 25% of the total enzyme production (Gopinath et al. 2017). Commercial amylases are used to

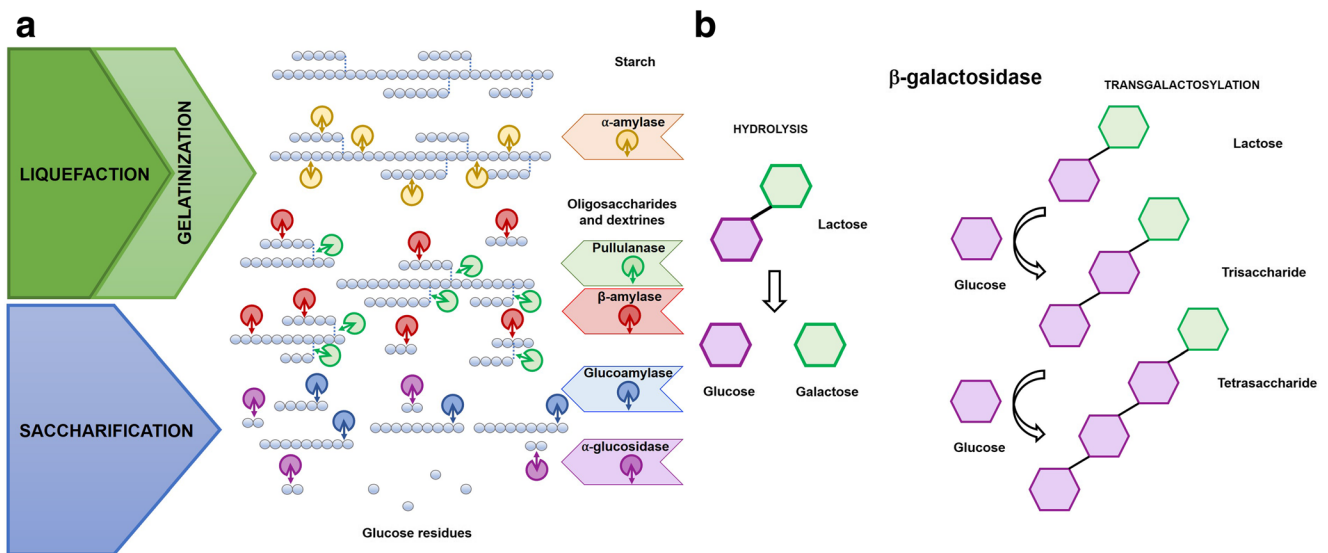


Fig. 2 Schematic representation of glycoside hydrolase activities. **a** General industrial processes of enzymatic hydrolysis of starch. **b** Hydrolysis and transgalactosylation activity of β -galactosidase

convert starch in glucose syrups, cyclodextrins, high-fructose corn syrups, and maltose syrups and to reduce the viscosity of sugar syrups (Chapman et al. 2018). Also, this type of enzyme can be used as a flavor enhancement and anti-staling agent in the baking industry, for reduction of high molecular mass branched dextrans, to obtain crystalline glucose, for reduction of cloudiness in juices, and for solubilization and saccharification of starch (Sivaramakrishnan et al. 2006; De Souza and de Oliveira 2010; Gopinath et al. 2017). Amyolytic LAB have been used in the processing of fermented products using natural starch sources, such as corn or wheat, in the raw material to obtain lactic acid with a single fermentation step, making the process efficient and cheaper. Some strains of *Lactobacillus* spp. produce extracellular amylases which directly hydrolyze starch. For example, *Lactobacillus amylophilus* GV6 was evaluated for its amyolytic activity and produced both amylase and pullulanase (Vishnu et al. 2006).

Glucoamylase

Glucoamylases have exoamylase activity that release β -glucose as the major end product from the nonreducing end of starch. They are used in a number of food and beverage industries to produce bioethanol, crystalline glucose, high glucose, and high fructose syrups, in the production of light beer and in the baking industry to reduce staling and improve bread crust color (Kumar and Satyanarayana 2009). Glucoamylases are enzymes widely distributed in all living organisms. The most used in the industry come from fungi; nevertheless, those with high importance are prokaryotic enzymes, such as LAB enzymes, due to their resistance to high temperatures.

Lactase

Lactase or β -galactosidase (EC 3.2.1.23) is an enzyme that hydrolyzes lactose to release glucose and galactose. Lactase has been isolated from diverse sources such as yeast, bacteria, fungi, plants, or recombinant sources. β -Galactosidase is commonly produced in bacteria classified as GRAS for application in the dairy industry. LAB are a food-grade source of lactase. LAB crude extract contains this activity without the need for enzyme purification, which is an economical and efficient option for the industry. Strains which produce lactase include *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus coryniformis*, *Lactobacillus helveticus*, *Lactobacillus reuteri*, and *Carnobacterium piscicola* BA (Asp et al. 1980; Coombs and Brenchley 1999; Goulas et al. 2007; Gosling et al. 2010; Lamsal 2012). This enzyme is one of the most important for biotechnological processing in the food industry. Its principal use is to produce lactose-free products for lactose-intolerant consumers and to improve the creaminess of ice creams. Lactose is hygroscopic and forms crystals in some products. In frozen desserts, β -galactosidase can be used to prevent undesirable crystallization. β -Galactosidase is also used to cleave lactose to produce sweet syrups that are used in bakery applications or to improve the sweetness of some products. Additionally, this enzyme is used to mitigate the environmental impact of lactose in whey produced as a by-product of the cheese industry, as lactose is associated with high biological and chemical oxygen demand causing severe water pollution. Dairy whey treated with lactase can be used in the production of ethanol (Panesar et al. 2010). In the presence of lactose, β -galactosidase can have dual activity (hydrolysis or

transglycosylation), in which lactose or released monosaccharides (glucose and galactose) can be used as galactosyl acceptors forming a series of disaccharides, trisaccharides, and higher oligosaccharides called galactooligosaccharides (GOS). GOS by-products can be used as prebiotics. Some infant formulas are supplemented with GOS to mimic the benefits of human milk oligosaccharides (Chen and Gänzle 2017). These GOS can promote the growth of good bacteria such as *Lactobacillus* and *Bifidobacterium*, reducing the attachment of pathogenic strains to the intestinal mucosa. Furthermore, GOS act as an energy source and dietary fiber for intestinal cells, which provides multiple benefits for the microbiome in the human gut (Panesar et al. 2013).

β -Glucosidase

β -Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of arylglucosides, alkylglucosides, cellobiose, and celooligosaccharides through the hydrolysis of glycosidic bonds and removal of glucopyranosyl residues from the non-reducing end of β -glucosides. The enzymes from different microbial sources exhibit a high degree of variability in regard to substrate specificity, inducers, and cellular location (Sestelo et al. 2004). β -Glucosidases are widely used in the industry to produce ethanol from cellulosic waste. In the food industry, β -glucosidases are used to release aromatic compounds from glucoside precursors and produce desirable flavor and aroma in fruits and fermented products. The β -glucosidase activities of *Lactobacillus plantarum*, *L. pentosus*, *L. brevis*, and *Pediococcus pentosaceus* are used to eliminate the bitterness of green olives through hydrolysis of oleuropein, a phenolic glucoside, releasing glucose and a phenolic group (Ghabbour et al. 2011). In fermented soy milk, β -glucosidase activity of strains like *Streptococcus thermophilus*, *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. plantarum*, and *L. fermentum* is desirable to obtain genistein and daidzein due to their reported benefits over human health (Michlmayr and Kneifel 2014). Species like *Lactobacillus*, *Leuconostoc*, and *Weissella* are used in the fermentation of cassava; however, the most commonly used strain is *L. plantarum*. The β -glucosidase activity of these strains eliminates linamarin, a toxic cyanogenic glucoside (Nout and Sarkar 1999).

