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Antibiotic Resistance in Lactic Acid Bacteria

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Abstract

Most starter cultures belong to the lactic acid bacteria group (LAB) and recognized as safe by the US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA). However, LAB may act as intrinsic or extrinsic reservoirs for antibiotic resistance (AR) genes. This fact may not constitute a safety concern itself, as the resistance gene transfer is vertical. Nevertheless, external genetic elements may induce changes that favor the horizontal transfer transmission of resistance from pathogens as well as from the human intestinal microbiota, which represents a severe safety issue. Some genus of AR LAB includes *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* isolated from fermented meat and milk products. Currently, the WHO recommends that LAB used in the food industry should be free of resistance. Therefore, the objective of this chapter is to present an overview of the LAB antibiotic resistance and some methods to determine the same.

Keywords: lactic acid bacteria, antibiotic resistance transfer, extrinsic genes, QPS strains, and GRAS

1. Introduction

The antimicrobial resistance has become one of the main safety issues for humanity, and several organizations, such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the US Food and Drug Administration (FDA), and the European Food Safety Authority (EFSA) among others, have raised an awareness on this issue. The antimicrobial resistance can take place when microorganisms (bacteria, fungi, viruses, and parasites) are continuously exposed to antimicrobials (antibiotics, antivirals, antifungals, etc.), and as a result of an adaptation process, some microorganisms can survive and grow in the presence

of the antimicrobial, which in normal conditions would inactivate them [1, 2]. In particular, antibiotics are drugs used to treat bacterial infections in humans and animals, preventing the reproduction of bacteria or inactivating them through several mechanisms (**Table 1**), either inhibiting the synthesis of the cell wall or the cytoplasmic membrane, blocking the protein synthesis or the DNA copying processes, altering the metabolism, or acting directly against the bacterial resistance pathway [3–5]. The use of antibiotics in humans (cephalosporins, broad-spectrum penicillins, and fluoroquinolones) has increased 36% from the years 2000 to 2010, mainly due to their inappropriate prescription and consumption for the treatment of viral instead of bacterial infections [3, 6]. This fact may be correlated with the global report on antimicrobial resistance that points over 700,000 human deaths each year associated to antimicrobial resistance, with a raising scenery to 10 million deaths each year by 2050 [2, 7].

The antimicrobial resistance involves several mechanisms associated to the presence of resistant genes that allow the direct inactivation of the active antimicrobial molecule as well as the loss of susceptibility to the antimicrobial by modification of the target site or reduction of the antimicrobial uptake [6]. Therefore, antimicrobials become ineffective, and resistant microorganisms can survive and transfer their resistant machinery to other microorganisms and

<i>Site of action</i>	<i>Mode of action</i>	<i>Antibiotics</i>
Cell wall	Inhibition of precursors for the peptidoglycan synthesis	Fosfomycin Cyloserine
	Stopping the transport of cell wall precursors through the membrane cell	Bacitracin Mueridomycins
	Blocking the polymerization and crosslinking processes of wall peptidoglycan at the level of penicillin binding proteins (PBP's)	β -Lactams (penicillin derivatives, cephalosporins, etc.), glycopeptides (vancomycin and teicoplanin)
Cytoplasmic membrane	Increasing the membrane permeability with the subsequent loss of small metabolites.	Polymyxins
	Depolarization of cytoplasmic membrane that reduces the protein and DNA synthesis	Lipopeptides (daptomycin)
	Alteration of the membrane by formation of pores	Ionophores (valinomycin, tirocydins) and gramicidins
Protein synthesis	Inactivation of the protein activation process	Mupirocin,
	Inhibition of the protein synthesis initiation	Oxazolidiones and aminoglycosides
	Blocking the tRNA amino acid complex to ribosomes	Tetracycline and glycylcyclines
	Modification of the protein final elongation stages by blocking the peptidyl transferase on the 50S ribosome subunit	Amphenicols, lincosamides, macrolides, ketolides
DNA synthesis	Alteration of the DNA copying processes at the DNA-dependent RNA polymerase	Nitroimidazoles and nitrofurans
	Inactivation final DNA coiling process	Quinolones
Resistance mechanisms	Protects against bacterial enzyme β -lactamases that provide resistance to β -lactam antibiotics and/or blocks the antibiotic active efflux process.	Clavulanic acid, sulbactam and tazobactam

Table 1. Antibiotics: Site and mode of action [3–5].

become a threat to public health [1]. The presence of antimicrobial-resistant microorganisms not only affects both the human and animal health but also increases the risk for spread and contamination of foods, crops, livestock, and aquaculture [3].

In particular, the FAO claims that 27 different antimicrobial classes are being frequently used in animals without an accurate reporting system to collect data related to their use and control [2]. Therefore, the WHO partners initiated a campaign all around the world in 2017, to raise the awareness of the antimicrobial resistance as part of a global program [1, 2]. The campaign constitutes a global action that involves governments, health professionals, food and feed industrialists, and the society to learn about antibiotic and antimicrobial resistance. It also includes some guidelines for the prevention and control of resistant *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonads* in health-care facilities. Additionally, the WHO recommends to farmers and the food industry sector to stop using antibiotics in healthy animals, in order to preserve the effectiveness of antibiotics currently used in human medicine [1–3, 8]. The global action plan on antimicrobial resistance points out that this issue has become an increasingly serious threat to public health and to the sustainable food production, where a rapid and effective response should involve the society and governments, as well as the health, food, and agriculture sectors and environmental specialists to promote practices that avoid the spread of antimicrobial resistance among common pathogens, especially those responsible for nosocomial and common infections [1, 2, 8].

The growing world population results in an increased demand for food, where antimicrobials such as antibiotics and fungicides are frequently used to treat infections in food-producing animals (cattle, swine, poultry, and fish), as well as in crops, to prevent diseases and as growth promoters [3]. This practice is frequently seen in developing countries where unauthorized high amounts of antibiotics are used that have been associated to the occurrence of multiple antibiotic-resistant *Enterococcus* and *Lactobacillus* strains from Indian poultry [2, 3, 9]. The FAO also reports that 90% of antibiotics may be excreted into the water and soil thus contaminating the environment, with the consequent exposure increment and development of AR microorganisms that can transfer their resistant genes to other microorganisms [2]. For instance, bacterial populations from the intestine of animals exposed to antibiotics (tetracycline, penicillin, sulfonamide, and polymyxins) were five times more likely to be resistant [6]. The resistant microorganisms can be spread to humans from contaminated foods and water or from the environment [2, 3]. Various practices such as adequate animal vaccination and the use of additives that promote health and efficiency of feed conversion, in combination with good hygiene and husbandry practices would reduce the need for antimicrobials and antibiotics for food production [7, 8].

