Chapter 2 Importance of peptidoglycan hydrolases, bactericidal enzymes produced by lactic acid bacteria, in the reduction of antibiotic

Capítulo 2 Importancia de las hidrolasas de peptidoglucano, enzimas bactericidas producidas por bacterias acido lácticas, en la disminución de la resistencia a antibióticos

LOPEZ-ZAMUDIO, Amairany†*, MENDOZA-GARCÍA, Patricia Guillermina, PEÑA-MONTES, Carolina and FONSECA-BARRERA, Itzel del Carmen

Unidad de Investigación y Desarrollo de Alimentos, Tecnológico Nacional de México/ I. T. Veracruz, Av. Miguel Ángel de Quevedo No. 2779, Col. Formando hogar, 91987 Veracruz, Ver, Mexico

ID 1st Author: *Amairany, Lopez-Zamudio* / **ORC ID:** 0000-0002-7765-3312, **CVU CONACYT ID**: 894981

ID 1st Co-author: *Patricia Guillermina, Mendoza-García* / **ORC ID:** 0000-00001-6838-0861, **CVU CONACYT ID**: 270773

ID 2nd Co-author: *Carolina, Peña-Montes* / **ORC ID:** 0000-0002-4767, **CVU CONACYT ID**: 277236

ID 3rd Co-author: *Itzel Del Carmen, Fonseca-Barrera* /**ORC ID:** 0000-0003-3562-9899, **CVU CONACYT ID**: 950657

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A. Lopez, P. Mendoza, C. Peña and I. Fonseca

patricia.mg@veracruz.tecnm.mx

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Abstract

The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) highlight in their global action, the problem of antimicrobial resistance (AMR), focusing on the concern about the effect every time minor of antibiotics, which is considered a threat in human medicine, veterinary, food sector and environment. In recent years, the interest in generating new technological alternatives for this problem has increased; this is the case of bioactive metabolites obtained from bacteria, viruses and fungi, such as protein molecules with bactericidal activity, as bacteriocins and enzymes and non-protein origin diacetyl and reuterin. Peptidoglycan hydrolases (PGH), also called autolysins, are enzymes involved in various cellular functions. These enzymes can hydrolyze the peptidoglycan bonds in a controlled way, and they are classified as N- acetylmuramidases, N-acetylglucosaminases, N-acetylmuramoyl-L-alanine amidases and peptidases. PGH are secreted by the pathway dependent on the General Secretion Pathway (Sec) or by the Double Arginine Translocation System (TAT) and have molecular weights in a range of 27 kDa to 137 kDa. Their importance liesin being used as bactericidal compounds, inhibiting the growth of bacteria of clinical relevance, which are currently a global public health problem.

Antibiotic resistance, Bactericidal enzymes, Peptidoglycan hydrolases

Resumen

La Organización Mundial de la Salud (OMS) y la Organización de las Naciones Unidas para la Alimentación (FAO) destacan en su plan de acción mundial el problema de la resistencia a los antimicrobianos (RAM), centrándose en la preocupación por el efecto cada vez menor de los antibióticos, lo cual se considera una amenaza en medicina humana, veterinaria, sector alimentario y medio ambiental. En los últimos años se ha incrementado el interés por generar nuevas alternativas tecnológicas para esta problemática; tal ha sido el caso de metabolitos bioactivos obtenidos de bacterias, virus y hongos, como proteínas con actividad bactericida como, bacteriocinas, diacetilo, reuterina y enzimas. Las peptidoglucano hidrolasas (PGH) también denominadas autolisinas, son enzimas involucradas en diversas funciones celulares, estas enzimas hidrolizando de manera controlada los enlaces del peptidoglucano y se clasifican en N-acetilmuramidasas, N-acetilglucosaminasas, N-acetilmuramoil-L-alanina amidasas y en peptidasas. Las son secretadas mediante la vía dependiente de Secreción (Sec) o mediante el sistema de translocación doble de arginina (TAT) y tienen pesos moleculares en un rango de 27 kDa a 137 kDa.

Resistencia a antibioticos, Enzimas bactericidad, Peptidoglucano hidrolasas

Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of microorganisms represented by several genera, with metabolic, morphological and physiological characteristics in common (Gálvez *et al*., 2007). They have diverse applications, due to the relevant role they play in fermentation in the food industry. Hence, they are widely used for the preservation of various food products, their usefulness is based on providing sensory characteristics such as flavor, odor, texture and consistency, in addition to increasing its nutritional value and safety (Carr *et al*., 2002; Azadnia *et al*., 2011). The end products of LAB metabolism involved in antibacterial capacity are organic acids as lactic, acetic and propionic acid; in addition can also produce hydrogen peroxide, carbon dioxide, reuterin, reutericicline, 2-pyrrolidone, 5-carboxylic acid, bacteriocins, peptidoglycan hydrolases (PGH), among others (Hernández *et al*., 2005). As a consequence, LAB historically have been used to preserve food, in addition to being GRAS (generally recognized as safe). Amog these antibacterial compounds, PGH are enzymes involved in diverse cellular functions since they hydrolyze in a controlled way the peptidoglycan bonds, then they can be used as bactericidal compounds, inhibiting the growth of bacteria thatrepresent a public health problem, due to this they have generated great interest (Turner *et al*., 2004).

1. Antibiotics

Molecules of natural, synthetic or semi-synthetic origin, capable of inducing the death or inhibiting the growth of bacteria and fungi, and the replication of viruses are defined as antimicrobials, within this category are antibiotics effective to stop the growth (bacteriostatic) or produce death (bactericidal) in bacteria by various mechanisms, exerting a specific action on some structure or function (Vignoli and Seija, 2008).

In the twentieth century, the use of antibiotics increased as treatment and prevention of diseases caused by infectious agents in humans, but also in the agricultural, veterinary and food sector (Alós, 2015).