Proteases

Stemming from their diverse biochemical processes, LAB have gained significant attention for their proteolytic activities to enhance many desirable qualities of foods as alternatives to negatively perceived genetically modified organisms (GMOs) (van Hylckama Vlieg and Hugenholtz 2007; Lim et al. 2019). Their diverse functionality in protein hydrolysis is noted by their species- and strain-specific differences in LAB proteolytic systems, consisting of three main components: (i) an

extracellular proteinase which hydrolyzes large proteins into di-, tri-, and oligopeptides; (ii) specific transporters in the cell wall which then transport these peptides into the cell; and (iii) intracellular peptidases which generate individual amino acids (AAs) to meet the metabolic requirements of the bacteria and fulfill their multiple auxotrophies (Calderon et al. 2003; Gobbetti et al. 2005).

Each component of this system offers some degree of versatility contributing to the overall properties of a strain for food production. For example, extracellular protease activity is dependent on specificity for a given protein substrate, extent and rate of reaction, degree of proteolysis, pH, and more and may be secreted depending on the type of organism (Broadbent and Steele 2007; Lim et al. 2019). Secreted bacterial proteases have been shown to degrade casein and release antimicrobial peptides in other Gram-positive bacteria leading to competitive advantages in food matrices, which suggest potential similar implications for LAB proteases for inhibition of food pathogens (Ouertani et al. 2018). Additionally, LAB transporters have specificity in terms of the size, hydrophobicity, and composition of the peptides translocated across the membrane, which may impact the availability of bioactive proteins (Kunji et al. 1996; Gobbetti et al. 2005; Venegas-Ortega et al. 2019). Intracellular peptidases contribute to AA production which can then be enzymatically converted into flavor compounds and is dependent on the type (endopeptidase or exopeptidase) and specificity of peptidases (Smit et al. 2005; Broadbent and Steele 2007; Liu et al. 2010). A summary of the LAB proteolytic system is depicted in Fig. 3.

Furthermore, the regulation of proteolytic activity is dependent on the conditions of a food matrix, such as availability of protein substrate, pH, temperature, and bacterial growth phase (Guédon et al. 2001; Gitton et al. 2005; Venegas-Ortega et al. 2019). In particular, low quantities of peptides and amino acids in a matrix are reported to induce gene expression of proteases involved in the degradation and transport of exogenous peptides and AAs to support their own nutritional requirements and energy production (Hebert et al. 2000; Pescuma et al. 2011; Biscola et al. 2018). It has also been demonstrated using genomic sequencing that all LAB encode many described peptidases, emphasizing the significance of these proteins for bacterial survival (Liu et al. 2010).

Selection of various proteolytic LAB strains and species with desirable proteolytic activities offers vast opportunities in the food industry in the subjects of flavor, nutrient bioavailability, and protein antigenicity. It is well established that LAB protease activity plays a significant role in the organoleptic properties of fermented dairy products and other foods, including flavor, mouthfeel, and taste (Kunji et al. 1996). Specifically, the AAs produced by intracellular peptidase activity are further converted to alcohols, aldehydes, carboxylic acids, esters, and sulfur compounds and are especially potent when derived from specific AAs, including methionine,

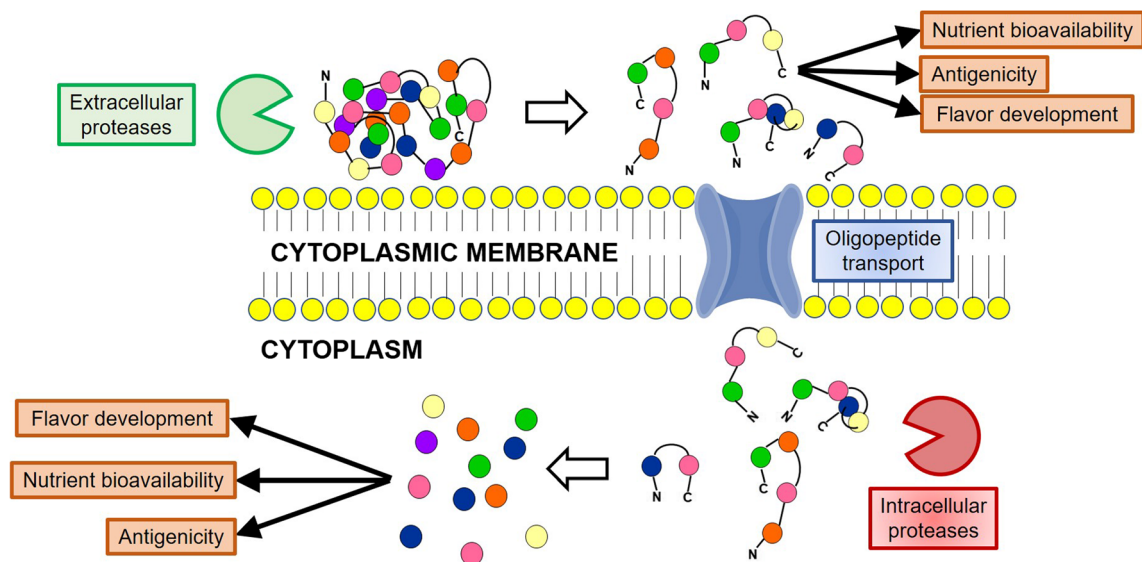


Fig. 3 The LAB proteolytic system consists of extracellular proteinases, peptide transporters, and intracellular peptidases, which lead to production of amino acids and peptides with a diverse range of beneficial effects

phenylalanine, and branched-chain amino acids (BCAAs; Smit et al. 2005). The distinctive nutritional requirements of individual strains contribute to the perceived flavor profile by a consumer. Described more thoroughly by Smit et al. (2005), two strains utilizing different degradative pathways involved in “proto-cooperation” can complete the metabolic incompetencies of one another allowing for more balanced flavor profiles. For example, bitterness flavor defects developed from hydrophobic peptides in cheddar and gouda cheese have been reported using some strains; however, the combination of strains with high PepN activity (an intracellular peptidase), such as *L. lactis*, degrades bitter peptides eliminating off-flavors for flavor balance (van Hylckama Vlieg and Hugenholtz 2007). In dairy products, LAB also degrade casein aiding in texture development of foods (Broadbent and Steele 2007). The most common LAB starters in cheese include *Lactococcus lactis*, *Lactobacillus* sp., *Streptococcus thermophilus*, and *Leuconostoc mesenteroides* (Smit et al. 2005).

Flavor development is also attributed to LAB proteolysis in other foods, including wine, cereal-derived foods, and fermented sausages (Fadda et al. 2010). During vinification, LAB (primarily *Lactobacilli*) generate AAs, such as methionine, that are converted to volatile sulfur compounds and contribute to the flavor complexity of wine, including chocolate and fruity aromas (Pripis-Nicolau et al. 2004; Cappello et al. 2017). Further, LAB can reduce haze formation (a wine defect) resulting from grape protein precipitation during yeast fermentation (Matthews et al. 2004). Cereal-based products, such as sourdough bread, rely significantly on LAB not only for acidification, but proteolysis for volatile flavor development (Thiele et al. 2002; Arendt et al. 2007). Ricciardi et al. (2005) have described the LAB populations in sourdough as 88%

facultatively heterofermentative and 12% heterofermentative (Ricciardi et al. 2005). Other studies have shown that the type of fermentation alters the volatile flavor composition in pizza dough and affects consumer acceptance with preference toward doughs fermented using diverse metabolic strains (Gaglio et al. 2018). Taken together, these findings suggest the incorporation of multiple types of LAB with various fermentative and proteolytic ability may yield more desirable flavor formation in foods.

