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ANTIMICROBIAL RESISTANCE OF LACTIC ACID BACTERIA IN FERMENTED FOOD

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Abstract

Lactic acid bacteria (LAB) have a long history of safe use in fermented food production and consumption that support their generally recognized as safe (GRAS) and qualified presumption of safety (QPS) status provided by US Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), respectively. FDA Antimicrobial resistance (AMR) is one of the leading public health issues which is closely related to farm animals, environment and food of animal origin.

Safety aspects of microbiota related to fermented food products are evaluated during last decades. Resistance in LAB is enhanced by the large numbers of LAB in fermented products and in the gastro-intestinal tract, but also from other bacteria in the environment. Once a LAB becomes resistant, amplified determinant can be transmitted to another host. Therefore, checking for signs of transferable antibiotic resistance in starter strains and bacteria used as feed and food additives is essential. The determination of the antibiotic resistance profiles of LAB is mostly based on the use of numerous phenotypic methods. However, there is no consensus on breakpoints for most antimicrobials. The confusion in this area is primarily due to the fact that different methods are used to define resistance (E-test, determination of minimum inhibitory concentration (MIC), disk diffusion or Kirby-Bauer method, and microdilution), thus preventing direct comparison of results. Phenotypic assays have now been complemented by molecular methods in which bacterial strains are directly screened for the presence of antibiotic resistance determinants. Generally, these methods include amplification by polymerase chain reaction (PCR) with specific primers for single or multiplex antibiotic resistance genes, real time PCR or the use of DNA microarrays containing large collections of antibiotic resistance genes. The existing genetic studies used to confirm the transmission of known resistance determinants are hampered by many experimental factors and thus show variable results. Two of the most commonly observed resistance genes in LAB found so far are tet(M) for tetracycline resistance and erm(B) for erythromycin, followed with cat genes coding for chloramphenicol resistance. The complex issue of AMR requires a wide multidisciplinary approach to predict and avoid the undesirable public health consequences along the whole food-producing chain.

Strategies for reduction of AMR in fermented food microbiota should be based on prudent use of antimicrobials in food animals and application of competitive starter cultures in food fermentation.

***Key words**: Antimicrobial resistance, Lactic acid bacteria, Fermented food, Phenotypic and genotypic methods, Veterinary public health.*

1. Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria comprising about 20 genera within the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*. The capacity of LAB to ferment sugars to lactic acid is the basic principle underlying their use in the production of fermented foods. However, apart from lactic acid, other metabolic products of LAB have positive physiological properties in terms of extending the shelf life of finished products and affecting their sensory attributes, as well as in terms of inhibiting the growth and multiplication of pathogenic and spoilage microorganisms (Vesković and Đukić, [1]). LAB play a

recognized role in fermented foods preservation and safety, thus promoting final products microbial stability. The preservation ability of LAB is based on competition for nutrients and the production of antimicrobial active metabolites such as organic acids (mainly lactic acid and acetic acid), hydrogen peroxide and peptidic compounds (bacteriocins) (Vesković and Đukić, [1]; Vesković Moračanin *et al.*, [2]). Today, the huge global food and beverage market offers a varied range of commercial fermented products obtained through lactic acid fermentation (meat, milk, vegetable, fruit and bakery industries etc.) carried out by certain LAB species commonly including species within the genera: *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Lactococcus* and *Weissella* (Fontana *et al.*, [3]). LAB have been used all over the world in various traditional and industrial food fermentations. The traditional production of fermented foods relies on the activity of "wild" microflora, with lactic acid fermentation taking place as a spontaneous uncontrolled process, thus resulting in non-uniform product quality with no pronounced intensity of sensory attributes. In contrast, increased demand for these products, the need for a standardized cost-effective production, and safe food production have triggered the use of active LAB starter cultures.