2. Classification of antibiotics

Antibiotics have been divided into three major groups (Table 2.1), according to their spectrum, mechanism of action (Figure 1) and pharmacokinetics-pharmacodynamics (Seija & Vignoli, 2008).

Table 2.1 Classification of antibiotics

Classification	Subclassification	Diana
Spectrum	Broad	Active against a large number of differentspecies and genera.
	Reduced	They are active against a limited number of species.
Mechanism of action	Attack on the bacterial wall.	
	Protein synthesis inhibitors.	
	DNA replication inhibitors.	
	Cytoplasmic membrane inhibitors.	
	Metabolic pathway inhibitors.	
Pharmacokinetics/ pharmacodynamics	Beta-lactams	Inhibits last stage of bacterial cell wallsynthesis.
	Penicillin	It prevents cell wall synthesis by inhibitingthe enzyme transpeptidase.
	Cephalosporins	Interferes with peptidoglycan synthesis.
	Monobactam	Interferes with cell wall synthesis.
	Carbapenems	Inhibits the synthesis and assembly of thelast stage of cell wall peptidoglycan.
	Beta-lactams associated with inhibitors of beta-lactamases	Inhibits bacterial beta-lactamase enzymes.
	Glycopeptides	Inhibits the synthesis and assembly of thesecond stage of cell wall peptidoglycan.
	Aminoglycosides	It interferes with the correct reading of thegenetic code.
	Macrolides	Interferes with a block in transpeptidationand translocation reactions.
	Quinolones	Inhibit DNA and RNA synthesis by interacting with DNA gyrase and topoisomerase IV.

Source: Seija & Vignoli (2008)

3. Worlwide problem of antibiotic use

Antibiotic resistance (AR) is a natural expression of bacterial evolution and genetics, first reported in 1912 (Cabrera *et al*., 2007). Some factors that favor AR are the inappropriate use of antimicrobials (Alós, 2015), the low quality of active compounds, the lack or deficiency of infection prevention and control programs, the inability of laboratories to detect resistance, as well as inadequate surveillanceand insufficient regulation of antibiotic use (Fariña, 2016). These factors are particularly important in countries where legislation is inadequate, surveillance and monitoring of antimicrobial use is lacking, and prevention and control of antimicrobial resistance (AMR) is also weak (WHO, 2017). As this problem has increased, epidemiological surveillance has categorized bacteria that are resistantto multiple antimicrobial agents, naming them by the absence of sensitivity into multidrug-resistant, extremely resistant, and resistant to all antimicrobials. According to Magiorakos *et al*. (2012), "Multiple Drug Resistance (MDR) is defined as the absence of sensitivity to at least one drug in three or more of the antibiotic categories; Extensively Drug-Resistant (XDR) refers to the absence of sensitivity to at least one agent in all but two or fewer of the antimicrobial categories; and resistance to all antimicrobials is defined as resistance to all antibiotic categories."

In recent years, the incidence of multidrug-resistant microorganisms has increased considerably. In addition, ineffective treatments must be applied that prolong the time of agony of the sick, forcing the administration of expensive drugs and increasing the time of hospitalization and risk of mortality (Fariña, 2016).

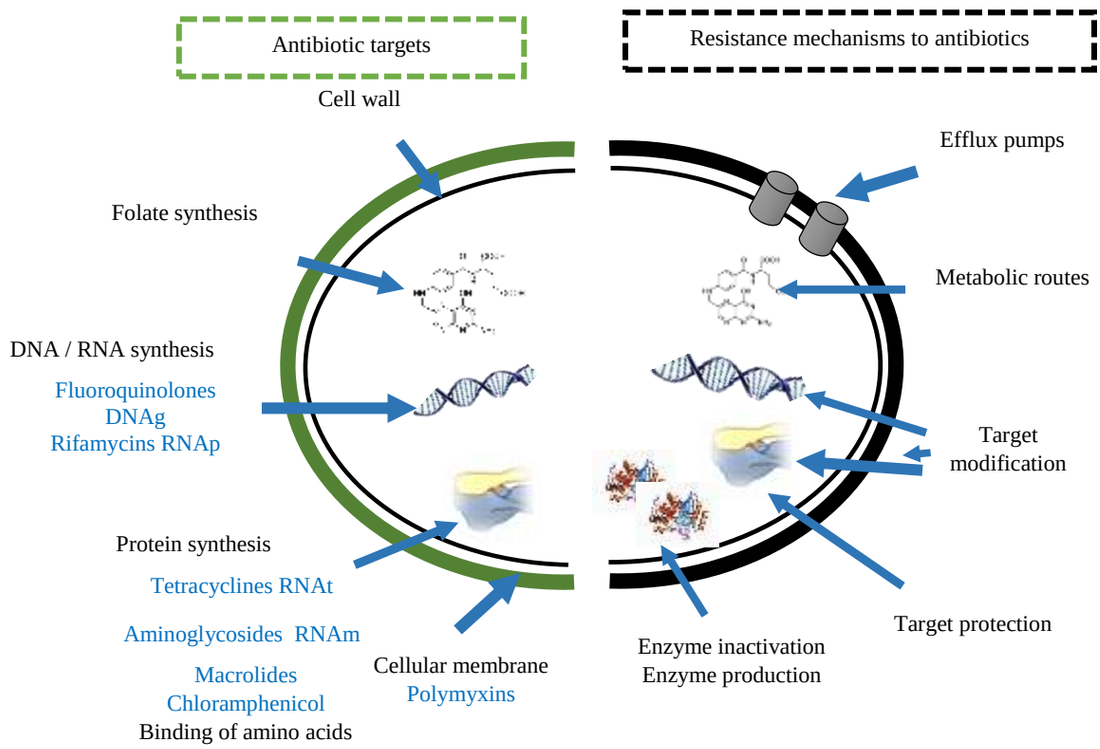
4. Mechanism of bacterial resistance to antibiotics

AR is the result of mutations and the exchange of genetic material by transferring resistance genes through mechanisms such as transformation, conjugation, transduction, and transposition. The transfer of genes from free DNA of a previously lysed bacterium to another is called transformation, while the transfer of genetic material contained in plasmids from one bacterium to another via pili is defined as conjugation (Levy, 2004, Prescott *et al*., 2002). Transduction is described as the transfer of genetic material from one bacterium to another by a phage. The last mechanism is transposition, which occurs by a displacement of a section of DNA between one genetic location (donor site) and another (acceptor site) (Levy, 1998; Levy, 2004; Prescott *et al*., 2004).