Another growing biotechnological application of LAB proteolytic activity is through increasing nutrient bioavailability. This can occur through transformation of poorly absorbed products or upcycling of agroindustrial waste products for either human or animal consumption. For example, bone powder is a rich source of collagen protein and calcium that is difficult to absorb in humans due to the complex triple-helix structure of collagen. Fermentation by LAB with proteolytic activity not only improves bioavailability of these nutrients and produces bioactive peptides, but may offer additional antioxidant properties (Han et al. 2018). The fish processing industry also holds promise to utilize LAB proteolytic activity for valorization of its large amount of waste production (Rustad et al. 2011). Furthermore, LAB proteolysis can be used to improve bioavailability of current fishmeal products and lower production costs of fish rearing (Lukic et al. 2019). Similar concepts are also applied in the area of animal feed supplementation which requires a precise balance of AAs for optimal development and production of animals, such as lysine, methionine, or threonine, which are also key AAs produced by LAB proteolysis (Lim et al. 2019). LAB proteases can release these amino acids extracellularly and are therefore suitable for use in feed supplements (Rustad et al. 2011). LAB proteolytic activity also correlates to bioactive peptide release from complex proteins, which act as beneficial mediators in

humans. These bioactive peptides include angiotensin I-converting enzyme (ACE)-inhibitory peptides, antimicrobial, antithrombotic, and immune modulatory peptides, which have important benefits to human health (Venegas-Ortega et al. 2019).

Mitigation of immunoreactive proteins through application of the LAB proteolytic system in foods holds additional promise and opportunities for further research. An application of this idea in dairy foods was investigated by Biscola et al. (2018) who screened and optimized conditions for LAB proteolytic degradation of immunoreactive proteins in milk using proteolysis kinetics. They concluded that LAB proteolysis improved milk digestibility but may simultaneously reduce immunogenic epitopes (Biscola et al. 2018). However, further antigenicity assays would be required to determine whether antigenicity of milk proteins was reduced. With the increasing demand for gluten-free products, LAB have also been applied to degrade gluten in pasta and breads to reduce gluten intolerance of foods (Di Cagno et al. 2005). These works may indicate additional value of LAB proteolysis.

Selection of LAB starter cultures based on their diverse metabolic processes may enhance organoleptic properties, reduce immunoreactivity, and increase nutritional properties for diversification of foods. Further means for prediction of these processes in foods would offer new tools for advancement of the food industry in respect to proteases.

Lipase, esterase, and phospholipase

Enzymes including lipases, esterases, and phospholipases have also been identified in numerous LAB. Lipases (triacylglycerol lipase, EC 3.1.1.3) are a group of enzymes capable of catalyzing hydrolysis of long-chain triacylglycerols to release free fatty acids and glycerol into the lipid–water interface. Lipases have a common α/β hydrolase fold and catalytic triad. Inside of a lipase, one or more α helices linking the main structure of the lipase function as a “lid” to cover the active site of lipase. In the presence of a lipid–water interface, the “lid” reveals the active site and the enzyme becomes active. Substrates can then bind to the binding sites of lipases and catalytic reaction can occur. Generally, the catalytic mechanism of lipases starts with an acylation step, in which a proton is transferred between different residues of the catalytic triad to activate the hydroxyl group of the catalytic serine inside the lipase. The activated serine hydroxyl group then attacks the carbonyl group of substrates to form an acyl-enzyme intermediate. The tetrahedral intermediate is stabilized by another important structural component of lipases called the oxyanion hole. In the diacylation step, water (or alcohol) attacks the intermediate complex via hydrolysis (or alcoholysis) to release the product and regenerate the enzyme (Castillo et al. 2015; Angajala et al. 2016). The equilibrium of reactions is

controlled by water activity of the reaction medium (Borrelli and Trono 2015). Under low water activity, lipases can also catalyze esterification, interesterification, acidolysis, alcoholysis, and aminolysis reactions (Kanasawud et al. 1992; Joseph et al. 2008; Bajaj et al. 2010). Esterases (carboxyl ester hydrolases, EC 3.1.1.1 and EC 3.1.1.2) are enzymes that catalyze the cleavage and formation of ester bonds (Bornscheuer 2002). Esterases also have the typical serine-histidine-aspartate catalytic triad and follow a similar hydrolysis mechanism as described for lipases (Rauwerdink and Kazlauskas 2015). Unlike lipases, the substrates of esterases are only triglycerides composed of short-chain fatty acids and water-soluble substrates in aqueous solutions. Phospholipases are interfacial enzymes which hydrolyze hydrophobic ester linkages of phospholipids (a major component of all biological membranes) rather than triglycerides (De Maria et al. 2007; Borrelli and Trono 2015). The phospholipases A1 (EC 3.1.1.32), A2 (EC 3.1.1.4), C (EC 3.1.4.3), and D (EC 3.1.4.4) hydrolyze phospholipids at different sites to release a variety of products. Phospholipases A1 also have a similar triad as lipase but shorter surface loops which give this enzyme different substrate specificity (Aoki et al. 2007; Borrelli and Trono 2015). Phospholipases A2 use the histidine-aspartate dyad as their catalytic residue and the intermediate of the reaction is stabilized by calcium ions (Schaloske and Dennis 2006). Phospholipases C from eukaryotic and bacterial sources use the common ping-pong reaction mechanism, in which the phosphate group from the substrate covalently binds to the nucleophilic amino acid at the active site of the enzyme to form an intermediate (Fukami et al. 2010; Borrelli and Trono 2015). All phospholipase D enzymes have a conserved sequence motif, H(X)K(X)₄D. The nucleophilic histidine from that motif attacks substrate to form the intermediate. Finally, histidine acquires a proton from a water molecule to regenerate the enzyme (Gottlin et al. 1998; Borrelli and Trono 2015). Among those three types of enzymes, lipases and esterase have been purified and characterized in many LAB, while less is understood about LAB phospholipase production (Esteban-Torres et al. 2014).

Lipases and esterases from LAB play an important role in fermented food production, like cheese and meat production, winemaking, and novel food product development. Both lipases and esterases from LAB contribute to aroma and flavor development and cheese production. Meyers et al. (1996) found that LAB lipases are produced as intracellular enzymes, which exhibited weak lipolytic activity compared with lipases from other microorganisms such as *Pseudomonas*, *Acinetobacter*, and *Candida*. Thus, they suggested that most lipases used for industrial application were derived from other microorganisms for efficient production and greater activity. However, they also stated that lipases from LAB have broader application in food products because many LAB are GRAS (Meyers et al. 1996). In a recent study,

50.3% of 137 LAB isolated from dairy products exhibited lipolytic activities predominately in the extracellular fraction, which suggests the extracellular nature of select LAB lipases. In dairy products, lipases released into the food matrices hydrolyze milk fat and contribute to the flavor development and texture modification of fermented dairy products. However, the type of lipolytic activity contributed by lipases, esterases, or a combination of both was not determined in this study (García-Cano et al. 2019). In another study, the addition of *Lactobacillus plantarum* CCFM12, a strain with high esterase activity, notably enhanced the generation of ethyl esters and corresponding fruity flavor in Camembert cheese (Hong et al. 2018). Di Cagno et al. (2006) also found the addition of adjunct cultures containing LAB isolated from cheese and sourdough significantly increased the concentration of free fatty acids in the production of Caciotta cheese (Di Cagno et al. 2006). Out of 17 strains studied, *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus casei* subsp. *pseudoplantarum* 2742 showed the greatest lipolytic activity. These strains may also supply potential candidates for adjunct cultures in cheesemaking to improve cheese flavor and accelerate ripening. A halotolerant lipase, Lp_3562, from *Lactobacillus plantarum* was purified and may be especially attractive for use in the food industry given that it is stable under 25% NaCl, exhibited greatest activity at 40 °C, good activity at refrigeration temperature, and activity under acidic pH representative of fermented food, as summarized in Fig. 4 (Esteban-Torres et al. 2016).