Apart from their dominant presence in fermented food products, these bacteria are widely found in nature, including the gastrointestinal and urogenital tracts of humans and animals (Carr *et al.*, [4]). Further, certain LAB are also used as probiotics added to confer health benefits to consumers (Ouwehand *et al.*, [5]) or to improve animal production (Brashears *et al.*, [6]). In this respect, lactic acid bacteria species are economically very important to the food and feed industry. LAB have a long history of safe use in fermented food production and consumption that support their GRAS (generally recognized as safe) and QPS (qualified presumption of safety) status provided by FDA and EFSA, respectively.

Antimicrobial resistance (AMR) is one of the leading public health issues which is closely related to farm animals, environment and food of animal origin (Zdolec *et al.*, [7]). According to the European Commission (EC, [8]), has been estimated that somewhere from one to ten million tons of antibiotics have been released into the biosphere over the last 60 years. Testing of antibiotic resistance of LAB has not been extensively investigated until recently, in contrast to pathogenic species (Resch *et al.*, [9]; EFSA, [10]). However, interest in LAB and their antimicrobial resistances has increased in recent years, due to the observed horizontal transmission of antibiotic resistant determinants that can be transferred between bacterial species and also from beneficial bacteria to pathogens (Devirgiliis *et al.*, [11]).

In general, low occurrence of hazards determinants was found in food LAB, however the transfer of antimicrobial resistance should be more studied *in vitro* and *in vivo*. In any case, the absence of mobile AMR determinants in LAB must be the prerequisite for introducing LAB strains in fermented food production as starter cultures (Talon and Leroy, [12]). Therefore, in addition to the need for their complete physiological and technological characterization, the demand to test LAB starter and probiotic strains for transmissible AMR is fully justified. It is vital to clearly define limit values used to classify strains as resistant or susceptible. Testing is particularly important in view of the results that contribute to distinguishing between natural (non-specific, non-transmissible) and acquired resistance using a procedure that involves comparison of antimicrobial resistance profiles for a large number of LAB originating from different sources (Teuber *et al.*, [13]).

Antimicrobial drug resistance profiles in bacteria as commensal microorganisms in an ecosystem, such as LAB, serve as indicators of the selective pressure placed on these microorganisms under habitat contamination with antimicrobial agents. During the production process, raw milk and meat can frequently be contaminated with materials containing LAB resistant to antimicrobials. This is how resistance genes are transferred to end products, primarily raw-milk cheeses or fermented sausages.

2. Antimicrobial resistance (AMR)

2.1. AMR - definition and types

AMR is the result of interactions between the microbial cell, its environment and the antimicrobial agent. In general, antimicrobial resistance is the capacity of a microorganism to resist the growth inhibitory or killing activity of an antimicrobial beyond the normal susceptibility of the specific bacterial species (Acar and Röstel, [14]; Mathur and Singh, [15]). AMR in bacteria is the ability of bacteria to survive and grow in the presence of chemical molecules that would normally kill them or limit their growth. In other words, resistance in microorganisms is their acquired trait resulting from adaptation to changing environmental conditions (Rodriguez-Rojas *et al.*, [16]).

Since the discovery of penicillin in 1928 and the introduction of antimicrobial substances (sulfonamides) in the 1930s (Davies and Davies, [17]), antibiotics have played a vital role in protecting human health and extending the human life span. The optimism of the early period of their discovery has been tempered very soon by the emergence of antibiotic-resistant strains of bacteria. However, the interaction between antibiotics and microorganisms is not due to the introduction of antibiotics into medical practice, but rather it is a common relationship whose existence dates back to the time microorganisms first appeared in nature. Given that antibiotics are basically substances of microbial origin secreted by microorganisms to protect against

other types of microorganisms in their environment, antibiotic resistance has therefore existed from time immemorial. What has changed over time is the fact that the massive use of natural and synthetic antibiotics for therapeutic and prophylactic purposes has led to an intense selective pressure on microorganisms and the rapid formation of their new mechanisms of resistance. Bacteria have changed their genetic basis due to rapid multiplication, higher mutation rates and resistance gene acquisition, thus causing the selection and expansion of resistant strains.