Bacteria have developed several mechanisms to resist the action of antibiotics as shown in Figure 2.1, among them are active expulsion, decreased cell wall permeability, enzymes production and binding to an essential protein (Couvalin, 1988; Prescott *et al*., 2004). The production of enzymes such as beta-lactamases are enzymes that hydrolyze beta-lactam antimicrobial agents. In gram-negative bacteria, beta-lactams enter the cell through porins and find beta-lactamases in the periplasmic space. Beta-lactamases destroy beta-lactam molecules before they have a chance to reach their target penicillin-binding proteins (PBPs). In contrast, in gram-positive bacteria, beta-lactamases are secreted extracellularly into the surrounding environment. It destroys beta-lactam molecules before they have a chance to enter the cell (Prescott *et al*., 2004).

Gram-negative bacteria can produce adenylating, phosphorylating or acetylating enzymes that modify an aminoglycoside to inactivate it. For example, chloramphenicol acetyltransferase is produced by gram-negative bacteria that modify chloramphenicol to inactivate it. Gram-negative bacteria can become resistant to beta-lactam antibiotics developing permeability barriers. Altered porins usually cause this in the outer membrane that no longer allows the entry and transit of antibiotic molecules into the cell. When beta-lactams cannot reach PBPs, the cell is resistant (Couvalin, 1988).

Figure 2.1 Antibiotic targets of action and resistance mechanisms



Source: Mandigan (2009)

PBPs in both gram-positive and gram-negative bacteria can be mutated so that beta-lactams cannot bind to them; therefore, the cell is resistant to antimicrobial agents. Mutations in the chromosomal genes for DNA gyrase and topoisomerase IV confer resistance to quinolones.

A wide variety of efflux pumps provide antimicrobial resistance to both gram-positive and gram-negative bacteria. The active efflux of antibiotics is mediated by transmembrane proteins inserted in the cytoplasmic membrane, and, in the case of gram-negative organisms, it also involves components in the outer membrane and periplasm. These proteins form channels that actively export an antimicrobial agent out of the cell as quickly as it enters. Some microorganisms develop an altered metabolic pathway that bypasses the reaction inhibited by the antimicrobial. Mutations that inactivate thymidylate synthetase block the conversion of deoxyuridylate to thymidylate. These mutants require exogenous thymine or thymidine for DNA synthesis and are therefore resistant to antagonists of the folate pathway such as sulfonamides and trimethoprim, to name a few (Couvalin, 1988).

5. Examples of recent cases of antibiotic resistance

Resistance to antibiotics has increased significantly in the last decade in the world, with the following cases standing out:

a) In Poland, between 2014 and 2015, *Acinetobacter baumannii* strains were isolated from blood cultures in hospitalized patients with pneumonia showing resistance to fluoroquinolones, amikacin, trimethoprim/sulfamethoxazole, imipenem, meropenem, cephalosporins and tetracyclines (Hernandez *et al*., 2018).

b) In other study, 46 potentially pathogenic *Pseudomonas aeruginosa* strains were isolated from agricultural water samples, which showed high rates of resistance to ampicillin, ceftriaxone, chloramphenicol, cefotaxime cephalothin, nitrofurantoin, kanamycin, streptomycin and tetracycline (Gutierrez *et al.*, 2017).

c) In Michoacán, Mexico 34 samples of *Escherichia coli* were isolated from cows with mastitis showing resistance to amikacin, ampicillin, levofloxacin, cephalothin, cefotaxime, ceftriaxone, chloramphenicol, gentamicin, netilmicin, nitrofurantoin, cefepime, trimethoprim sulfamethoxazole, tetracycline, kanamycin and streptomycin (Jiménez *et al*., 2017).

d) In another case in Mexico at the National Institute of Pediatrics, from 149 cultures of urine culture, blood culture, non-surgical wound and vaginal exudate samples, strains of Enterococcus faecalis and Enterococcus faecium were isolated in the period from January to December 2016; *Enterococcus faecalis* and *Enterococcus faecium* strains, which showed resistance to ampicillin, streptomycin, penicillin, vancomycin, gentamicin, erythromycin and quinupristin and dalfopristin (Arredondo-García *et al*., 2018).

e) At the National Institute of Rehabilitation "Luis Guillermo Ibarra Ibarra" in Mexico City, 11 strains of *Staphylococcus aureus* and 12 strains of coagulase-negative *Staphylococcus* from surgical wounds, bronchial secretions, blood cultures, catheter tips and bone cultures, collected during the period from February to July 2018 were studied showing methicillin resistance (MRSA) (Garcia *et al.*, 2019).

6. Worlwide action plan on antibiotic resistance

To guide and promote research and development (R&D) of new alternatives to the global problem of AR, the WHO in 2017 published the first list of priority AR pathogens, which includes 12 families of bacteria, using specific criteria to list them such as the degree of lethality of infections, whether or not treatment requires long hospitalization, the frequency of contagion in the community, consider whether or not they can prevent infections, number of therapeutic options that exist, and presence of R&D of new antibiotics to treat infections (WHO, 2017).

The list is divided into three categories, critical, high or medium priority. The critical priority group includes multidrug-resistant bacteria that can cause serious and lethal infections. The second and third tier encompasses other bacteria that manifest increasing drug resistance and cause common diseases (Table 2.2) (WHO, 2017).