Lipases also produce distinct flavors in fermented meat products. Studies have shown that some LAB species, such as *Lactobacillus plantarum*, exhibit strong lipolytic activity and contribute to the development of flavor and aroma in

fermented meat products (Leuschner 1998). Dinçer and Kıvanç (2018) found that 25 LAB strains isolated from pastirma, a traditional Turkish fermented meat product, showed lipolytic activity. Among these bacteria, *Lactobacillus plantarum*-150 showed the greatest activity. These LAB may contribute to product quality, improve flavor, and accelerate maturation in cured meat production (Dinçer and Kıvanç 2018).

In winemaking, malolactic fermentation (MLF) is normally carried out by LAB, including *Lactobacillus* spp., *Pediococcus* spp., and *Oenococcus* spp. During MLF, esterases from LAB catalyze the hydrolysis and synthesis of esters (Davis et al. 1985; Carr et al. 2002).

LAB with high esterase activity are also used in the production of novel foods or food supplements. Guglielmetti et al. (2008) screened food-grade bacteria starters with high esterase activity. These bacteria were then used to ferment a food product containing a high amount of chlorogenic acid naturally present in food. In consequence, chlorogenic acid was hydrolyzed by esterase to produce a large amount of caffeic acid and result in an alimentary product exhibiting high antioxidant powder with probiotic potential (Guglielmetti et al. 2008). LAB are also used to produce conjugated fatty acids with the lipase-catalyzed triacylglycerol hydrolysis (Kuhl et al. 2016). For instance, some conjugated linoleic acid (CLA) isomers are used in medicine, dietary supplements, and nutraceuticals for their physiological properties, like anti-carcinogenesis, anti-atherosclerosis, and body fat reduction. LAB can produce CLA from linoleic acid and such a biological reaction is a safe isomer-selective process. *Lactobacillus plantarum* AKU 1009a is a potential CLA producer of multiple isomers from different oil sources (Ogawa et al. 2005).

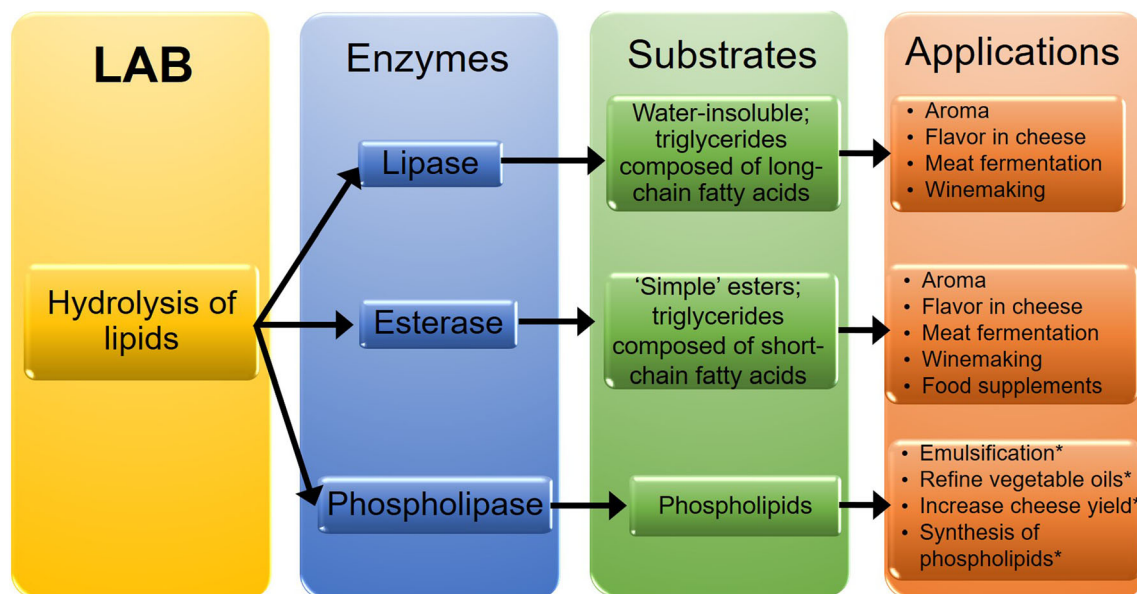


Fig. 4 Lipolytic activities of LAB are useful in a variety of industrial applications. The asterisk (*) indicates the role of phospholipases from other bacteria and yeasts, as the applications of LAB phospholipases are still under investigation

Currently, few, if any, studies have focused on the application of phospholipases from LAB; however, phospholipases from other sources have been employed in the food industry. Egg yolk treated with phospholipase A₂ improves emulsification properties possibly due to interaction enhancement between proteins and phospholipids (Dutilh and Groger 1981; van Kampen and Egmond 2000). Phospholipase A1 is also used in the physical refining of vegetable oils, in which the majority of phospholipids are removed by water. Phospholipases are then used to remove the remaining “nonhydratable” phospholipids (Clausen 2001; De Maria et al. 2007). Some phospholipases have shown good emulsification activity in wheat dough. Phospholipases work together with lipases to replace emulsifiers, like DATEM (diacetyl tartaric acid esters of mono- and diglycerides; it is an anionic oil-in-water emulsifier) and SSL (sodium stearyl lactylate; emulsifier with anti-staling and dough-strengthening properties), by acting on different substrates in the flour to produce optimal gas bubble stability. A known phospholipase under the trade name Lipopan F™ from *Fusarium oxysporum* is being sold by Novozymes A/S for baking applications (De Maria et al. 2007). The phospholipase A1 under the trade name YieldMAX™ from the fungi *Fusarium* sp. is used to hydrolyze milk phospholipids prior to the addition of rennet during cheese production for improvement of cheese yield through higher fat retention (Nielsen 2000). Phospholipases also play a role in synthesizing phosphatidylserine (PS) which is known to improve memory and enhance mood and is used in drug delivery application, although PS is low in availability and is high in extraction cost (Zhou et al. 2017). On the other hand, some authors have used a phospholipase D (PLD) to synthesize PS using L-serine and soybean lecithin as substrates (Choojit et al. 2016; Zhou et al. 2017).

Phytases

Phytases belong to the class of phosphomonoesterases (EC 3.1.3.8) which act to release inorganic phosphorus and myo-inositol phosphate intermediates from phytate or phytic acid (myo-inositol (1,2,3,4,5,6) hexakisphosphate), the major phosphate storage unit in plant-derived food, seeds, and pollen. Phytases can be classified based on the position of first phosphate hydrolyzed, such as 3-phytases (EC 3.1.3.8) that initiate dephosphorylation at the 3 position of phytic acid and 6-phytases (EC 3.1.4.26) at position 6. Phytic acid is a polyanionic chelating agent that forms complexes with several divalent ions of major nutritional importance, such as Ca²⁺, Mg²⁺, Zn²⁺, Cu²⁺, Fe²⁺, and Mn²⁺. Phytic acid is considered an anti-nutritional factor as it chelates minerals; interacts with proteins and amino acids at acid and alkaline pH, affecting their digestion; and inhibits the activity of digestive enzymes (Konietzny and Greiner 2004). Phytases are widely distributed enzymes in plants, microorganisms, and in some animal

tissues that have been used in animal feed to improve the nutritional value and to reduce phosphorus pollution from animal waste. Microbial phytases are most promising for biotechnological application (Jain et al. 2016). Bacterial phytases show distinct biochemical characteristics that differ from fungal phytases. Bacterial phytases are resistant to proteolysis, have an optimal pH range from acidic to alkaline, require Ca²⁺ ions, and have high substrate specificity. In bacteria, phytase production is an inducible process and its expression is subject to complex regulation (Konietzny and Greiner 2004). Phytase production is induced in the presence of agricultural residues which contain phytic acid, such as wheat bran, wheat straw, rice bran, etc. (Haefner et al. 2005). Bacterial phytases are used in the food and feed industry for improving nutritional quality, bread making, and promotion of plant growth (Jain et al. 2016). Monogastric animals are unable to utilize phytic acid due to the lack of adequate phytase levels in the gastrointestinal track. The excreted phytic acid from these animals presents a problem for pollution. Commercial phytases added to feed on farms can replace the inorganic phosphorus used as a feed supplement, contributing significantly to the reduction of environmental pollution.