In recent years, the use of antibiotics in human medicine has significantly increased (a 36% increase between 2000 and 2010), mostly in developing countries (Van Boeckel *et al.*, [18]). However, apart from the justified use of antibiotics, there have been frequent records of antibiotic misuse which has reduced the numbers of susceptible bacterial strains, leading to the emergence of new resistant strains. Moreover, the use of antibiotics has been on the rise in animal husbandry as well, with overuse and improper use causing the emergence of resistant species/strains in the food chain (Teale, [19]). The largest absolute increase in antibiotic use has been seen in cephalosporins, broad-spectrum penicillins and fluoroquinolones (Vesković *et al.*, [20]). In fact, the last European Surveillance of Veterinary Antimicrobial Consumption (ESVAC, [21]) report, regarding the overall sales in 2011 for 25 countries, states that the largest proportions, expressed as mg/PCU, were accounted for: tetracyclines (37%), penicillins (23%), sulfonamides (11%) and polymyxins (7%). The World Health Organization report (WHO, 2014 [22]) provided a comprehensive picture of increasing antibiotic resistance across an ever-increasing range of infectious agents and their growing threat to public health. It should also be noted that the use of antimicrobials is not restricted to animal husbandry but also occurs in horticulture, for example the use of aminoglycosides in apple growing (Teale, [19]). Therefore, antibiotic use for therapeutic, prophylactic and subtherapeutic purposes calls for an integrated approach in the fields of human medicine and animal health as well as in the environmental protection field (Janković *et al.*, [23]; Berkner *et al.*, [24]).

Today, there are more than 15 classes of antibiotics targeting major physiological and metabolic functions of bacterial cells. For instance, β-lactams (penicillin's, cephalosporin's), carbapenems, daptomycin, monobactams and glycopeptides (vancomycin and teicoplanin) inhibit cell wall synthesis; macrolides (erythromycin, clarithromycin and azithromycin), aminoglycosides (streptomycin, gentamicin, amikacin), clindamycin, chloramphenicol, oxazolidinones, streptogramins, ketolides, lincosamides and tetracycline's inhibit protein synthesis; rifampicin inhibits RNA synthesis; fluoroquinolones inhibit DNA gyrase or DNA replication; trimethoprim and sulfamethoxazole inhibit folate synthesis (Berkowitz, [25]; Levy and Marshall, [26]). Of particular concern today is the fact that none of these classes of antibiotics has avoided the development of resistance mechanisms in bacteria.

It is necessary to differentiate between natural or intrinsic and acquired (transmissible) resistance. Resistance to a given antimicrobial can be intrinsic to a bacterial species or genus (natural resistance), and it refers to the ability of a microorganism to survive in the presence of an antimicrobial agent, due to innate resistance characteristics i.e. due to the distinctive feature of a bacterial species found in all its strains. Since it is generally consistent and inheritable, intrinsic resistance is predictable.

Intrinsic resistance of a bacterial cell is presumed to present a minimal potential for horizontal spread of resistance (between different bacterial species), as was demonstrated for example with the chromosomal vancomycin resistance determinant of the *Lactobacillus rhamnosus* strain GG (Tynkkynen *et al.*, [27]). In contrast, resistance is considered to be acquired when a strain of a normally susceptible species becomes resistant to an antimicrobial drug (EC, [28]). In other words, acquired resistance is a characteristic of certain strains within the species. Acquired resistance to an antimicrobial results either from mutation in the bacterial genome or from the uptake of extra genes encoding resistance mechanism. It is not typical of most strains of certain species and is, thus, unpredictable (Gunell, [29]). Acquired antimicrobial resistance is a specific attribute of microorganisms, chiefly those whose primary habitats include environments that are regularly challenged with antibiotics (human and animal intestines) (Teuber *et al.*, [13]). Acquired resistance is considered as having a higher potential for horizontal spread of antibiotic resistance, since the resistance genes are present on mobile genetic elements (plasmids and transposons) (Devirgiliis *et al.*, [11]; Đukić *et al.*, [30]).

Antimicrobial drug resistance profiles in bacteria as commensal microorganisms in an ecosystem, such as LAB, serve as indicators of the selective pressure placed on these microorganisms under habitat contamination with antimicrobial agents. During the production process, raw milk and meat can frequently be contaminated with materials containing LAB resistant to antimicrobials. This is how resistance genes are transferred to end products, primarily raw-milk cheeses or fermented sausages.