Table 2.2 WHO list of priority multi-resistant pathogens for R&D of new antibiotics

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Carbapenem-resistant *Acinetobacter baumannii* Carbapenem-resistant *Pseudomonas aeruginosa* Carbapenem-resistant *Enterobacteriaceae*, producers of extended- spectrum beta-lactamases.	*Enterococcus faecium*, resistant to vancomycin *Staphylococcus aureus*, methicillin-resistant, intermediate susceptible and vancomycin resistant *Helicobacter pylori*, resistant to clarithromycin *Campylobacter spp.*, resistant to fluoroquinolones *Salmonellae*, resistant tofluoroquinolones *Neisseria gonorrhoeae*, cephalosporin-resistant, resistant to fluoroquinolones	*Streptococcus pneumoniae*, notpenicillin sensitive Ampicillin-resistant *Hemophilusinfluenzae* Fluoroquinolone-resistant *Shigella spp.*

Source: WHO (2017)

7. Development of the presence of antimicrobial resistance in Mexico

In 1973, 493 strains of *Salmonella typhi* were isolated and studied during an outbreak in the laboratories of the Hospital de Infectología del Centro Médico La Raza (IMSS), showing resistance to chloramphenicol (CM), tetracycline (TC), streptomycin (SM) and sulfonamides (SU) (Olarte *et al*., 1973).

In 1981-1982, 22 strains of enterotoxigenic *Escherichia coli* with resistance to ampicillin, tetracycline, streptomycin and kanamycin were isolated from children at the University Hospital of Puebla (Martínez *et al*., 1987).

Other studies in Mexico City over three decades, 1960, 1970 and 1980, have isolated strains of *Shigellaspp.*, *Salmonella spp.* and *E. coli* (Estrada-Garcia *et al*., 2005; Santos *et al*., 1989) that showed resistance to ampicillin, and decreased resistance to furazolidone.

Studies conducted in 1996 for *Enterococcus sp.* in Mexico showed high levels of resistance to gentamicin and *Enterococcus faecalis* showed resistance to ampicillin and imipenem. Also, in 2007, resistance to vancomycin was reported in these pathogens (Sifuentes-Osornio *et al*., 1996).

In 1998, it was observed that strains *of Streptococcus pneumoniae* had begun to show resistance to penicillin and to cephalosporins, macrolides, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol and tetracyclines (Silva *et al*., 1998).

BLEE-producing strains of *Klebsiella pneumoniae*, *E. cloacae, E. coli* and *Serratia marcescens* were isolated from several hospitals in Mexico between 2001-2008. In other studies, *Streptococcus pneumoniae* strains were found to have increased resistance to erythromycin, chloramphenicol, trimethoprim/sulfamethoxazole and vancomycin. In addition, reports have shown increasing resistance to trimethoprim/sulfamethoxazole and erythromycin in 10 Latin American countries, including Mexico (Quinones-Falconi *et al*., 2010; Bautista-Márquez *et al*., 2013).

In 2009, multidrug-resistant *Salmonella typhimurium* strains and the production of an AmpC-type beta-lactamase were reported in Yucatan (Wiesner *et al*., 2009; Zaidi *et al*., 2007).

Likewise, methicillin resistance has been described in *Staphylococcus aureus* strains. In 2010, the first report of resistance to linezolid was made in Mexico (Villaseñor-Sierra *et al*., 2012).

In 2011, increasing resistance to clarithromycin was reported in *Helicobacter pylori* in Mexico City (Ayala *et al*., 2011) and in 2012 antimicrobial resistance in *Campylobacter spp.* isolated in Sonora, San Luis Potosi, Michoacan and Yucatan (Zaidi *et al*., 2012).

8. Alternative for controlling antibiotic use

The WHO and FAO proposals seek to promote R&D in projects aimed at solving the problem of AR.

Therefore, work has been proposed derived from the use of microorganisms with antimicrobial activity,such as fungi such as *Aspergillus ochraceus* 3MCMC3 isolated from *Rhizophora mangle* roots that inhibit the growth of *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14036, and *Salmonella typhimurium* ATCC 14036, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus* ATCC 9634 and *Staphylococcus aureus* ATCC 25923 (Castillo-Machalskis *et al*., 2007).

Another example are the studies of bacteriophages ØA392 against infections caused by imipenem- resistant *Pseudomonas aeruginosa* (Wang *et al*., 2006) or the phages Φ H5 and Φ A72 that inhibit the growth of *Staphylococcus aureus* in milk (García-Suarez *et al*., 2008). Also, LAB with antimicrobial activity such as *Lactobacillus acidophilus* ATCC 33200, *Limosilactobacillus fermentum* ATCC 9338, *Lacticaseibacillus casei* ATCC 27139, *Lactiplantibacillusplantarum* ATCC 10776 have been used, *Lactobacillus bulgaricus* ATCC 11842 and *Lactobacillus helveticus* ATCC 15807 that have bactericidal activity against *Escherichia coli, Salmonella enteritidis* and *Shigella dysenteriae*, which were isolated and identified from clinical samples (Larre *et al*., 2007).

9. Proteins of lactic acid bacteria with biological activities

In addition to their technological function, LAB have the ability to inhibit the growth of certain altering and/or harmful microorganisms in food or even within the community. LAB have a primary antimicrobial effect, this is due to the competition for the substrate and the production of various metabolites such as organic acids; as well as ethanol, CO_2, H_2O_2, diacetyl, acetaldehyde and other oxygen metabolites. Also, LAB produce ribosomal antimicrobial protein compounds such as bacteriocins and enzymes such as PGH (Cintas *et al*., 2000).

The reserve of organic acids mainly lactic and acetic acid reduces the pH of the environment, this causes the inhibition of Gram-positive and Gram-negative bacteria, such is the case of *Limosilactobacillus fermentum* (QDC32) *Lacticaseibacillus casei* (QDC31), which have an inhibitory effect against *Salmonella typhimurium*. This effect is attributed to the penetration of lactic acid in a non-dissociated form in the cellular membrane, which decreases the pH in the cellular interior and provokes the dissociation, giving place to the liberation of H+ and the corresponding anion; so that, both ions interfere in the metabolism and inhibit the cellular growth, since all the efforts of the cell are to expel the ions (Requena, 1995; Urrego *et al*., 2005).