Phytase supplementation leads to improved availability of mineral and trace elements (Gupta et al. 2015). Phytic acid inhibits iron absorption in cereal- and legume-based complementary foods, causing iron deficiency. Some food processing methods such as cooking, germination, hydrothermal treatment, fermentation, and soaking are shown to reduce considerable amounts of phytic acid in legumes. Phytases can be added to food during processing to reduce the phytic acid content, such as in rice, wheat, oat, maize, and sorghum flours. It is well known that phytases are used in the breadmaking industry to improve the volume, texture, and sensory quality of the bread and reduce the fermentation period (Schlemmer et al. 2009). During this fermentation, the phytate is partially hydrolyzed by the endogenous cereal phytase. However, high-fiber content breads contain high phytic acid level due to slow enzymatic activity of phytase. This enzymatic action depends on many factors such as fermentation time, temperature, pH, starter culture, mineral content, and leavening agent. Bread fermented with some *Bifidobacterium* strains show no sensorial changes in bread with significant reductions in phytic acid levels (Sanz-Penella et al. 2009). The strains *Bifidobacterium breve* and *Bifidobacterium longum* show high hydrolysis of phytic acid in the fermentation of whole wheat dough and minor acidity compared to dough containing *Lactobacillus plantarum* as a commercial starter (Palacios et al. 2008).

The LAB strains *Lactobacillus sanfranciscensis* LS40 and LS41 and *Lactobacillus plantarum* CF1 have been selected for the production of gluten-free bread showing a high phytase activity. These strains have shown to increase the free amino acid content; increase the concentration of some ions, including as Ca²⁺, Mg²⁺, and Zn²⁺; and enhance the nutritional value

of gluten-free bread (Di Cagno et al. 2008). Some LAB strains isolated from Lithuanian sourdoughs have been evaluated for their phytase activity in extra- and intracellular fractions. *Lactobacillus panis*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, and *Pediococcus pentosaceus* showed phytase activity. The greatest activity was displayed by *L. panis* in the extracellular fraction. These LAB strains are promising for use in food fermentation focusing in phytic acid reduction (Nuobariene et al. 2015). Sreeramulu et al. (1996) evaluated 19 LAB strains of the genera *Lactobacillus* and *Streptococcus* for the production of phytases. Most of them showed phytase activity. *Lactobacillus amylovorus* B4552 produced the maximum amount of phytase using glucose and inorganic phosphate in the culture media. LAB are a good source of phytases and have strong advantages for use in food fermentation as most of these bacteria are GRAS, can increase the shelf life of the final product, and provide other qualities mentioned above. Nevertheless, the predominant commercial phytases belong to the genus *Bacillus* due to their higher enzymatic activity, greater thermal resistance, and biochemical characteristics desirable for the industry.

Biotechnological tools to improve enzyme production in LAB

Although there are many sources of enzymes in nature, those that come from microorganisms are the most used in the industry due to their low cost and high production yield. Microorganisms are one of the few sources that meet most, if not all, of the desirable characteristics and industrial demands for enzymes. For example, only specific α -amylase enzymes derived from microbial origin have all of the desirable characteristics for industrial use, such as thermostability, pH tolerance, calcium independency, oxidant stability, high hydrolytic efficiency, and a broad spectrum of activity. In the industry, enzyme thermostability is one of the most desirable traits which is found in extremophile bacteria; however, the isolation and culture of these microorganisms is not an easy feat. Therefore, multiple biotechnological tools to enhance the thermostability of enzymes and other traits from nonextremophile bacteria have been developed. Microbial enzymes may be modified through protein engineering, chemical and genetical modifications, immobilization like adsorption, covalent attachment, entrapment, cross-linking, and encapsulation (Dey et al. 2016).

The successful production of proteins from LAB requires diverse processes including fermentative production, optimization, purification, enzyme characterization, stabilization, immobilization, and more. In many cases, the amount of a protein of interest produced by the LAB is low. Production efficiency is dependent on a variety of factors, including culture conditions, presence of antibiotics, metabolic stress, and more. The use of molecular biological techniques such as

cloning, expression, conjugation, etc. in heterologous systems is an option for production, opening up opportunities for further studies and potential applications in food.

Gene transference to enhance the activities in LAB

Specific gene transfer is one option to improve enzyme production efficiency and enhance activity. At least three main processes have been used in LAB: transformation, transduction, and conjugation. Conjugation is a natural mechanism by which genes can be transferred from one LAB to another, maintaining the food-grade status of the host strain. In contrast to conjugation, transformation is a slower method, which requires free DNA. The use of conjugation has been reported in the production of bacteriocins. The pMRC01 plasmid is the most used in *Lactococcus* genus for conjugation and is of food grade (Fallico et al. 2009). For example, in the strain *Lactococcus lactis* DPC5552, the presence of native lacticin 48 combined with the acquisition of lacticin 3147 through conjugation has a synergistic effect, which increases cell permeability and the spectrum of inhibition without diminishing the acidification capacity of the producing cell (O'Sullivan et al. 2003; Fallico et al. 2009). Over 30 *Lactococcus* strains produce various types of bacteriocins, resulting in a significant increase in the antibacterial spectra (Trotter et al. 2004). Gene transformation in different LAB has been used to promote higher production or greater utilization of bacterial lipases and esterases and to characterize and optimize the growth conditions for these enzymes. Vogel et al. (1990) developed an electroporation-based transformation system for *Lactobacillus curvatus* Lc2-c. The lipase gene from *Staphylococcus hyicus* was transformed and expressed in a staphylococcal vector plasmid, pLipPS1, into *L. curvatus* Lc2, a potential raw sausage starter (Vogel et al. 1990). Transformants exhibited high lipase activity compared to the wild-type strain and contributed to flavor and aroma formation in the prepared raw sausage. Drouault et al. (2000) successfully expressed the *Staphylococcus hyicus* lipase gene into *Lactococcus lactis* at a high level under the control of the inducible promoter PnisA. The *S. hyicus* lip gene encodes a lipase with broad substrate specificity and high phospholipase activity. They also found that treatment with *L. lactis* expressing *S. hyicus* Lip enhanced lipid digestion and corrected steatorrhea in pigs with induced pancreatic insufficiency (Drouault et al. 2000; Drouault et al. 2002).

Genes and protein analysis in silico

Other approaches are used to find new proteins with potential applications in the industry and overcome multiple inconvenient factors, such as uncultured microorganisms, cryptic genes, and low expression using the information contained in genes and genomes. Using the information contained on

genomic databases for the analysis and prediction of genetic and structural information allows for comparison of similarities, clustering patterns, putative functions, and interactions with other proteins in silico. Comparative genomics is a technique that allows for identification of putative genes in silico using databases of complete genomes. Metagenomics is a useful tool to find potential genes from uncultured microorganisms. This technique has opportunities to find new promissory genes in metagenomic libraries including databases, sequences, phylogenetic analysis, and biochemistry characterization (Zhang et al. 2017).

In recent years, many proteins have been studied using new analytical technologies such as “genomic mining.” For example, several tools have been created to search bacteriocins from metagenomic and whole-genome sequencing data (Blin et al. 2013; van Heel et al. 2018). For example, BAGEL4 is an open access software which exclusively uses genomic mining of bacteriocins and allows you to search gene clusters that code for ribosomally synthesized peptides. Over 150 LAB genomes have been deposited and analyzed in this software (van Heel et al. 2018). At least 785 putative bacteriocin clusters have been reported including posttranslationally modified peptides (van Heel et al. 2018). Bactibase is another bacteriocin software that shows predicted physicochemical properties of 230 peptides, allowing for rapid prediction of structure, function, and target organisms (Hammami et al. 2010). Other software has been developed such as RIPPMiner (Agrawal et al. 2017), PRISM (Skinnider et al. 2017), and antiSMASH (Blin et al. 2019); however, their focus is on antibiotics with diverse structures and bioactivities and secondary metabolites, rather than specific clusters of bacteriocins. Another example is the software HyPe (a peptidoglycan hydrolase prediction tool) that was developed for identification and classification of novel peptidoglycan hydrolases from metagenomic and genomic data. Predictions through these tools allow for easier characterization and application. For proteolytic enzymes, MEROPS is a database containing protease, proteinases, and proteolytic enzyme information, which describes the nomenclature, sequence identification, structure, classification, and references of each enzyme. More than 4000 individual proteases are present in the database. Each protease is assigned to a family on the basis of statistically significant similarities in amino acid sequence, and the families that are thought to be homologous are grouped together in a clan (Rawlings et al. 2018).