Lactic acid bacteria (LAB) constitute one of the most important groups of microorganisms present in several habitats; they are in large numbers in the gastrointestinal tract of animals and humans and form part of the microbiota in several foods. Historically, LAB have been recognized as safe with a GRAS (generally recognized as safe) and QPS (qualified presumption of safety) status given by the FDA and EFSA authorities. However, the recent detection of antibiotic-resistant LAB and the continuous exposure to environmental conditions may promote that LAB became as intrinsic or extrinsic reservoirs for AR genes, which can be horizontally

transmissible to pathogens through the food chain [3, 6]. The resistance to a specific antimicrobial may be intrinsic (when a microorganism does not possess target sites for the antimicrobial) or acquired. The acquired resistance is more complex and involves the presence of enzymes that inactivate the antimicrobial, posttranscriptional, or posttranslational modifications of the target site or reduction uptake and active efflux of the antimicrobial; those mechanisms derive from the gain of exogenous DNA or the mutation of indigenous DNA [4, 9, 10]. In general the AR genes can be horizontally transferred from one microorganism to another by transduction (via bacteriophages) or by transformation between microorganisms (when released DNA is taken up by other microorganism). However, it is claimed that the primary mechanism to acquire resistant is by direct cell to cell contact or conjugation between different genera of bacteria, especially when the resistant genes are present on mobile genetic elements such as plasmids and transposons [5, 10, 11]. LAB are highly adaptable and capable of developing resistance to antibiotics; most AR studies were focused on pathogenic microorganisms, but recently some investigators have questioned the safety of commensal LAB as some strains of *Lactococcus lactis*, *Enterococci*, and *Lactobacillus* isolated from fermented foods showed genes conferring resistance to tetracycline, erythromycin, and vancomycin [12].

Bacterial resistance to antibiotics is an emerging public concern that may compromise the efficacy of agents used for the treatment of infectious diseases [13]. Therefore, the objective of this chapter is to present an overview of the LAB antibiotic resistance and some methods to determine this characteristic, as per the FAO/OMS guideline for testing food-related bacteria and probiotics for resistance patterns.

2. Lactic acid bacteria

The term lactic acid bacteria refers to a taxonomically diverse group of Gram-positive bacteria, facultative anaerobic, nonspore-forming, nonmotile, and acid-tolerant cocci, coccobacilli, or rods that appear as single cells or forming couples, tetrads, or long chains, with common metabolism and physiology capable of fermenting sugars primarily into lactic acid. LAB species are found in two phyla, the *Firmicutes* and the *Actinobacteria*; for the first the genus, *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* that are low G + C (31–49%) belong to the *Bacilli* class and the *Lactobacillales* order. While, the *Bifidobacterium* genus with a high G + C content (58–61%) belongs to the *Actinobacteria* phylum [6, 14, 15].

This bacterial group is classified into homofermentative and heterofermentative according to the end products derived from the glucose metabolism. The homofermentative converts glucose mainly into lactic acid by the Embden-Meyerhof pathway, while the heterofermentative LAB transforms glucose into lactic acid, carbon dioxide, and ethanol or acetic acid by the 6-phosphogluconate pathway. LAB are capable of inhibiting the growth of spoilage and pathogenic bacteria based on the competition for nutrients and adhesion niches due to their great acid tolerance and ability to adapt to redox changes [14, 15]. In addition LAB are capable to produce antimicrobial metabolites such as lactic and acetic acids, ethanol, hydrogen peroxide, diacetyl, antifungals (short-chain fatty acids derived from lipolysis reactions), antimicrobial

peptides known as bacteriocins, and other antibacterial proteins like peptidoglycan hydrolases (PGH) capable to cleave the peptidoglycan cell wall of Gram-positive and Gram-negative bacteria [6, 14]. Bacteriocins are ribosomal antimicrobial peptides active against closely related and non-related sensitive bacterial strains by forming pores in the cytoplasmic membrane and responsible for the reduction of microbial LAB competitors under stress conditions. Several studies have demonstrated the potential of bacteriocins to be applied for food preservation and in the pharmaceutical industry for their action against spoilage microorganisms and pathogens such *Listeria monocytogenes* and *Staphylococcus aureus* [16–18].

LAB have been safely used for centuries in numerous indigenous food fermentations up to the actual modern industry in the elaboration processes for dairy products, vegetables, meats, coffee, cocoa, silages, sourdough bread, and wine, as LAB contribute to the taste, flavor, and texture of those fermented products but also inhibit the development of spoilage and pathogenic microorganism by acidification and production of antimicrobials [14, 19]. Therefore, LAB are widely employed as starter cultures in the food industry to accelerate ripening or to control the adventitious microbiota for elaboration and preservation of several fermented foods including dairy (hard- and semihard-type cheeses, yogurt, butter, and cream), meats, sourdough bread, and vegetables. LAB contribute to the taste, flavor, and texture of those fermented products as a result of several reactions, including lipolysis, proteolysis, and conversion of lactose in citrate and pyruvate intermediates that can be converted to various aromatic compounds, such as diacetyl, acetoin, acetaldehyde, and acetic acid. Proteolytic processes induces the accumulation of small peptides and free amino acids that are further transformed into alcohols, aldehydes, acids, and esters responsible for the flavor profile and organoleptic characteristics of fermented foods [14]. In addition some LAB strains such as *Lactococcus lactis*, *Lactobacillus sakei*, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* can produce exopolysaccharides (EPS) that not only confer protection to the cell producer but can be applied in the food industry as thickeners to increase viscosity and firmness, improving texture and mouthfeel of yogurt and other low-fat milk products. The EPS produced by LAB range from 10 to >2000 kDa and can be classified as homo- or heteropolysaccharides according to their monomer composition, where galactose, glucose, and rhamnose are the most common monomers [20].