In the case of heterofermentative LAB, one of the final products of fermentation is CO2 and sometimes it is obtained by decarboxylation of amino acids, this product promotes an anaerobic environment,reduces the pH and helps to destroy the integrity of the cell wall (Ouwehand, 1998; Mora-Villalobos. *et al*; 2020). Another metabolite with antimicrobial activity is diacetyl, which is a fermentation product of citrate,has been shown to have antimicrobial activity at the level of 200 μg/ml for yeasts and Gram-negative bacteria and at 300 μg/ml for non-lactic Gram-positive bacteria (Axelsson, 2000; Ouwehand, 1998).

In addition, they can produce hydrogen peroxide when oxygen is present, leading to peroxidation of membrane lipids by hydroxy radicals and a consequent susceptibility of the cell (Ouwehand, 1998). Among the protein compounds of ribosomal synthesis are bacteriocins which are peptides that are excreted into the extracellular medium and, in some cases, have a broad spectrum of action and activity against pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Clostridium botulinum* and *Salmonella* (Yang *et al*., 2012; Benmechernene *et al*., 2013). The main substances produced by LAB that exhibit antimicrobial activity are listed in Table 2.3.

From the molecular point of view, antimicrobial peptides exert their action on some of the following bacterial structures or functions, either by inhibiting the synthesis of the bacterial wall, altering the integrity of the cytoplasmic membrane, preventing protein synthesis or blocking the synthesis or functions of nucleic acids (Calvo & Martínez-Martínez, 2009). In addition to the production of peptides with antimicrobial activity (Lorenzen & Meisel, 2005), peptides with other biological activities such as immunomodulant (LeBlanc *et al*., 2002), anticancer (De Moreno de LeBlanc *et al*., 2005), hypocholesterolemic (Kawase *et al*., 2000), mineral carrier (Lorenzen & Meisel, 2005), regulator of intestinal and nervous system activity (Rokka *et al*., 1997) and antioxidant (Hernández-Ledesma *et a*l., 2005).

Table 2.3 Metabolites with antibacterial activity produced by LAB

Metabolite	Producing microorganism	Reference
Diacetyl	Most of the BAL	Montville & Winkowski, 1997
Reuterin	*L. reuteri* *L. coryniformis*	Magnusson & Schnürer, 2001
BLIS: Bacteriocin-likeand inhibitory substances	Most of the BAL	Montville & Winkowski, 1997
Bacteriocins	Most of the BAL	Nes, *et al*., 1996
Cyclic dipeptides:Cyclo-PhePro Cyclo-PheOHPro Cyclo-GlyLeu	*L. coryniformis* *L. plantarum* *L. pentosaceus*	Magnusson & Schnürer, 2001
Hydroxy acids 3-hydroxy-tetradecanoicacid 3-hydroxy-decanoic acid 3-hydroxy-5-cis- dodecanoic acid 3-hydroxydecanoic acid	*L. plantarum*	Magnusson *et al.*, 2003
Bioactive peptides	Most of the BAL	Visser *et al*., 1986.

10. Peptidoglucan hydrolases (PGH)

Enzymes with lytic activity as antibacterial agents have been used in the food industry, due to the advantages they provide to the product and the consumer. Among the advantages are the elimination of bacteria, their biocontrol and the treatment of infectious diseases caused by these microorganisms. Several authors report that LAB is an important source of PGH production, the most reported genera are *Pediococcus* and *Lactobacillus* (Turner *et al*., 2007).

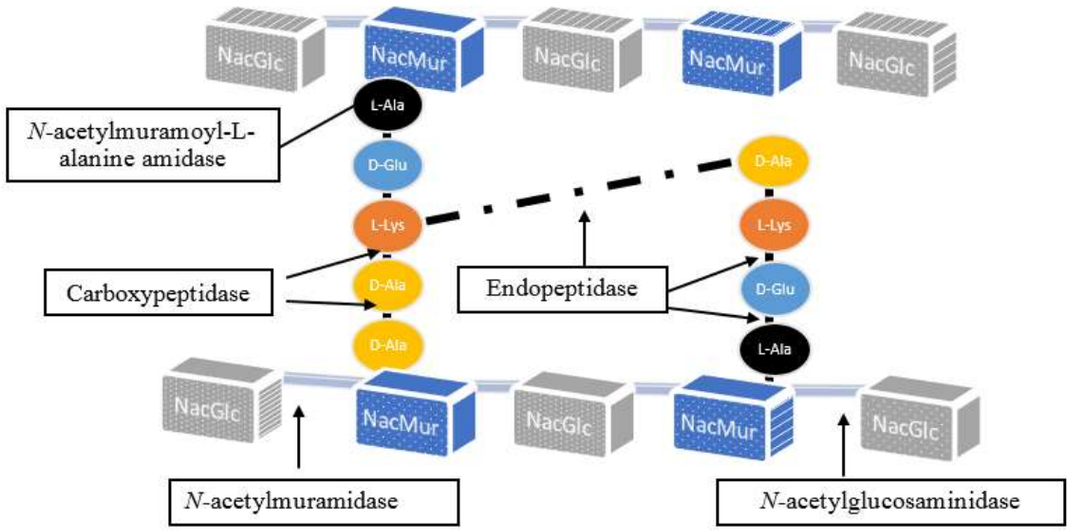
PGHs are enzymes involved in various cellular functions that require the cell wall; for example, during growth, cell division, regulation of cell wall growth, exchange of peptidoglycan units during growth, separation of daughter cells during division, flagella formation (in some cases) and autolysis, which is generally induced under adverse conditions such as lack of nutrients (Lortal & Chapot-Chartier, 2005;Vollmer *et al*., 1997). This regulation is performed by PGHs through the hydrolysis of covalent bondsof the PG, are located in the cell wall and act specifically on the basis of their incision site in the PG (Vollmer *et al*., 2008).