For example, the genome analysis of *Lactococcus lactis* spp. *lactis* B84 allows to identify four genes for starch hydrolysis putative activities: *amyL*, *amyY*, *glgP*, and *apu* coding for α -amylases, glycogen phosphorylase, and amylopullulanase, respectively. Both genes *amyL* and *amyY* were transcribed to mRNA but not *glgP* and *apu*, what makes them good candidates of study in the process of regulation, cloning, expression, and characterization (Petrov et al. 2008).

Regulation and molecular characterization in LAB

Some studies have focused on protein production and optimization of growth conditions, including culture medium composition, temperature, and pH. Other research has focused on nitrogen source, carbon, and mineral modification to increase production yields (Kanmani et al. 2013). Another strategy commonly used to increase production is genetic modification; however, it is necessary to understand the molecular regulation and expression of each gene for this technique.

Savijoki et al. (2006) described the regulation of the proteolytic system in LAB. Nitrogen concentration regulates the proteolytic activity because di- and tripeptides affect molecules involved in the transcriptional regulation of the oligopeptide transport (Opp) system in *L. lactis*. Nitrogen-limiting conditions in the culture media increase the level of expression of six transcriptional units, including *prtP* (cell involve proteinase), *prtM* (membrane-bound lipoprotein), *opp-pepO1* (oligopeptide permease for endopeptidase PepO1), *pepD* (dipeptidases D), *pepN* (aminopeptidase), *pepC* (aminopeptidase), and *pepX* (X-prolyl dipeptidyl aminopeptidase; Guédon et al. 2001). Through proteomic techniques, it was observed that Opp, PepO1, PepN, PepC, and PepF (endopeptidase) and the substrate-binding protein (OptS) system increase after LAB were grown in free amino acid and peptide culture medium (Gitton et al. 2005). The proteolytic mechanism described for *L. lactis* involves the breakdown casein by PrtP and a PrtM protein that is a chaperone essential for autocatalytic maturation of PrtP (the *prtM* gene is divergent to the gene *prtP*). Through this mechanism, casein is broken down into peptides containing 4–18 amino acid residues, which can be transported into the cell by Opp oligopeptide transport system. Other small peptides, such as di-, tri-, and tetrapeptides, are transported by Dpp and DtpT (specific peptide transporters), which display high affinity for tripeptides and hydrophilic, charged di- and tripeptides, respectively (Hagting et al. 1994; Foucaud et al. 1995; Sanz et al. 2003). Intracellular peptidases, such as PepO (prolidase), PepR (prolinase), PepI (proline iminopeptidase), PepN, PepC, PepF, PepX, PepT (tripeptidase), PepD, and PepV (dipeptidases V), can cleave amino acids from polypeptides and di-, tri-, and tetrapeptides. Finally, CodY, a transcriptional repressor, senses the pool of specific amino acids such as isoleucine, leucine, and valine, which are cofactors, repressing the gene expression of the proteolytic system in *L. lactis* (Savijoki et al. 2006).

Another example of regulation in LAB is the bacteriocin system, which are synthesized ribosomally, involving transcription and translation. The genes which encode bacteriocins are organized in operons located in either chromosomes or plasmids (Drider et al. 2006). Four genes are required for class IIa bacteriocin production: (a) the structural pre-bacteriocin gene, (b) the immunity gene, (c) the ABC

secretion system gene, and (d) the inductor gene (Nes et al. 2002). Bacteriocin biosynthesis begins with the inactive pre-peptide formation containing a leader peptide in the N-terminal and is modified through cleavage during transportation in the plasmatic membrane, obtaining the mature bacteriocin (Chen and Hoover 2003). The enterocin A operon has been reported in *Enterococcus faecium*, which contains the pre-enterocin gene (*entA*), immunity gene (*entI*), inductor gene (*entF*, a peptide pheromone gene), extracellular transport genes (*entT* and *entD*, which are ABC transporter and accessory genes, respectively), and genes for regulation of bacteriocin synthesis (*entK* and *entR*, or histidine protein kinase genes and the response regulator genes, respectively; Rahmeh et al. 2018). In contrast, lantibiotics additionally possess bacteriocin modification genes. The system can be activated by a variety of factors, including nutrient competition with other bacteria, stress due to temperature or pH, and quorum sensing (Blanchard et al. 2016). The biosynthesis of lantibiotics is regulated by a signal transduction system containing two or three components. The regulation mediated by two-component systems plays important roles in sensing and adapting to the environment. This phenomenon has been studied for nisin of *L. lactis*. In these systems, bacteriocins have a dual function since they have antimicrobial activity and act as a signaling molecule to their own synthesis (Kleerebezem 2004). The three-component system that controls the synthesis of enterocin A in *E. faecium* is regulated by a population census mechanism. This includes a histidine kinase protein (HPK) located in the cytoplasmic membrane to sense and translate extracellular signals; a cytoplasmic response regulator (RR) which mediates an adaptive response that is usually a change in gene expression; and an inductor factor (IF), which is detected by HPK protein. The system is initiated as a consequence of slow accumulation and excess of IF during cell growth. HPK detects this increased concentration and initiates a signaling cascade that activates the transcription of genes involved in enterocin A synthesis (Franz et al. 2007). This regulation has also been described for other bacteriocins (class II), such as sakacin P and sakacin A from *Lactobacillus sakei* (Barbosa et al. 2014).

PGHs are another protein group with an extensive regulation system. These enzymes are potentially lethal to bacteria because they degrade the bacterial cell wall. However, PGH expression must be highly regulated to prevent cell lysis of the PGH-producing cell. Regulation and expression of lytic enzymes occurs at multiple levels via transcription, posttranslational regulation, and more (Vollmer et al. 2008). In *Bacillus subtilis*, genetic regulation by two enzymes, LytC (N-acetylmuramyl-L-alanine amidase) and LytD (N-acetylglucosaminidase), is related to autolysis, in which the greatest activity of PGH occurs in the stationary growth phase. The production of these proteins corresponds with the morphological and physiological changes of LAB, such as