Antibiotic resistance genes can be transferred from one bacterial cell to another (horizontal transmission of genetic material) via a number of mechanisms. The transfers of DNA by transduction (via bacteriophages) or by transformation (when DNA is released from one bacterium and taken up by another) are not believed to be relevant mechanisms of antibiotic resistance transfer (Ammor and Mayo, [31]; Đukić *et al.*, [30]).

By contrast, conjugation i.e. the direct cell-to-cell contact can potentially achieve horizontal gene transfer, as it has been shown to be a genetic information transfer mechanism with a broad host range (Courvalin, [32]). Conjugation is thought to be the major mode of transfer of antibiotic resistance genes (Salyers, [33]).

The evolution of antimicrobial resistance in microbial communities is enhanced by horizontal transfer of resistance genes over species and genus borders by conjugative plasmids, transposons, the possession of integrons and insertion elements, as well as lytic bacteriophages and prophages (Davies, [34]). Such genetic changes enhance the defence mechanism of bacteria. Resistance genes can originate from microorganisms as natural antimicrobial producers harbouring these genes for self-protection (Davies, [35]). Another potential source of resistance genes are genes whose products are involved in bacterial metabolism. Such genes may have been exposed to "smart" mutations that have changed the substrate spectrum (in mutation, the substrate is an antimicrobial in relation to previous substrates for biosynthesis or biodegradation) (Bulajić and Mijačević, [36]).

Since molecular analysis of resistance genes localized on plasmids and transposons shows identical genetic elements in humans and animals, it seems likely that food products of animal origin serve as transmission pathways for resistant bacteria i.e. antimicrobial resistance determinants. Over the last years, scientists have been seeking the answer to the question of whether commensal bacteria from food products can transfer resistance genes to intestinal bacteria in humans during their passage through the digestive tract. In certain pathogenic and potentially pathogenic bacteria, such as staphylococci and enterococci, the development of highly resistant bacterial clones has prompted the antimicrobial resistance crisis (Neu, [37]). In the case of vancomycin resistant enterococci, there still remains no useful antimicrobial for a successful treatment (Jett *et al.*, [38]).

2.2. Mechanisms of antibiotic resistance

During more than 60 years of global antimicrobial use, several resistance mechanisms have been identified, viz. enzymatic degradation of antibiotics, antibiotic target modification, changing the bacterial cell wall permeability and alternative pathways to escape the activity (Levy, [39]; Verraes *et al.*, [40]).

In the presence of certain resistance genes, bacteria can avoid antimicrobial agents through any of the three mechanisms:

- direct inactivation of the active molecule;
- loss of bacterial susceptibility to the antimicrobial by modification of the target of action; and
- reduction of the drug concentration that reaches the target molecule without modification of the compound itself (efflux pump) (Fraqueza, [41]).

The antibiotic defence mechanisms of intrinsic resistance are, in most of cases, related to the presence of low affinity targets, absence of targets, innate production of enzymes that inactivate the drug, inaccessibility of the drug into the bacterial cell by decreased drug uptake or extrusion by efflux of drug (Kumar and Schweizer [42]).

Bacteria that have resistance genes located on mobile genetic determinants pose a threat to public health (often referred to as "reservoirs") and enable the spread of these genes, especially if the environment contains numerous microbiota (Salyers *et al.*, [43]; Haug *et al.*, [44]). Today, many researchers have emphasized the hypothesis that commensal bacteria, primarily lactic acid bacteria, can serve as reservoirs of antibiotic resistance genes (Perreten *et al.*, [45]; Levy and Salyers, [46]). This is why the population of commensals is highly important in identifying mechanisms of persistence and spread of resistance genes in the microbial world. Accordingly, the food colonized by bacteria with transmissible antibiotic resistance genes has been specifically addressed. Antibioresistance of foodborne bacteria has aroused great interest because they may act as reservoirs for antibiotic resistance genes (Talon and Leroy [12]).