The characterized PGHs have a modular structural organization with two domains: a catalytic domain containing the active site of the enzyme and a cell wall binding domain (Turner *et al*., 2004; Lortal & Chapot-Chartier, 2005).

11. Classification of peptidoglycan hydrolases

The classification of PGHs depends on the type of bond they hydrolyze in the PG, as shown in Figure 2.2. N-acetylglucosaminases hydrolyze the β-1,4 bond of the glucan chain, leaving a reducing N-acetylglucosamine end. N-acetylmuramidases hydrolyze the β-1,4 bond of the glucan chain, leaving a free reducing N-acetylmuramic acid end, also called lysozymes. In case of forming a 1,6-anhydro ring on the N-acetylmuramic they are called lytic transglucosylases. N-acetylmuramoyl-L-alanine amidases break the amide bond between the N-acetylmuramic acid and the L-alanine of the peptide. Peptidases are able to hydrolyze the last amino acid of the carboxyl end of the peptides also called carboxypeptidases or to completely break the bridges formed by the peptides and are called endopeptidases (Layec *et al*., 2008; Vollmer *et al*., 2008).

Figure 2.2 Classification of peptidoglycan hydrolases according to their specificity



Source: Layec et al.,2008.

12. Catalytic domains of peptidoglycan hydrolases

The specificity of PGHs depends on their catalytic domain, as mentioned above in most cases they are composed of two domains: a catalytic domain containing the active site of the enzyme and a cell wall binding domain composed of several amino acid repeats (Diaz *et al*., 1991; Joris *et al*., 1992; Layec *et al*., 2008). BALs belong to the phylum Firmicutes, which is characterized by extensive PGH expression, so far 14 catalytic domains and 27 surface association domains have been described (Finn *et al*., 2006).

13. Catalytic domains

Catalytic domains are specialized for cleavage of a specific peptidoglycan bond, 14 catalytic domains have been described and are listed in Table 2.4. PGHs are composed of a single catalytic domain, however, enzymes have been studied that exhibit multiple distinct or identical catalytic domains associated with one or more substrates/binding domains. An example is the major PGH of *S. aureus*, a bifunctional autolysin named Atl (Oshida *et al*., 1995). Atl is initially produced as a 138 kDa protein with an amidase domain and a glucosaminidase domain after proteolytic processing that generates two major PGHs: a 62 kDa N-acetylmuramyl-L-alanine amidase and a 51 kDa N-acetylglucosaminidase, conferred by glucosaminidase and amidase_2 (Oshida *et al*., 1995; Komatsuzawa *et al*., 1997).

Table 2.4 Catalytic domains of peptidoglycan hydrolases

N-acetylmuramoyl- **L-alanine amidase**	**Endopeptidase**	**Carboxypeptidase**	***N-acetylglucosami-nidase***	***N-acetylmurami-dasa***
Amidase 2 (PF01510)	Peptidase M23 (PF01551)	Peptidase_S66 (PF02016)	Glucosaminidase (PF01832)	Glico_hydro_25 (PF01183)
Amidase 3 (PF01520)	CHAP (PF05257)	VanY (PF02557)		SLT (PF01464)
Amidase 5 (PF05382)		Peptidase_S11 (PF02113)		Transglucosylase (PF06737)
CHAP (PF05257)		Peptidase_S13 (PF00768)		

Source: Layec et al., 2008

Lactococcus lactis has three known N-acetylglucosaminidases (AcmA, AcmB and AcmC) and one hypothetical one (AcmD), two of which (AcmA and AcmD) have three peptidoglycan-binding LysM domains (Huard *et al*., 2003; Huard *et al*., 2004). The presence of three LysM domains has been shown to be optimal for AcmA activity because variant proteins with fewer or more LysM domains exhibit lower activity (Steen *et al*., 2005).

The level of hydrolytic enzyme activity is not only a result of the efficiency of the catalytic domains, but is also controlled by the cell wall binding domains (Layec *et al*., 2008).

14. Cell wall binding domains

Cell wall binding domains are of great importance for the catalytic efficiency of PGHs. One of their main functions is the binding of proteins to the cell wall and the targeting of the enzyme to its site of action (Braun *et al*., 1997; Janecek *et al*., 2000). Their number may be essential for efficient binding of PGHs to the cell wall. For example, the copy number of the choline-binding domain (ChBD/CW_binding_1), LysM and SH3, has been shown to be important for high catalytic efficiency of PGHs, since their inactivity leads to strongly reduced (Eckert *et al*., 2006) or even deficient PGH activity (Sass & Bierbaum, 2007).

N-acetylmuramidase LytC from *S. pneumoniae* contains a choline-binding domain by which it binds to the teichoic acid of the cell wall, which is essential for its activity (Monterroso *et al*., 2005).

Another case is the G5 domain, which induces binding to N-acetylglucosamine, is found in certain hydrolases that can cleave oligosaccharides in their environment to provide carbon sources (Clarke *et al*.,1995) or the SH3 domain which is associated with the survival of pathogens within the invaded cell (Layec *et al*., 2008).

Table 2.5 shows 27 surface domains that can be found in bacteria such as *Pediococcus, Lactobacillus, Oenococcus*, among others.