flagellum formation and motility, which are co-regulated by sigma D (SigD) factor. The SigD factor affects the expression level of PGHs (Serizawa et al. 2004). One type of posttranslational regulation is proteolytic processing which optimizes activity. In *S. aureus*, the PGH AtlA is a 138-kDa proenzyme (Pro-AtlA) that is cleaved to generate two extracellular enzymes with greater activity: a 51-kDa N-acetylglucosaminidase and a 62-kDa N-acetylmuramyl-L-alanine amidase (Bourgeois et al. 2009). In addition, other regulation mechanisms have been proposed, such as substrate conformational changes and covalent modifications (Bera et al. 2005). For example, the *Streptococcus mutans* Smu0630 (AtlA) protein that participates in cell separation, biofilm formation, and autolysis has a multigene operon whose transcription is regulated by at least three promoters. Specifically, when genes adjacent to the *atlA* gene are deleted, such as the *thmA* gene that is involved in pore formation, the PGH phenotype changes, reducing biofilm formation and autolysis (Ahn and Burne 2006). In general, PGHs may have a signal peptide (depending on cell function), as well as one or two catalytic domains and one or more noncatalytic or substrate-binding domains. Noncatalytic domains conserved in PGHs are commonly referred to as CBDs (cell-wall-binding domains) and at least seven different types are reported: CHAP (cysteine- and histidine-dependent amidase/peptidase), GW domain for noncovalent binding to lipoteichoic acid, SH3 domain, PlyPSA domain, FtsN domain, choline-binding domain, and LysM domain (Vollmer et al. 2008). Among the most important are CHAP domains that are related to histidine- and cysteine-dependent amidase/peptidase activity. Cysteine plays an important role in the mechanism of action of N-acetylmuramyl-L-alanine amidase (Zou and Hou 2010). In *Lactococcus lactis* MG1363, the AcmA bacteriolysin is involved in cell division and produced during the stationary phase. AcmA has two domains: N-acetylmuramyl-L-alanine amidase domain in the N-terminal region and specific peptide-binding domains against *L. lactis* and other Gram-positive bacteria in the C-terminal region (Steen et al. 2005). In *Pediococcus acidilactici* ATCC8042, a bifunctional PGH has been reported. It has two catalytic domains: one with N-acetylglucosaminidase activity and the other with N-acetylmuramyl-L-alanine amidase activity, and the last one contains a substrate-binding site. This property of dual activity gives the protein to have a broad spectrum of inhibition (García-Cano et al. 2015).

On the other hand, the regulation of phytase genes is not completely understood. Phytase genes are widely distributed among bacteria, fungi, cereal crops, and animal tissues. These genes can be classified in three groups: *PAPhy* gene (plant kingdom), *HAPhy* gene (bacteria, fungi, and plants), and *MINPhy* gene (endoplasmic reticulum). Each gene has specific structural and catalytic properties, and for this reason, they can use phytate as a substrate in diverse environments

(Vashishth et al. 2017). The expression of this type of enzyme depends on different factors, such as carbon source, phytic acid, and inorganic and organic phosphate, among others (De Angelis et al. 2003).

Cloning and expression of biotechnological proteins from LAB

The study of a specific gene function and the adjacent genes provides information about the induction or repression of a specific gene in nature. Cloning and expression of those genes under selected specific promoters allows for the discovery of novel activities, characterization and improvement of the yield of production, and stability of desirable genes. For example, error-prone PCR techniques permit variations of a specific protein, random generation of proteins with broad activity, and proteins with greater stability and major activity, which all induce direct evolution. The protein design uses the knowledge in the structure, folding, and function of a protein to make desired changes using site-directed mutagenesis (Liu et al. 2012).

PGH enzyme overproduction is an example application to use cloning and expression systems. The heterologous production of this type of enzyme is complicated because it can be toxic for the host system, via degradation of peptidoglycan. Although the most commonly used species for cloning and expression is *E. coli*, LABs have also been used, such as *Lactococcus lactis* (Song et al. 2017). Several authors have reported the use of numerous cloning and expression systems; however, the most common is the pET expression system (Novagen, USA). In some cases, using these systems, the protein of interest is found intracellularly and it is necessary to lyse the cells by physical or chemical methods (Anzengruber et al. 2014). This is likely due to the enzymes containing an active catalytic domain and a domain that binds to the cell wall (Vollmer et al. 2008). Other authors indicate that in order to prevent the formation of inclusion bodies and host degradation, it is necessary to decrease the expression temperature, which is generally 37 °C (Xu et al. 2015).

Cloning and expression of phytases has been reported in a few bacteria. The phytase gene (*appA*) from *E. coli* was cloned and expressed successfully into *L. lactis*. The use of a probiotic strain with phytase activity can increase phosphorus bioavailability and improve nutrient digestibility and absorption, while maintaining probiotic status and delivering specific proteins and molecules into the human gut (Majidzadeh Heravi et al. 2016).

Despite these challenges, subcloning and expression of bacteriocin genes or gene clusters into strains which lack bacteriocin production or into bacteriocin-producing strains as additional copies of the biosynthesis genes have been used successfully to enhance the antibacterial spectra or provide synergistic effects. This has been reported in *Lactococcus*

lactis DPC3147 which produces lacticin 3147, a broad-spectrum class I bacteriocin. Lacticin 3147 is composed of two peptides which are encoded on two divergent operons. The addition of gene copies, which include biosynthetic production machinery and the regulator gene *ltnR* via subcloning, increased the level of production of lacticin (Cotter et al. 2006). The heterologous production of class II bacteriocins depends on several factors, such as the host strain, expression and secretion system, plasmid stability, number of copies, and the presence of bacteriocin immunity genes. Expression systems have either inducible or constitutive promoters (such as the nisin inducible promoter, P_{nisA}); however, inducible systems have been more successful, due to the control over expression. In general, the strategies used depend on the type of cloned gene, which could be involved in biosynthesis, fusion (to help the transport of bacteriocin), or in the production of secretion signals (Borrero et al. 2011). Other heterologous systems include yeast, which are useful for large-scale production of bacteriocins. Enterocin A, which shows antibacterial activity when introduced in a LAB, has significantly increased production and antibacterial activity when expressed in different yeasts such as *Pichia pastoris*, *Kluyveromyces lactis*, *Hansenula polymorpha*, and *Arxula adeninivorans* (Borrero et al. 2012). Although recombination techniques improve production levels and bacteriocin activity, some governmental agencies classify them as GMOs, which may limit their application in the food industry.

Biochemical characterization of proteins from LAB

The biochemical characterization of any protein provides insight on the most favorable conditions to have enzymatic activity so that factors which negatively affect or inhibit the activity may be avoided. This information helps specify the most suitable areas of application for each specific enzyme and contributes to the understanding of the relationship between structure and function of each particular enzyme.

One example of how biochemical characterization guides the selection of an enzyme's use is an intracellular glucoamylase from *Lactobacillus amylovorus*. This enzyme showed greater affinity for starch and dextrin and low affinity for maltose and maltotriose. Furthermore, it was inhibited by 10 mM of glucose, but not EDTA or other metal chelators. However, Cu²⁺ and Pb²⁺ inhibited glucoamylase activity. These findings, combined with the fact that the enzyme is thermolabile at 55 °C, suggest that this type of enzyme has potential for application in the brewing industry (James and Lee 1996). Another example reported by Coombs and Brenchley (1999) is a β-galactosidase identified, cloned, and expressed from *Carnobacterium piscicola* BA. A cluster of three open frames was identified with one gene related to α-glucosidase and two genes for β-galactosidases. The *bgab* encoded a β-galactosidase enzyme with 76.8 kDa and activity

at 4 °C and required 10% of glycerol to maintain the activity, its optimal temperature for activity was 30 °C, and it lost the activity at 40 °C exposed during 10 min. The K_m was 1.7 mM and the V_{max} was 450 mmol/min mg for *O*-nitrophenyl β -galactopyranoside (Coombs and Brenchley 1999). Additionally, a putative gene to encode a 66.31-kDa oligosaccharide α -1,6-glucosidase (α -amylase) with 572 amino acid residues was identified in *Lactobacillus plantarum* LL441. This enzyme was cloned, expressed, and purified and showed hydrolytic activity over 4-nitrophenyl- α -d-glucopyranoside, with high activity at pH 5 and 6 and between 20 and 42 °C (optimum 30 °C). The enzyme had specificity for isomaltose activity with V_{max} and K_m values of 40.64 μ mol/min mg and 6.22 mM, respectively (Delgado et al. 2017). These findings suggest that those enzymes should be used to hydrolase lactose and degrade polysaccharides, for production of glucose, fructose syrups, maltodextrins, etc.