Generally, the food chain is considered a major transmission pathway for resistant bacteria between human and animal populations (Witte, [47]; von Wright, [48]). Antibiotic resistant bacteria in the food chain are commonly sustained through faecal contamination or recontamination due to incompetent or improper heat treatment during the food production process. After such a product is consumed, resistant bacteria colonize the digestive tract (Singer *et al.*, [49]).

In conclusion, the transmission of antibiotic resistance via the food chain is the same as for food borne pathogens (Figure 1).

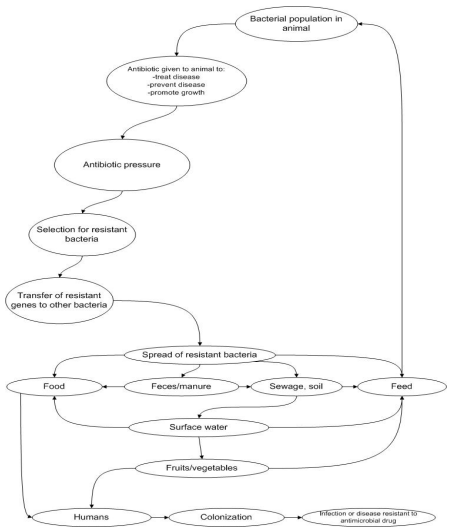

Figure 1. Possible transmission routes of antibiotic resistance bacteria from animals to humans (Modified from Khachatourians, [51]; Claycamp and Hooberman, [52])

Undisputedly, the presence of resistant strains of microorganisms isolated from food products, as either causative agents of alimentary diseases or commensal bacteria, and the potential to transfer resistant determinants to much more pathogenic species, pose a hazard. Therefore, EFSA has asked its Panel on Biological Hazards to identify, from a public health perspective, the extent to which food serves as a source of antimicrobial-resistant microorganisms or antimicrobial resistance genes, to rank the identified risk and to identify potential control options for reducing the risk (EFSA, [50]). The WHO report on global surveillance of antimicrobial resistance (WHO, [22]) claims that there is an association between antibiotic use in feed production and the emergence of resistance in mutual pathogenic microorganisms.

2.3 Antibiotic resistance in LAB

Testing of antibiotic resistance of LAB has not been extensively investigated until recently, in contrast to the situation with pathogenic species and their antibiotic resistance. However, interest in LAB and their antibiotic resistances has increased in recent years, due to the observed horizontal transmission of antibiotic resistant determinants that can be transferred between bacterial species and also from beneficial bacteria to pathogens (Devirgiliis *et al.*, [11]). The determination of antibiotic resistance of LAB as the dominant microbial community in fermented products is highly important in terms of identifying and understanding mechanisms of persistence and spread of resistance genes in the microbial world (Levy and Miller, [53]; Mathur and Singh, [15]). Therefore, in addition to the need for their complete physiological and technological characterization, the demand to test LAB starter and probiotic strains for transmissible antimicrobial resistance is fully justified. It is vital to clearly define limit values used to classify strains as resistant or susceptible. Testing is particularly important in view of the results that contribute to distinguishing between natural (non - specific, non - transmissible) and acquired resistance using a procedure that involves comparison of antimicrobial resistance profiles for a large number of LAB originating from different sources (Teuber *et al.*, [13]).

Resistance in LAB is enhanced by the large numbers of LAB in fermented products and in the gastrointestinal tract, but also from other bacteria in the environment. Once a LAB becomes resistant, amplified determinant can be transmitted to another host. Therefore, checking for signs of transferable antibiotic resistance in starter strains and bacteria used as feed and food additives is essential.

LAB possesses a broad spectrum of intrinsic and acquired antibiotic resistance (Table 1). An overview of antibiotic resistances reported together with potentially transferable resistance determinants in the food-associated LAB is given in Table 2. The antibiotic resistance profiles of *Lactobacillus*, *Lactococcus*, *Streptococcus, Pediococcus, Leuconostoc* and *Bifidobacterium* are quite different (although clear-cut species-specific patterns have not been observed) (Ammor *et al.*, [54]).