Some LAB are present in the respiratory, gastrointestinal, and genital tracts of humans and animals and therefore used as probiotics for healthiness improvement related to their influence on the immune system for the prevention and control of some infections during pregnancy or as part of the treatment for antibiotic-derived diarrhea, constipation, and intestinal inflammation, also to manage allergies and lactose intolerance and prevention of urinary infections [21–23]. The WHO and FAO describe the probiotics as live microorganisms that in adequate amounts confer health benefits for the host [24]. Several strains of *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, *Pediococcus*, and *Propionibacteria* present in foods and in dietary supplements are commonly used as probiotics and considered desirable members of the intestinal microbiota that can be used to deliver vaccines and other metabolites directly in the gastrointestinal tract [21]. Consumption of LAB probiotics may help for modulation of the immune system and reduction of pathogens, thereby, improving the gut functionality. Other health benefits associated to the consumption of LAB probiotics include an antihypertensive

effect, reduction in the serum cholesterol level, antioxidant effect, protection against colon cancer, reduction in the allergy symptoms, reduction in dental caries, and reduction in the obesity index [21, 22]. In addition, secondary metabolites with health-promoting properties include the antihypertensive angiotensin-converting enzyme produced through the proteolytic system of *Lactobacillus helveticus*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* [14, 22].

LAB are considered naturally resistant to several antibiotics and may have the potential to acquire resistance to other antimicrobials or to disseminate the resistance to pathogens present in the gastrointestinal tract of animals and humans [9]. For instance, Shao et al. [11] demonstrated that two isolates of *L. plantarum* possessed the *aadA* and *ant(6)* genes associated to the resistance to streptomycin, and the overexposure to this antibiotic dramatically increased the minimum inhibitory concentration (MIC) and increased a cross-resistance to other antibiotics from the same class. On the other hand, the presence of 6% strains isolated from some pharmaceutical and dairy products from Egypt with tetracycline [*tet(M)*] and/or erythromycin [*erm(B)*] resistant genes has been reported [21]. In a similar study, a high incidence of *Lactobacillus* resistant to vancomycin (58%), erythromycin (10.8%), tetracycline (4.3%), gentamicin (48%), and ciprofloxacin (26%) was reported in Turkish fermented dairy products [13]. However, studies made by Flores and Mayo [25] indicate that no transfer of the tetracycline [*tet(M)*] and erythromycin [*erm(B)*] resistant genes from *S. thermophilus* to *L. delbrueckii* was detected during the production and storage of yogurt. Furthermore, the food chain can facilitate the transmission of antibiotic-resistant bacteria between animals, foods, and humans, being the fermented milk and meat products the most common vehicle for antibiotic-resistant bacteria to the indigenous flora of the gastrointestinal tract, as these products are consumed without a thermal treatment [12]. Even though that some reports confirm the transmission of resistant determinants, the two most common resistant genes in LAB are tetracycline [*tet(M)*] and erythromycin [*erm(B)*] resistant genes, followed by *cat* genes coding for chloramphenicol resistance [26]. Considering the wide range of potential applications of LAB in the industry and in the human and animal health, there is a need of their detailed examination that involves the detection of AR genes.

3. Transfer mechanisms of antibiotic resistance genes

For antibiotics to function and inhibit microbial growth, they must be at the proper concentration so that they can cross the cellular wall and interact with their target. As previously mentioned, AR is the capacity that has a microorganism to resist the inhibitory activity of an antibiotic beyond the normal susceptibility of similar bacterial species [27]. On the other hand, the different mechanisms of AR are based on the modification of the antibiotic target site as well as on the reduction of the antibiotic concentration that manages to get the cell target.

LAB are considered carriers of resistance genes that could propagate their genes within the food chain between food and humans, as well as to the environment through different mechanisms [27–30]. According to the FAO and WHO [24], it is important to determine whether starter or probiotic cultures intended for human or animal consumption have mobile resistance genes that could be transferred to other microorganisms [6, 31]. In addition, some authors have demonstrated that the use of antibiotics in animals destined for consumption, either as growth

promoters or pathogen inhibitors, is directly related to the presence of AR microbiota in the human gastrointestinal tract [27, 32]. On the other hand, Gad et al. [21] isolated some *Lactobacillus*, *Streptococcus*, and *Lactococcus* strains from both pharmaceutical and probiotic dairy products, but the AR tests from the pharmaceutical probiotic isolates were free of resistance genes, unlike the LAB isolated from dairy products that showed resistance profiles comparable to those from pathogens such as *Staphylococcus spp.*, *Escherichia coli*, and *Salmonella spp.* Furthermore, some *Enterococcus faecium* strains have demonstrated the transference of vancomycin resistant genes from to *Lactobacillus acidophilus* La5 “in vitro” and “in vivo” studies in the gut mice [33].

Exposure to antibiotics may allow bacteria to develop different mechanisms to counteract the bactericidal effect; a single bacterium can develop different types of resistance; these systems include an intrinsic or innate and the acquired resistance mode. Among these, the mechanism that prevails within bacteria varies according to the nature of the antibiotic, the target site, the bacterial species, and/or whether the resistance gene is part of the chromosome or mobile elements such as plasmids or transposons [12, 19, 28].

3.1. Mechanisms of resistance in LAB

Two relevant elements must be present for the antibiotic-target interaction, first the antibiotic must recognize the target, and the concentration of the antibiotic in the target must be sufficient to inhibit the bacterial growth. A resistance mechanism conduces to the antibiotic failure to inhibit the bacterial growth due to an inefficient antibiotic-target interaction, which can be classified as passive and active. The passive mechanism can only be transferred to other cells by clonal transfer that involves modifications of the target site or decrease in antimicrobial absorption, without affecting the antibiotic structure; this resistance is also known as intrinsic resistance. In contrast, the active mechanism involves the reduction on the concentration of the intracellular antibiotic by modification or degradation of its structure with enzymes or through the action of efflux pumps [34, 35].

Figure 1 shows the mechanisms by which some bacteria can show resistance to antibiotics that involves (1) modification of the antibiotic by enzymatic complexes that prevent the antibiotic-target interaction, (2) enzymatic degradation of intra- or extracellular antibiotics, and (3) reduction in the intracellular antibiotic concentration through the activation of flow pumps or due to the change in the cell wall permeability [19].