Furthermore, biochemical characterization of lipases/esterases helps to determine the best conditions during the fermentation process for maximum activity. For example, different esters in wine often act synergistically to influence wine aroma. Sumby et al. (2009) found that esterase activity has a significant influence on the flavor profile of the wine. The esterase from *Oenococcus oeni*, EstB28, was cloned, expressed, and characterized, showing optimal activity at temperature 40 °C, pH 5.0, and 28% ethanol. Moreover, the esterase showed high activity under winemaking conditions (10–20 °C, pH 3.5, and 14% ethanol; Sumby et al. 2009). Matthews et al. (2007) characterized esterase activities of nine LAB strains under wine-like conditions, including the presence of ethanol, acidity, and low temperature. The strains they selected had greater activities toward short-chain esters (C2–C8) compared to long-chain esters (C10–C18; Matthews et al. 2007). Others have shown that concentrations of branched hydroxylated esters increased after MLF under all experimental conditions and were dependent on LAB strains used (Gammacurta et al. 2018). However, the level of most other compounds like ethyl fatty acid esters, higher alcohol acetates, and methyl esters was only slightly impacted by LAB strains. These findings suggest that esterases from different LAB strains may have different substrate preferences, which may be important for esterase application (Matthews et al. 2007; Gammacurta et al. 2018). Pérez-Martín et al. (2013) examined esterase activity of 243 LAB from wines made from different red grape varieties and observed strain-specific differences in the degree of esterase activity, which had different effects on wine aroma. The biochemical characterization of these LAB esterases may offer insight into how they affect the final aroma of wine and how to utilize esterases to manipulate the flavor profile of wine (Pérez-Martín et al. 2013).

Biochemical characterization of bacteriocins is extensive and depends on multiple factors due to the properties of these peptides. It has been reported that bacteriocin production

depends on the physiological activity and biomass of the strain. The composition of the culture medium with the relationship between carbon, nitrogen, and phosphorus; the fermentation conditions; temperature; growth time; initial and final pH; agitation; aeration; and more may affect bacteriocin production (Wu et al. 2004). Bacteriocin production by LAB is usually associated with the growth phase and is stopped at the end of the exponential phase (sometimes before growth ends). This can be attributed to the adsorption of the bacteriocin to the producing cells or to degradation by proteases (Quintero-Salazar et al. 2006).

Many bacteriocins have been characterized according to different parameters such as temperature and pH, because by definition they must resist high temperatures (90–120 °C for 15–20 min). On the other hand, the pH ranges are more varied and the stability of bacteriocins can be between pH 3 and pH 10. Also, bacteriocin characterization includes treatment with different proteases to confirm that activity is from protein origin (Martinez et al. 2013). To determine the molecular weight of bacteriocins, mass spectrometry (MALDI-TOF/MS or ESI/MS) is used in addition with traditional techniques such as two-dimensional polyacrylamide gel electrophoresis (Ghadbane et al. 2013). Additionally, it is possible to use circular dichroism or nuclear magnetic resonance to confirm the structure of the bacteriocin and determine the amino acids involved in folding and activity (Baindara et al. 2016).

The biochemical characterization of PGHs is complex due to the wide variety of domains and structures reported. Unlike bacteriocins, these proteins are subject to regulation by activators or inhibitors. For example, two PGHs from *Enterococcus faecalis* were visualized by SDS-PAGE and reported as different molecular masses (54 and 72 kDa); however, upon identification by LC/ESI-MS/MS, the molecular weight differed from that observed by electrophoresis. By mass spectrometry, the proteins had molecular weights of 54 and 72 kDa, whereas the proteins had molecular weights of 80 and 86 kDa by electrophoresis. The 80-kDa protein showed optimal activity at pH 6 and retained 50% activity after 30 min at 90 °C with NaCl 300 mM. The 86-kDa PGH displayed optimal activity at pH 6 and retained 50% of the activity after a 70 °C treatment for 30 min in NaCl 500 mM (García-Cano et al. 2014). These enzymes can be used as therapeutic agents, for prevention of animal infection or as a food preservative. Another PGH characterized with dual antibacterial activity was cloned, expressed, and purified by molecular exclusion chromatography from *Pediococcus acidilactici* ATCC8042. The results showed one protein in 99 kDa with N-acetylglucoamidase and N-acetylmuramidase activity. The optimal activity was at pH 6 and the pH stability range was from pH 5 to 7. The optimal temperature activity was 60 °C and disappeared after incubating at 70 °C for 1 h. EDTA did not show inhibition over PGH activity; however, EGTA and Zn^{2+} ion decreased activity by 100%. The PGH showed

antibacterial activity against many pathogenic strains and LAB (García-Cano et al. 2015). Another LAB, isolated from meat products, with PGH activity was *L. sakei*. Using LC/MALDI-TOF/TOF, a 77-kDa PGH was identified and had putative activity of N-acetyl-muramoyl-L-alanine amidase. The protein has a catalytic domain and five LytM domains, which are substrate-binding sites. This protein was purified using molecular exclusion chromatography. Pure PGH showed pH stability between pH 7 and 9, with a maximum activity at pH 8. The optimal temperature was 37 °C, which was lowered after incubating at 90 °C for 1 h. The PGH was partially inhibited by EDTA and EGTA and showed inhibitory activity against LAB and pathogenic strains as *Salmonella typhimurium* and *Staphylococcus aureus* (García-Cano and Ponce-Alquicira 2015).

Protease activity is dependent on the substrate specificity, hydrolysis site, etc. In general, aminopeptidases N (PepN) and the proline peptidase (PepX) recently have been of interest for applications in the food industry. In *Lactobacillus helveticus* ATCC 12046, a fusion protein with both activities (PepN-L1-PepX) was developed. After purification, the fusion enzyme showed optimum temperature at 35 °C and was stable for 14 days at 50 °C. Additionally, it showed antioxidative capacity (5.8 µg/ml) and the reducing reagent β-mercaptoethanol did not affect over the activity (Stressler et al. 2016). Biochemical characterization of proteases is also important for selection of strains that influence flavor. Aminopeptidase A (PepA) is an intracellular exopeptidase, which cleaves glutamyl/aspartyl residues from the N-terminal end of peptides. This enzyme was cloned, expressed, and characterized with a 480-kDa molecular mass, optimum pH of activity in pH 6–6.5, and temperature optimum between 60 and 65 °C. This aminopeptidase can be used and applied for the production of protein hydrolysates, increasing amino acid concentration, which results in a savory taste (umami; Stressler et al. 2016). Additionally, phytase proteins have a promising biotechnological application. Some phytases have been reported in LAB (for example in *L. plantarum*, *L. acidophilus*, and *Leuconostoc mesenteroides* subsp. *mesenteroides*); however, only a few phytases from LAB has been characterized. The phytase of *Lactobacillus sanfranciscensis* CB1 was purified and characterized a monomeric protein showing a 50-kDa molecular weight. The intracellular phytase showed optimal activity at pH 4 and 45 °C. It was inhibited by divalent ions (Hg²⁺ and Fe²⁺) and by serine-protease inhibitor (PMSF). The thermostability was for 30 min at 70 °C and the calculated *D* value (decimal reduction time) was 10 min at 80 °C. These properties of phytases may provide some health benefits, through increasing the iron bioavailability and nonmetal chelator compounds due to the hydrolysis of phytate that

releases phosphate, upgrading the nutritional quality of foods and feeds (De Angelis et al. 2003).

Conclusion

Lactic acid bacteria represent a unique source of industrially relevant peptides and enzymes that have coexisted with humans since the domestication of animals and have helped in making our food safer and more digestible. However, we have presented the current issues and the need for new and more specialized applications, which requires a better understanding of the physical properties and genetics of these strains. The new era of bioinformatics and biotechnological tools allows for the detection of potentially important compounds encoded in the genes of all bacteria for application in foods, medicine, and waste reduction at an industrial level. The use of LAB as a source of these peptides and enzymes or as a heterologous host has significant advantages in the food industry because most LAB have GRAS status and also inhibit the growth of undesirable bacteria. Understanding and implementation of these LAB and their enzymes allow for better adaptation under diverse production conditions and generation of novel products, posing a large opportunity for research and industry.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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