Table 2.5 BAL cell wall binding domains

Binding domains	Joint sites	XFAM access number
Big_4	Variety of bacterial surface proteins.	PF07532
CBM_5_12	Carbohydrate Binding Modulus (CBM)	PF02839
ChW	Proteins containing ChW repeats (tryptophan)	PF07538
Collagen	Triple helix repeat proteins	PF01391
CpL_7	The CW_7 repeats form a cell wall binding motif.	PF08230
Cu_amine_oxidN1	Oxidation of primary amines to aldehydes	PF07833
Cw_binding_1	Repeats in P15057 recognition of choline-containing cell walls	PF01473
Cw_binding_2	SlpA and Cwp2 domains for the binding of PSII, acell wal component.	PF04122
DUF1142	Prophage tail proteins that probably act asendopeptidases.	PF06605
DUF1958	Prokaryotic penicillin binding protein 4.	PF09211
Erfk_YbiS_YhnG	YkuD, ErfK / YbiS / YcfS / YnhG Protein	PF03734
FG_GAP	Extracellular FG-GAP repeat found in alphaintegrins.	PF01839
GBS_Bsp_like	GBS Bsp-like repeat, group B streptococcus(GBS) protein.	PF08481
G5	G5 domain, extracellular proteins, PG metabolismproteins	PF07501
LysM	LysM domain (lysine motif) bacterial cell walldegradation.	PF01476
PBP5_C	Penicillin-binding protein 5, C-terminal domain,D-alanyl-D-alanine carboxypeptidase.	PF07943
PG_bindling_1	Putative peptidoglycan binding domain, it iscomposed of three alpha helices.	PF01471
fago bolin	SPP1 phage holin, holin proteins of the bacteriophage group dsDNA *Siphidoviridae*	PF04688
SH3_2	SH3 variant domain, protein involved intransduction.	PF07653
SH3_3	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF08239
SH3_4	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF06347
SH3_5	Bacterial SH3 domain, hypothetical bacterialproteins of unknown function	PF08460
SLpA	Protein A domain of the surface layer,bacterial cell surface proteins	PF03217
SLH	Cell wall pyruvoylated polymers: teichoic acids, teicuronic acids, lipoteichoic acids or lipoglycans.	PF00395
SPOR	Bacterial SPOR domains bind to the peptidoglycan	PF05036
TMP	WXXh motif repeat where X can be any residueand h is a hydrophobic residue.	PF05017
YSIRK_signl	Motif SIRKxxxGxxS transmembrane domain	PF04650

Source: Modified from Layec et al.,2008

15. PGH production by lactic acid bacteria

Several authors have reported that LAB are an important source of PGH and can be used to control pathogens in the food industry and in hospitals, due to their antimicrobial activity. The genus with the most reported production of PGH is *Lactobacillus* (Cibik & Chapot-Chartier, 2004; Turner *et al*., 2004; Yokoi *et al*., 2005; Donovan & Foster-Frey 2006). Furthermore, it has been found that one species can produce two or even three enzymes with this lytic activity (Baker *et al*., 2006).

Studies of this type of enzyme begin with lysozyme which is a muramidase (N-acetyl muramidase, E.C. 3.2.1.17), discovered by Alexander Fleming, catalogued as a food additive, with a molecular weight of 14.31 kDa and is composed of a sequence of 129 amino acid residues, with an isoelectric point (pI) of 10.7. This enzyme prevents the growth of *Oenococcus oeni, Clostridium tyrobutyricum, Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*, (Jollés & Jollés, 1984).

In *Leuconostoc mesenteroides subsp. mesenteroides* isolated from dairy products, a glucosidase and an N-acetyl-muramyl-L-alanine amidase with lytic activity of 41 and 52 kDa are expressed according to specificity analysis (Cibik *et al*., 2001).

It has also been reported the characterization of a PGH produced mainly during Stationary phase of *Clostridium perfringens*, named as ACP, which has a modular structure with three domains: a signal peptide domain, an N-terminal domain with repeated sequences and a C-terminal catalytic domain with a molecular weight of 122.388 kDa with a pI of 8.79 (Camiade *et al.*, 2010).

In another study, Donovan et al. 2006 investigated *Streptococcus agalactiae* bacteriophage B30 endolysin with lytic activity against the three main pathogens causing mastitis in dairy cattle, i.e. *Streptococcus agalactiae*, *Streptococcus uberis* and *Staphylococcus aureus* (Baker *et al*., 2006; Pritchard *et al*., 2006; Donovan *et al*., 2006).

Lactobacillus gasseri JCM11 31 (*Lactobacillus acidophilus*) has two extracellular proteins of 55 and35 kDa with autolytic activity (by zymogram analysis), the optimum pH for lysis was in the range of 6.0 to 7.0 (Yokoi *et al*., 2004).

In 2005, the activity of PGH produced by *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus, Lactobacillus delbrueckii subsp. lactis, Lactobacillus acidophilus, Leuconostoc citreum and Lacticaseibacillus casei* with molecular weights of 18-55 kDa was reported (Lortal & Chapot-Chartier, 2005).

Two PGHs with muramidase activity have been identified in *Enterococcus hirae* ATCC9790; one of them, named SF muramidase, which has been shown to be an exoenzyme that progressively degrades glucan strands from its GlcNAc end and pesticin which is an endo N-acetylmuramidase (Vollmer *et al.*,2008).

In *Staphylococcus lugdunensis* a PGH (ALT) with N-acetylglucosaminidase and N-acetylmuramoyl-L- alanine amidase activities was identified with a molecular weight of 140.69 kDa, being the main autolysin of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Bourgeois *et al*., 2007).

In 2013 García-Cano et al. reported PGHs isolated from *Pediococcus acidilactici* ATCC 8042 with lytic activity against *Staphylococcus aureus* with molecular weights of 110 and 99 kDa in zymograms with substrate *M. lysodeikticus* ATCC 4698 (García-Cano *et al*., 2015). Likewise, a PGH from *Enterococcus faecalis* (AtlD) with molecular weight of 62 kDa with antibacterial activity against *Listeria monocytogenes, Staphylococcus aureus* and *Enterococcus* strains of clinical origin was reported (Serrano-Maldonado *et al*., 2018).

Cibik and Chapot-Chartier (2004) evaluated PGH enzymes from *Lactobacillus pentosus*, where they identified PGH in membrane proteins with molecular weights of 31, 58 and 112 kDa in the stationary phase of growth (16 h) . In cytosol proteins it was presented activity in 31 kDa and, in the crude extract were found enzymes with PGH activity with an approximate molecular weight of 31, 43, 58, 77, 95 and 112-kDa.