The main mechanism of resistance to antibiotics presented by LAB has been related with multidrug-resistant (MDR) efflux pumps involved in the expulsion of structurally unrelated compounds [31, 36]. Wachter-Rodarte et al. [37] analyzed LAB isolated from pozol (a traditional fermented maize beverage), identifying that MDR strains such as *Lactococcus lactis* and *Lactobacillus plantarum* present active efflux pumps, including the chromosomally encoded ABC type with the LmrA transporter (*lmrA* gene). On the other hand, Poelarends et al. [38] demonstrated that the presence of the LmrA transporter in *Lactococcus lactis* is associated with the innate resistance of 17 up to 21 clinically relevant antibiotics, including aminoglycosides (kanamycin and gentamicin), lincosamines (clindamycin), macrolides (erythromycin), quinolones (ciprofloxacin), and tetracyclines. Other authors such as Casado Muñoz et al. [39] reported that *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from fermented olives are resistant to cephalosporins, streptomycin, and kanamycin due to the

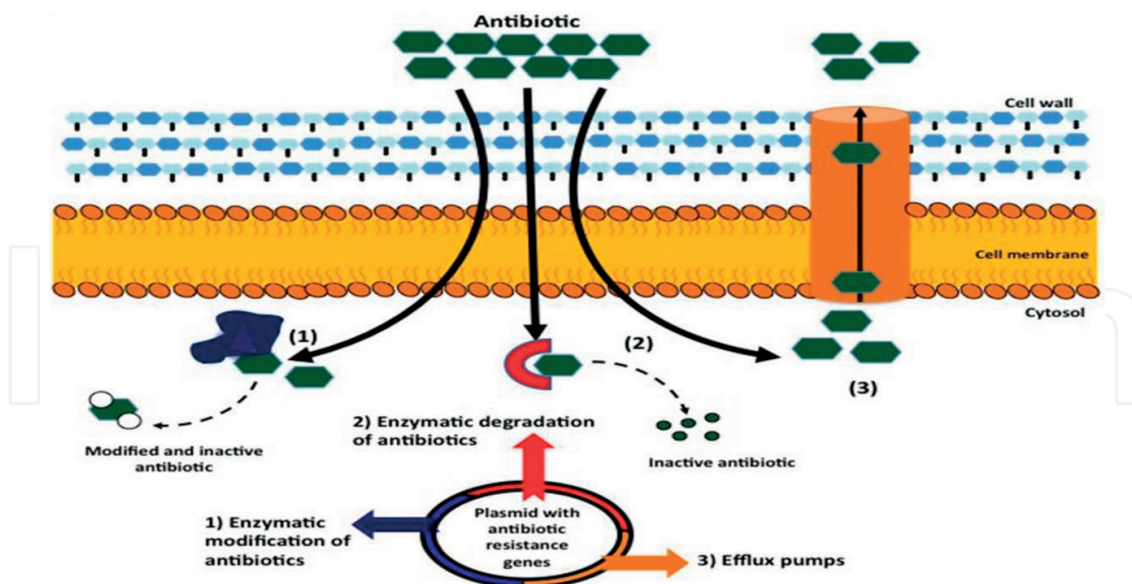


Figure 1. Mechanisms of antibiotic resistance in the LAB: (1) enzymatic modification, (2) enzymatic degradation, and (3) enzyme efflux pumps. Adapted from Sharma et al. [19].

variation of the cell wall permeability as their main mechanism of resistance; they also pointed that both strains presented a complex AcrAB-TolC system involved in MDR efflux pumps for β -lactams, fluoroquinolones, chloramphenicol, tetracycline, and other genes related with chromosomally encoded superfamily pumps *norA* and *Mde* that confer resistance to chloramphenicol and fluoroquinolones.

The resistance to aminoglycosides in LAB has not been reported, although in recent years LAB isolated from farm origin show resistant to gentamicin, kanamycin, and streptomycin, whose resistance mechanism is associated to impaired transport or enzymatic inactivation by three main aminoglycoside-modifying enzymes (AMEs) as N-acetyltransferases (AACs), O-phosphotransferases (APHs), and O-nucleotidyltransferases (ANTs) encoded by MGEs (mobile genetic elements) like transposons and insertion sequences [40].

Some bacteria belonging to the genera *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Bifidobacterium* present both intrinsic or innate and extrinsic or acquired AR, which can be a factor of food safety as they can spread resistance to other bacteria by vertical (between species) or horizontal transference (between bacterial genera) [25, 29, 31, 41].

3.1.1. Intrinsic resistance

Intrinsic resistance is the natural or innate ability of a bacterium to survive the effect of antibiotics, as a result of mutations derived from changes in the bacterial physiological state or by the uncontrolled exposure to antibiotics [42]. Intrinsic resistance has a minimum propagation potential between bacterial genera, as resistance genes are located into the chromosome with a limited transference to other genus, which represents a low risk within nonpathogenic bacteria. Any gene responsible for intrinsic resistance could be disseminated and transferred to other bacteria if it is flanked by insertion sequences that may promote its mobilization [12]. For instance, *Bifidobacterium* strains are commonly used as starter cultures and/or prebiotics

in traditional and industrialized fermented foods although they have intrinsic resistance to quinolones (ciprofloxacin and nalidixic acid), mupirocin, tetracyclines, and aminoglycosides such as streptomycin; however, all the genes are located in the chromosome with a limited transference to other genus [28, 43]. It has been reported that some LAB genera have intrinsic resistance to bacitracin, vancomycin, kanamycin, teicoplanin, and quinolones [28]. This intrinsic resistance mechanisms presented by LAB include:

- Modification of the cell wall, commonly observed in the resistance to glycopeptides (vancomycin and teicoplanin) and non-ribosomal antibiotics (bacitracin). In particular, *Lactobacillus plantarum* and *Enterococcus faecium* present innate resistance to vancomycin, due to the substitution of D-alanine residues of the muramyl pentapeptide cell wall by D-lactate (high-level resistance) or D-serine (low-level resistance) in the chemical structure of the peptidoglycan, thus avoiding the antibiotic interaction [35, 41, 44].
- Enzymatic inactivation such as for aminoglycosides (neomycin, kanamycin, streptomycin) or quinolones (ciprofloxacin, norfloxacin, nalidixic acid) prevents the binding of these antibiotics with their specific targets, as observed for *Lactobacillus* and *Enterococcus* for the 16S rRNA of the 30S ribosomal bacterial subunit and DNA gyrase, respectively, that explains the intrinsic resistance to both groups of antibiotics [29, 40].