Lactobacilli are generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin and tetracycline, and more resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin) (Charteris *et al.*, [55]; Coppola *et al.*, [56]; Zhou *et al.*, [57]). However, resistant strains to these agents have also been identified (Flórez *et al.*, [58]). Resistance against aminoglycosides, such as neomycin, kanamycin, streptomycin and gentamicin has been observed more frequently among lactobacilli (Danielsen and Wind, [59]; Coppola *et al.*, [56]. Zhou *et al.*, [57]). Most common resistance genes detected in lactobacilli have been: tet(M), tet(W), tet(S) for tetracycline, and erm(B) and erm(C) for erythromycin resistance (Danielsen, [60]; Gevers *et al.*, [61]). A chloramphenicol-resistance (cat) gene has been found in many lactobacilli species of food origin, but not from lactobacilli isolated from dry-fermented sausages (Hummel *et al.*, [62]).

Table 1. Intrinsic antibiotic resistance profile of LAB (modified from Teuber *et al.*, [13])

Type of Bacteria	***Intrinsic Antibiotic Susceptibility***	***Intrinsic Antibiotic Resistance***
Bifidobacterium	Ampicillin, penicillin G, bacitracin, cephalosporin, chloramphenicol, erythromycin, clindamycin, nitrofurantoin, tetracycline	Vancomycin, gentamycin, fusidic acid, streptomycin, polymyxin B, trimethoprim, aminoglycosides, colistin, metronidazole
Lactococcus lactis	Amikacin, ampicillin, 1st generation cephalosporine, chloramphenicol, erythromycin, gentamicin, penicillin, imipenem, oxacillin, sulfonamide, tetracycline, vancomycin	Colistin, fosfomycin, pipemidic acid, rifamycin
Lactobacillus spp.	Chloramphenicol, streptomycin, gentamycin, penicillin G, tetracycline, erythromycin	Aminoglycosides, fluoroquinolones, glycopeptides, vancomycin

Bifidobacteria are usually very susceptible to Gram positive spectrum antibiotics (macrolides, bacitracin, erythromycin, lincomycin, novobiocin, teicoplanin and vancomycin), broad-spectrum antibiotics (rifampicin, spectinomycin and chloramphenicol) and beta-lactams (penicillin, ampicillin, amoxicillin, piperacillin) (Zhou *et al.*, [57]; Delgado *et al.*, [63]). Resistances - some being most likely intrinsic - exist towards: vancomycin, gentamicin, kanamycin, streptomycin, fusidic acid, trimethoprim, norfloxacin, nalidixic acid, metronidazole, polymyxin B and colistin. The mechanisms of resistances are unknown (Ammor *et al.*, [54]).

Pediococci are usually susceptible to penicillin G, imipenem, gentamicin, netilmicin, erythromycin, clindamycin, rifampin, chloramphenicol, daptomycin and ramoplanin. On the contrary, *Pediococcus* species are intrinsically resistant to glycopeptides (vancomycin and teicoplanin) and to streptomycin, kanamycin, tetracycline (especially *Pediococcus acidilactici*), doxycycline, ciprofloxacin, sulphamethoxazole and trimethoprim-sulphamethoxazole (Danielsen *et al.*, [64]); Zdolec *et al.*, [54]). In isolates *Pd. pentosaceus* isolated from fermented sausages antibiotic-resistant genes for tetracycline - tet(M) and for erythromycin - erm(B) have been detected (Federici *et al.*, [65]).

Leuconostoc species are mostly susceptible to rifampicin, chloramphenicol, erythromycin, clindamycin and tetracycline (Swenson *et al.*, 1990 [66]; Flórez *et al.*, [67]). On contrary, *Leuconostoc* spp. are resistant to glycopeptides (e.g. vancomycin), cefoxitin and metronidazole, and usually (at least partially) to nalidixic acid, gentamycin, kanamycin, streptomycin, nitrofurantoin, sulphadiazine and trimethoprim (Swenson *et al.*, [66]; Flórez *et al.*, [67]; Zdolec *et al.*, [68]) isolated from traditionally fermented sausages *Ln. mesenteroides* strain resistant to streptomycin, enrofloxacin, trimethoprim, nalidixic acid, metronidazole, gentamycin and kanamycin.