16. Recombinant peptidoglycan hydrolases

Recombinant PGH have been reported, where their expression, activity, as well as their physicochemical, biochemical, reaction and specificity properties have been evaluated. Some cases are mentioned below. Cloning of a PGH from *Lactobacillus gasseri* JCM11 31 T was performed in the *E. coli* XL1-Blue system using the plasmid vector pUC118. Two recombinant plasmids, holgaY and lysgaY, wereproduced. The PGH gene inserted into the LysgaY plasmid encoded for a protein of 310 amino acids, whose molecular weight was calculated to be 33.7 kDa and a pI 8.75.

The gene inserted into theholgaY plasmid, on the other hand, coded for a protein of 143 amino acids with calculated molecular mass and pI of 15.7 kDa and 9.25, respectively. Sequencing of the gene revealed significant homology with hypothetical muramidases from the phage *Lactobacillus* Badh, Lj965, Lj928, LL-H, mv4 and mv1(Yokoi *et al*., 2005).

The AtlL protein from *Staphylococcus lugdunensis* was characterized for the first time and cloned in *E.coli.* The atlL gene encodes a bifunctional protein with lytic activity N-acetylmuramoyl-L-alanine amidase and N-acetylglucosaminidase on peptidoglycan. Cloning was performed in the pBAD / His B expression system (Invitrogen), which was used to subclone and express the gene fragments in *E. coli.* The atlL gene encoded a protein of 1279 amino acids with a calculated molecular mass of 140.69 kDa (Bourgeois *et al*., 2009).

In another work, the pET system (pET System, Novagen) was used with the Escherichia coli strain BL21 (DE3) for the cloning and expression of the 99 kDa bifunctional PGH of Pediococcus acidilactici ATCC M8042.

CHAP k lysine CHAP k from bacteriophage K, was heterologously expressed in *Escherichia coli* BL21(DE3) using the pET28a vector, the gene encoded a protein with a calculated molecular mass of 19,701kDa, with a purity of 95% (Shan *et al*., 2020).

17. Applications of peptidoglycan hydrolases

It has been described concisely the mechanism of these enzymes mainly in the most studied which is the lysozyme, this enzyme uses its mechanism of action against Gram positive bacteria destroying the cell walls by hydrolysis of the β 1-4 bond between the N-acetyl-muramic acid and N- acetylglucosamine of the peptidoglycan, thus weakening the cell wall and causing the consequent cell lysis.

In the food industry, lysozyme is used in foods such as meats, sausages, fish, vegetables, fruits, wine and milk powder and is also used in cosmetics and in the pharmaceutical industry (Maidment, 2009; Nakimbugwe *et al*., 2006).

Egg lysozyme is listed as a food additive with the code E-1105, it has been used as a contact preservative for food surface such as fresh vegetables, fish, meat, fruits, shrimps and other foods (Mine *et al*., 2004). Lysostaphin is an enzyme produced by *Staphylococcus simulans* which has a bactericidal effect on *S. aureus* and this confers applications in food, veterinary and human medicine (Fedorov *et al*., 2003; Szweda *et al*., 2012; Turner *et al*., 2007).

18. PGHs with antimicrobial activity against resistant microorganisms

Antibiotic resistance represents a threat to health, food safety and development, the use of some enzymes with lytic activity as antimicrobial agents has increased in the food industry (Donovan *et al*., 2006; Garcia *et al*., 2019). Table 2.6 lists some HGPs that have shown activity against resistant microorganisms.

Table 2.6 PGH reported with antimicrobial activity

PGH	Isolated microorganism	Inhibited microorganism	Reference
Lysostaphin	*Staphylococcus simulans*	*Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)	Fedorov *et al*., 2003
HolgaY and lysgaY	*Lactobacillus gasseri* JCM 1130	*Lactobacillus gasseri, Streptococcus cremoris and Lactococcus lactis*	Yokoi *et al*., 2004
N-acetyl-muramidase	Bacteriophage B30	*Streptococcus agalactiae, Streptococcus uberis and Staphylococcus aureus*	Baker *et al*., 2006
AtlL	*Staphylococcus lugdunensis* ATCC 43809	*Micrococcus lysodeikticus, Bacillus subtilis and Staphylococcus lugdunensis*	Bourgeois *et al*., 2009
Endolysin	Profago LambdaSa2	*Streptococcus pyogenes, Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus equi*	Donovan *et al*., 2006
Acp	*Clostridium perfringens*	*Micrococcus lysodeikticus* ATCC4698, *Bacillus subtilis*, *Clostridium difficile* and *Clostridium perfringens*	Camiade *et al*., 2010
N-acetylglucosamidase	*Pediococcus acidilactici* 99 kDa	*Streptococcus pyogenes, Enterococcus faecium, Lactobacillus paracasei, Listeria monocytogenes, Pediococcus acidilactici ATCC 8042, Enterococcus faecalis and Staphylococcus aureus ATCC 6538*	García-Cano *et al*., 2015
AtlD	*Enterococcus faecalis*	*Enterococcus faecium, Listeria monocytogenes, Enterococcus faecalis,* *Micrococcus lysodeikticus* ATCC4698, *Enterococcus faecium, Enterococcus faecalis* ATCC, Enterococcus *faecalis, Listeria innocua*, *Staphylococcus aureus* ATCC 6538	Serrano-Maldonado *et al*.,2018.

19. Conclusions

The overuse of antibiotics is a global public health problem that must be addressed. Therefore, an alternative to this problem is the use of therapies with lytic enzymes such as PGH produced by BAL, which are efficient because they target one of the main structures of the cell necessary for life, the cell wall.

In addition, new bactericidal compounds of natural origin can be designed to replace antibiotics in the health, agricultural and agri-food sectors.

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