3.1.2. Extrinsic resistance

Extrinsic or acquired resistance is one in which bacteria can incorporate into their cellular structure mobile genetic material capable of conferring resistance to certain antibiotics. Unlike intrinsic resistance, the acquired resistance is only found in some traits or bacterial subpopulations. The gene propagation may occur between bacteria of different genera or between different organisms. The horizontal gene transfer (HGT) occurs when the bacteria is capable of acquiring new genes that can increase their intrinsic resistance spectrum, or they can transfer resistance to other microorganisms or directly to humans or animals, which is already considered a health risk, according to the WHO. Therefore, the protocols for the analysis of resistance genes in LAB are increasing as they have a high capacity to acquire AR and since they have a close relationship with food processing [6, 19, 31, 45, 46]. **Figure 2** shows the three main mechanisms of HGT; some of which are not considered relevant in the transfer of resistance to antibiotics in LAB, for example, transduction (through bacteriophages) and transformation (when DNA is released from one bacterium and is absorbed by another), as the conjugation is the primary mechanism observed in lactic acid bacteria [12, 19, 47, 48].

The conjugation is the transfer of mobile genetic material from plasmids or transposons through a tube of proteins, called sexual pilus [6]. Plasmids are extrachromosomal DNA molecules capable of autonomous replication and that may confer resistance to microorganisms against antibiotics and represent one of the main mobile elements for dissemination of antibiotic-resistant genes against β -lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides, and quinolones [29, 47, 48].

Plasmids have a large number of genetic determinants that may confer resistance by conjugation, and it is important to consider that a single bacterium can have multiple plasmids [49].

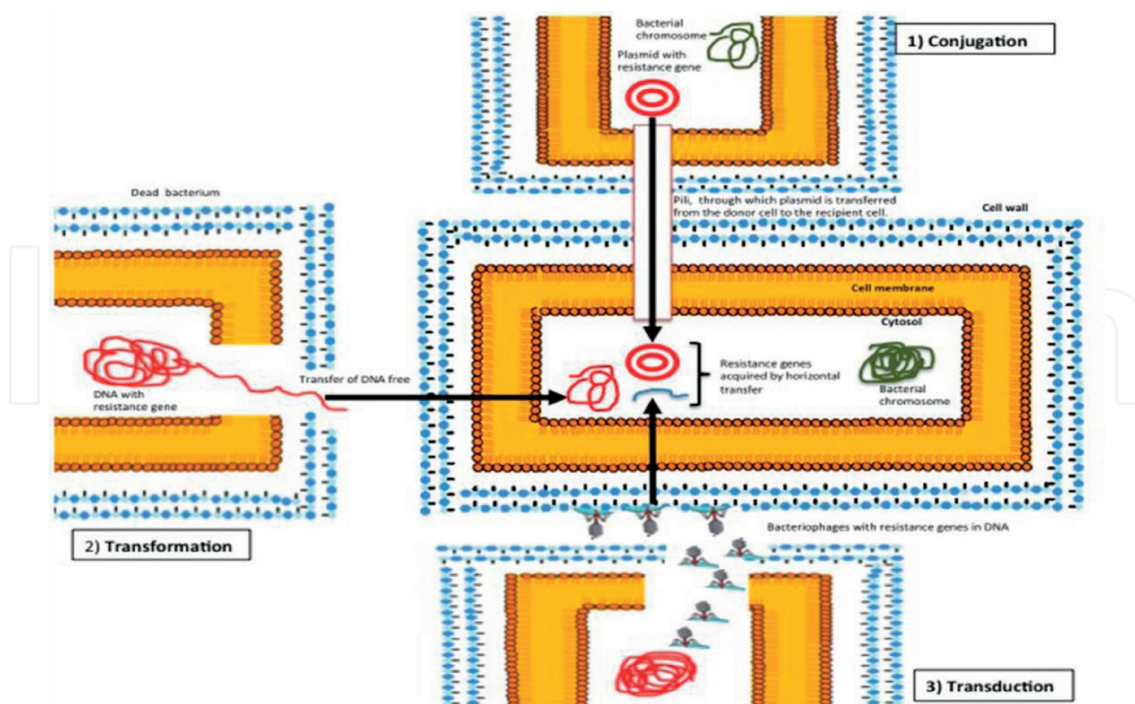


Figure 2. Mechanisms of horizontal gene transfer in the LAB. (1) conjugation is a process requiring the cell to cell contact via cell surface pili; (2) cell transformation by integrating extracellular DNA; (3) transduction, bacteriophages may transfer bacterial DNA from a previously infected donor cell to the recipient cell. Adapted from Sharma et al. [19] and Von Wintersdorff et al. [47].

Some authors indicate that the genetic diversity of resistance is proportional to the number of plasmids present in the environment, without forgetting that there are other mobile elements such as transposons and integrons, although these elements do not self-replicate and must be transported by an appropriate plasmid or phage [49, 50]. Some conjugative transposons used as vehicle of antibiotic resistance genes in LAB include Tn916, Tn918, Tn920, Tn925, Tn2702 (*E. faecalis*), Tn5233 (*E. faecium*), Tn5276, and Tn5301 (*Lactococcus lactis*) [19].

3.2. Resistance to antibiotics in LAB

As mentioned, the presence of resistance genes in LAB is considered a public health problem, so the EFSA through the panel of additives and products or substances used in animal feed (FEEDAP) developed a technical guide to identify the bacteria that show acquired resistance to antibiotics such as ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracyclines, and chloramphenicol [51]. Most LAB that present acquired resistance in the food production chain include the obligate homofermentative *Lactobacillus* genera (*L. helveticus*, *L. acidophilus*, *L. delbrueckii*), obligate heterofermentative *Lactobacillus* (*L. reuteri*, *L. fermentum*), heterofermentative *Lactobacillus* facultative (*L. plantarum*, *L. rhamnosus*, *L. paracasei*), *Lactococcus lactis*, *Streptococcus thermophilus*, *Pediococcus* spp., *Leuconostoc* spp., and *Enterococcus* spp. [31, 51]. On the other hand, LAB can be incorporated into food in the form of probiotic or starter cultures or they can be part of the natural microbiota of traditional fermented foods, but some authors have found that the vast majority of these bacteria are resistant to antibiotics [6, 19, 31, 40, 45]. **Table 2** shows some AR LAB isolated from traditional fermented foods, industrialized and probiotic recommended for improving the intestinal microbiota [20, 37, 49, 50].

In particular, the enterococcal and *Lactobacillus* genera may be associated to a health risk, as they carry innate and acquired resistance genes and because of their high residence in food and in the gastrointestinal microbiome of humans and animals [28, 36].