Investigated strains of *Lactococcus lactis* were sensitive to: amikacin, ampicillin, 1st generation cephalosporin, chloramphenicol, erythromycin, gentamicin, imipenem, oxacillin, penicillin, pipericillin, sulphonamide, tetracycline, trimethoprim/sulfomethoxazole, and vancomycin (De Fabrizio *et al.*, [69]). Intrinsic resistances were recorded towards colistin, fosfomycin, pipemidic acid and rifamycin. As with the lactobacilli, single strains of *L. lactis* have been shown resistant to: chloramphenicol, clindamycin, streptomycin, erythromycin and tetracycline (Flórez *et al.*, [67]). Indeed, *L. lactis* ssp. *lactis* K214, isolated from a raw-milk soft cheese, was found to contain at least three different plasmid-encoded

Table 2. Overview of antibiotic resistances reported in the food-associated LAB (Mathur and Singh, [15])

Foods	Species	Resistance
Raw meat products		
Poultry	*Lb. reuteri* G4	*cat*
Raw ground pork	*Lb. reuteri* 100-63	*erm*(T)
	Lb. plantarum caTC2R	Cm
Raw ground pork and beef	*Lb. sakei, Lb. curvatus,*	Tetracycline (69%)
	Lb. plantarum, Lb. brevis,	chloramphenicol (3%)
	Ln. mesenteroides	methicillin (85%)
Fermented products		
Raw milk soft cheese	*Lc. lactis* strain K214	*Str–tet* (S)–*cat*
Greek cheese	*Lb. acidophilus* ACA-DC 243	Penicillin
Yoghurt starter cultures	*S. thermophilus*	Neomycin, polymyxin B
	Lb. delbrueckii ssp. *bulgaricus*	
Nigerian fermented foods and beverages	*Lb. pentosus, Lb. acidophilus,*	Tetracycline (42.5%)
	Lb. casei, Lb. brevis,	Erythromycin (17.5%)
	Lb. plantarum, Lb. jensenii	Ampicillin (47.5%)
		Cloxacillin (80%)
		Penicillin (77.5%)
Fermented dry sausages	*Lactobacillus* species	Tetracycline
		Gentamicin (79%)
		Penicillin G (64%)
		Kanamycin (79%)
Turkish yoghurts	*S. thermophilus*	Vancomycin (65%)
European probiotic products	*Lb. acidophilus, Lb. rhamnosus,*	Tetracycline (26%)
	Lb. casei, Lb. johnsonnii,	Penicillin G (23%)
	Lb. plantarum, Lb. reuteri,	Erythromycin (16%)
	Lb. delbreukii spp. bulgaricus	Chloramphenicol (11%)
Others		
Maize silage	*Lb. plantarum* 5057	tet (M)

antibiotic resistance determinants (for tetracycline [tet(S)], chloramphenicol and streptomycin). Other tetracycline resistant strains have been shown to harbour the tet(M) gene (Ammor *et al.*, [54]).

Enterococci are widely distributed in the environment, and primarily are hosted in the gastrointestinal tract of humans and animals. Most important and dominant species are *Enterococcus faecalis and E. faecium* (Franz *et al.*, [70]). The presence of *E. faecalis* in foods is not always connected with faecal contamination meaning that enterococci are not considered as indicators of hygiene in food production and processing (Birollo *et al.*, [71]). The investigations suggest a high prevalence of (multiple) antibiotic resistant enterococci in foods, which nevertheless were mostly susceptible to the clinically relevant antibiotics ampicillin and vancomycin. In general, enterococci possess intrinsic antibiotic resistance to: cephalosporin's, ß-lactams, sulphonamides, and to certain levels of clindamycin and aminoglycosides, while acquired resistance exists to: chloramphenicol, erythromycin, clindamycin, aminoglycosides, tetracycline, ß-lactams, fluoroquinolones and glycopeptides (Giménez Pereira, [72]). Intrinsic resistance to ß-lactams as a general rule is not always demonstrated in food-related enterococci, as already mentioned for ampicillin (Jahan *et al.*, [73]; Zdolec *et al.*, [7]).