3.2.1. Enterococcus

Enterococci are widely distributed in vegetables, dairy products, prepared foods, and meat products and used as probiotics; however, they have intrinsic resistance to a large number of antibiotics such as β -lactams and aminoglycosides. In some cases, they can present profiles of resistance similar to enterococci considered nosocomial emergent pathogens which could present multiple drug resistance (MDR) with mechanisms of resistance that include modification of pharmacological targets, inactivation of therapeutic agents, overexpression of efflux pumps, and sophisticated adaptive response of cell envelope that promotes survival in the human host [41, 52].

Lactic acid bacteria	Antibiotic resistance*	Food
<i>Lactobacillus sakei</i> <i>Lactobacillus curvatus</i> <i>Leuconostoc mesenteroides</i>	Vancomycin, gentamicin, ampicillin erythromycin and tetracyclines.	Chorizo, fuet and sausage
<i>Lactobacillus sakei</i> <i>Pediococcus pentosaceus</i> <i>Lactobacillus plantarum</i> <i>Lactobacillus paraplantarum</i>	Streptomycin, gentamicin and tetracyclines.	Italian sausage
<i>Lactobacillus sakei</i> <i>Lactobacillus plantarum</i>	Chloramphenicol, quinupristin-dalfopristin, lincomycin, erythromycin (<i>ermA</i> , <i>ermB</i> and <i>ermC</i>), rifampicin, tetracycline (<i>tetM</i> , <i>tetO</i> , <i>tetS</i> , <i>tetW</i> , <i>tetK</i> and <i>tetL</i> genes), gentamicin, vancomycin and penicillin.	Traditional dry fermented sausages from the south Portugal
<i>Enterococcus faecium</i> <i>Enterococcus faecalis</i> <i>Lactobacillus reuteri</i> <i>Lactobacillus acidophilus</i> <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> <i>Lactobacillus johnsonii</i> <i>Lactobacillus plantarum</i>	Gentamicin, tetracycline (<i>tetM</i>), clindamycin, vancomycin (<i>vanA</i>), chloramphenicol (<i>cat gen</i>), ciprofloxacin, penicillin and nitrofurantoin.	Sausages
<i>Lactococcus lactis</i> K214	Chloramphenicol, streptomycin and tetracycline (<i>tetS</i> and <i>tetM</i>), erythromycin (<i>ermT</i>).	Raw milk cheese
<i>Streptococcus thermophilus</i>	Tetracyclines (<i>tetS</i>), erythromycin (<i>ermB</i>), clindamycin, streptomycin and neomycin.	Raw milk
<i>Lactobacillus pentosus</i> <i>Leuconostoc pseudomesenteroides</i>	Amoxicillin, ampicillin, chloramphenicol, gentamicin, erythromycin, streptomycin, vancomycin and teicoplanin.	Green olives
<i>Bifidobacterium</i> spp. <i>Lactobacillus</i> spp. <i>Streptococcus thermophilus</i>	Vancomycin, streptomycin, aztreonam, gentamicin, ciprofloxacin, gentamicin, and ciprofloxacin.	Commercial probiotics

*Italic letters indicate the resistance genes identified by Polymerase Chain Reaction (PCR).

Table 2. Lactic acid bacteria resistant to antibiotics isolated from food [20, 37, 49, 50].

Streptomycin was the first aminoglycoside reported for which resistance appeared in enterococcal strains (concentrations higher than 2000 µg/mL); this resistance is carried out by adenylation of streptomycin, by the action of the enzyme streptomycin adenylyltransferase, encoded by the *aadA* gene [35, 41]. Resistance to gentamicin, kanamycin, neomycin, and netilmicin (aminoglycosides as well) is mainly due to the production of the bifunctional enzyme 2'-phosphotransferase-6'-acetyltransferase, which promotes the ATP-dependent phosphorylation of aminoglycosides [41].

Strains of enterococci of clinical origin between 60 and 65% exhibit resistance to tetracyclines, although these antibiotics are not routinely used in the treatment of infections caused by these microorganisms. There are two fundamental mechanisms of resistance to tetracyclines in enterococci: flow pumps and protection of the ribosome, thus preventing the binding of the antibiotic. The *tetK* and *tetL* genes code for proteins associated to flow pumps responsible to remove the antibiotic outside of the cell, while the *tetM*, *tetO*, and *tetS* genes code for proteins that provide resistance to tetracyclines for ribosome protection. The *tetL* and *tetM* genes are the most frequent in the chromosome and mobile determinants [41, 52, 53]. Finally, vancomycin (glycopeptide) is the main cause of concern, since this antibiotic is considered at the last option for antibiotic therapy for the treatment of Gram-positive bacteria. The resistance to vancomycin in enterococci is varied, having described six genotypes called *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*, where the genotype *vanA* is more frequent in the *Enterococcus* genus [41].

3.2.2. *Lactobacillus*

In general, *Lactobacilli* have a high natural resistance to vancomycin, bacitracin, cefoxitin, metronidazole, nitrofurantoin, and sulfadiazine, as well as antibiotics that inhibit the synthesis of proteins such as chloramphenicol, erythromycin, quinupristin/dalfopristin, lincomycin, clindamycin, and tetracyclines [45]. Guo et al. [54] observed 85% of incidence of vancomycin resistance in food isolated *Lactobacillus* strains, especially in *Lactobacillus plantarum* and *Lactobacillus casei*, with the lower frequency for *Lactobacillus helveticus*, but these resistances are not transferable, as genes are located in the chromosome [54]. In addition, genes that code for resistance to tetracycline and erythromycin have been detected in different *Lactobacillus* species isolated of probiotics and foods [12, 31, 55].

The genus *Lactobacillus* is an excellent receptor for exogenous genes by conjugation, as demonstrated by Abriouel et al. [45] for the conjugative pAMβ1 plasmid found in *Lactobacillus plantarum* that could be obtained from enterococci and streptococci. *Lactobacillus* are commonly susceptible to antibiotics, such as penicillins (ampicillin, oxacillin, and piperacillin), inhibitors of β-lactamase, and cephalosporins (cephalothin and cefuroxime, ceftriaxone and cefoxitin), but in recent years some authors have reported resistance to penicillin G in some strains of *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, and *Lactobacillus plantarum* [45, 56]. Other studies demonstrated that *Lactobacillus rhamnosus* is safe to use as a starter or probiotic culture, despite having resistance genes to vancomycin, as these resistance is encoded into the chromosome [45, 48, 54].

3.3. Horizontal transfer of LAB to the intestinal microbiota

The horizontal gene transfer (HGT) involves the gene interchange between different bacteria through mobile DNA elements such as plasmids, conjugative transposons, integrons, and

bacteriophages [27, 47–49]. The transfer of resistance genes by HGT initiates from the farm animals that were treated with antibiotics used as growth promoters to prevent diseases, but these uncontrolled treatments may induce resistance in their intestinal microbiota; later this biota can reach foods and finally being transferred to the human [3]. Conjugation in food matrices has been reported from commensal bacterium (*Enterococcus faecalis* and *Lactococcus lactis*) to potentially pathogenic strains (*Listeria spp.*, *Salmonella spp.*, *Staphylococcus aureus*, and *E. coli*) in fermented milk [25, 27]. Also, the transfer of tetracycline resistance genes among LAB has been reported in fermented milk and fermented sausages [27]. Martínez and Baquero [34] report the HGT of tetracycline and vancomycin resistance genes in *Enterococcus faecalis* during the fermentation process of cheese and sausages. Bonham et al. [30] have demonstrated that aged cheeses contain AR *Lactobacillus* and *Lactococcus* that acquired the resistance through HGT induced by the strong condition of microbial selection during the food production and maturation process.

A wide diversity of AR species can be found in the human gastrointestinal tract that could be acquired AR genes by HGT; this fact is related to the metagenomic comparison showing that most resistance genes found in the human microbiome are those associated with approved antibiotics used in livestock, which supported the hypothesis that resistance genes can be transferred from the farm to consumers [48]. Therefore, the WHO indicates that the HGT genes can be a significant health problem, as most antibiotic resistance is acquired through the HGT [1].

4. Regulation of the use of LAB

The FDA categorizes microorganisms with the GRAS distinction after being evaluated in general aspects of safety, taxonomy, potential to produce pathogenicity toxins, resistance to antibiotics, and the historical background of food safety. LAB have a broad history of use in fermented foods and usually recognized as safe. However, the dissemination of AR genes puts the GRAS category in another context, especially for bacteria that present mobile genes of transfer such as *Lactobacillus*, since in the US there are still no guidelines that contemplate the type of resistance in microorganism used in food processing [57]. On the other hand, the EU commission regulates the safety of LAB used as starter or probiotic cultures in the European continent, through the EFSA that establishes guidelines for assigning qualified presumption of safety quality to the organisms since 2003. As previously mentioned, the term QPS is based on reasonable and qualified evidence to allow certain restrictions and may be analogous to the GRAS concept but with more rigid guidelines in which the reliable safety of the bacteria is verified, making clear the phrase “from farm to fork” [58]. The QPS status is given to a bacterium, by the EFSA BIOHAZ Panel (Biological Hazards) that must take into account the following aspects (**Figure 3**): (1) the identity of the taxonomic unit at the genus level; (2) documentation related to the LAB safety, based on scientific evidence and history of use; (3) pathogenicity, in which it is evaluated if any species of the genus has pathogenicity factors, if the information is available, the pathogenic strains are excluded; and (4) knowledge of the final use of the microorganism, identifying if the bacteria is part of the food chain or if it is used to produce other products [6, 58].

The list of QPS includes species of *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus*

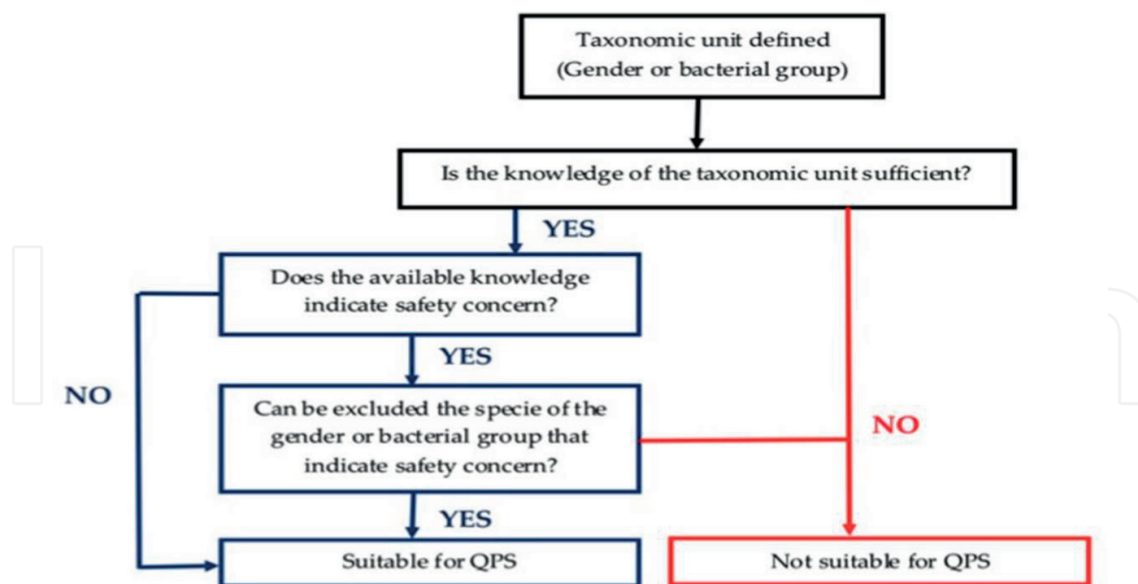


Figure 3. Scheme for assessing the suitability for qualified presumption of safety (QPS) status of a BAL. adapted from Laulund et al. [58].

alimentarius, *Leuconostoc lactis*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Pediococcus acidilactici*, *Pediococcus dextrinicus*, *Pediococcus pentosaceus*, *Lactococcus lactis*, and *Streptococcus thermophilus* [6]. In the case of *Enterococcus*, the QPS category cannot be assigned to all species; each specie must be individually analyzed [6].

5. Methods to identify antibiotic-resistant LAB

Most widely used antibiotic susceptibility testing methods are based on (1) phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the tested antibiotic and (2) molecular identification of resistant genotypes through polymerase chain reaction (PCR) [21, 25, 29, 39, 54]. The evaluation of phenotypic susceptibility to antibiotics in lactic acid bacteria should be done using recognized methods that allow the identification of the minimum inhibitory concentration (MIC) for the most commonly used antibiotics. Most LAB species used in food can be evaluated by the method described in ISO 10932: 2010 [59], considering the conditions and culture media for *Bifidobacteria* and LAB that do not belong to the genus enterococci [56, 57]. In case of having strains of *Enterococcus*, it is recommended to use the methods described by the Clinical and Laboratory Standards Institute [21, 60]. Some of the recommended methods to determine the MIC in LAB are the E-test, the Kirby-Bauer test (diffusion method), and the broth microdilution method (MDIL) [43]. In particular the cutoff values are known for the genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, and *Bifidobacteria*. The MDIL method is widely used to evaluate MIC for a large number of strains and antibiotics, although the method has some limitations, especially for those antibiotics for which a strain could quickly acquire resistance [43]. However, MIC evaluation in LAB is somewhat inconsistent among the researchers, mainly due to the lack of culture media that can ensure proper growth of LAB without interfering with the assay results. Therefore,

a complementary technique involves the search for AR genes using PCR techniques and microarrays [25, 29, 54]. Also, identifying the location of these genes allows to determine their potential transfer, while their sequencing can provide evidence of their bacterial taxa and identity of the genes, which helps to trace the origin of their genomes [29].

Functional metagenomics is an important approach in the investigation of antibiotic resistance genes (ARG) since it can be used to identify and characterize new ARG, including those not previously associated with antibiotic resistance [48, 61]. It is also one of the most recent techniques in the study of resistance in pure bacterial groups or more complex samples such as food; some works reported in the literature indicate the wide diversity of resistance systems that are present in food, considering the cultivable and not cultivable bacteria. Metagenomic studies help to understand the mechanisms of resistance in such a way that it allows direct applications in the identification of new drugs and the synthesis of novel and active antibiotic molecules [61].

5.1. Procedure to evaluate LAB resistant to antibiotic used in food

The FEEDAP Panel proposed a scheme to evaluate the resistance present in lactic acid bacteria that can be used as probiotic or starter cultures in food processing; as previously mentioned, it is essential to distinguish between the intrinsic and acquired resistance as part of the food safety of lactic acid bacteria [58, 62]. The correct identification of the bacteria (sequencing and comparison of the 16S rDNA gene in international databases) by molecular taxonomy is essential to evaluate the type of resistance, since the intrinsic resistance is specific for a specie or genus. Once the specie under study has been identified, the MIC (minimum inhibitory concentration)

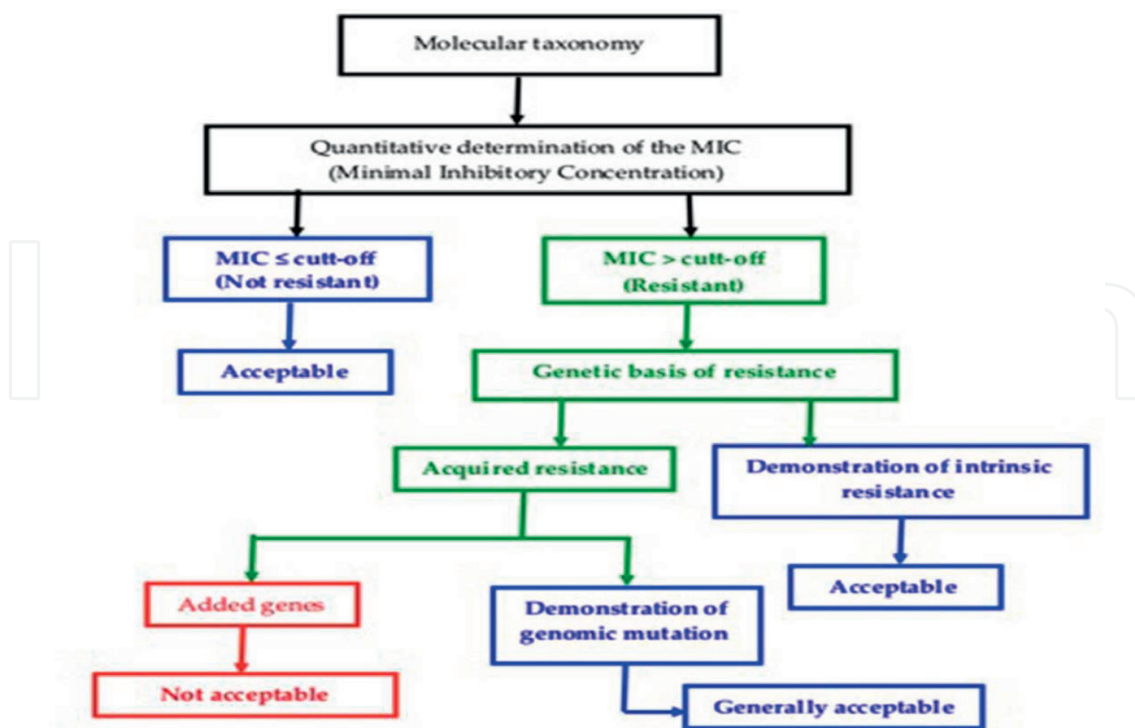


Figure 4. Proposed scheme for the antibiotic resistance assessment of lactic acid bacteria used as probiotic and starter culture. Adapted from Laulund et al. [58] and EFSA [62].

in which the LAB is sensitive to the antibiotic analyzed is determined. The bacterium can be considered safe when the MIC is lower than the cutoff level ($MIC < \text{cutoff}$). On the other hand, if the MIC value is above the cutoff value ($MIC > \text{cutoff}$), the bacterium is considered resistant to the antibiotic, and its resistance should be confirmed by molecular methods as PCR [39, 54, 62]. However, the resistance genes not always are expressed but can be transferred to other bacteria if the environmental conditions stimulate the expression of these genes [34]. If the bacteria have intrinsic resistance, it is considered acceptable for use in food. Otherwise, it must be demonstrated whether the acquired resistance is in mobile genetic material or was acquired in the process of mutation in the bacterial chromosome (also acceptable for use in foods). Finally, the bacteria are not accepted by any regulatory body for its application in food if it is demonstrated that the resistance is exogenous and easily transferable (**Figure 4**).

6. Conclusion

LAB are of great importance in the food industry for the preparation of fermented foods, in addition to being widely used as probiotics to regulate the intestinal microbiota in animals and humans. However, it is important to carry out the appropriate tests to identify the presence of antibiotic resistance genes that can be transferred horizontally to other microorganisms, whether pathogenic or those present in the gastrointestinal microbiota, which can cause a health problem because of the continuous exposure to the environmental conditions that favor the resistance spread that threatens the public health and the food production.

Conflict of interest

“No conflict of interest declared.”

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