2.4 Phenotypic methods

The determination of the antibiotic resistance profiles of LAB is mostly based on the use of numerous phenotypic methods. However, there is no consensus on breakpoints for most antimicrobials. The confusion in this area is primarily due to the fact that different methods are used to define resistance: Etest - based on antibiotic diffusion (Danielsen and Wind, [59]), agar and broth dilution or agar dilution methods for the determination of minimum inhibitory concentration (MIC) (Herrero *et al.*, [74]; Flórez *et al.*, [67]), disk diffusion or Kirby-Bauer method (Charteris *et al.*, [55]; Gevers *et al.*, [75]), and microdilution (Kushiro *et al.*, [76]; Klein *et al.*, [77]), thus preventing direct comparison of results. The Etest (Epsilometer Testprinzip, Ellipse gradient test - AB Biodisk) is a popular quantitative technique for determining antimicrobial susceptibility. It is based on the combined concepts of in vitro dilution and diffusion tests. In the assay, there is an immediate and effective release of the antimicrobials in a continuous exponential gradient when they are applied to an agar surface (Ribeiro *et al.*, [78]). The technique is accurate and reproducible because of the stability of the antibiotics (Sader *et al.*, [79]).

Breakpoint values (minimum inhibitory concentrations - MIC of antibiotic) that discriminate between resistant

Table 3. Microbiological breakpoint values (mg/L) (EFSA, [50])

Species/group	*Ampicillin*	*Vancomycin*	*Gentamicin*	*Kanamycin*	*Streptomycin*	*Erythromycin*	*Clindamycin*	*Tetracycline*	*Chloramphenicol*
Lactobacillus obligate homofermentative[a]	1	2	*16*	*16*	*16*	*1*	*1*	*4*	*4*
Lactobacillus acidophilus group	1	*2*	*16*	*64*	*16*	*1*	*1*	*4*	*4*
Lactobacillus obligate heterofermentative[b]	*2*	*n.r.*	*16*	*32*	*64*	*1*	*1*	*8*	*4*
Lactobacillus reuteri	*2*	*n.r.*	*8*	*64*	*64*	*1*	*1*	*16*	*4*
Lactobacillus fermentum	*1*	*n.r.*	*16*	*32*	*64*	*1*	*1*	*8*	*4*
Lactobacillus facultative heterofermentative[c]	*4*	*n.r.*	*16*	*64*	*64*	*1*	*1*	*8*	*4*
Lactobacillus plantarum/pentosus	*2*	*n.r.*	*16*	*64*	*n.r.*	*1*	*1*	*32*	*8*
Lactobacillus rhamnosus	*4*	*n.r.*	*16*	*64*	*32*	*1*	*1*	*8*	*4*
Lactobacillus casei/paracasei	*4*	*n.r.*	*32*	*64*	*64*	*1*	*1*	*4*	*4*
Bifidobacterium	*2*	*2*	*64*	*n.r.*	*128*	*1*	*1*	*8*	*4*
Pedicoccus	*4*	*n.r.*	*16*	*64*	*64*	*1*	*1*	*8*	*4*
Leuconostoc	*2*	*n.r.*	*16*	*16*	*64*	*1*	*1*	*8*	*4*
Lactococcus lactis	*2*	*4*	*32*	*64*	*32*	*1*	*1*	*4*	*8*
Streptococcus thermophilus	*2*	*4*	*32*	*64*	*64*	*2*	*2*	*4*	*4*
Propionibacterium	*2*	*4*	*64*	*64*	*64*	*0.5*	*0.25*	*2*	*2*

n.r.– not required.
[a]including *Lb. delbrueckii, Lb. helveticus*
[b]including *Lb. fermentum*
[c]including the homofermentative species *Lb. salivarius.*

and susceptible strains should be clearly defined, as well as distinguishing between intrinsic (non-specific, non-transferable) and acquired, potentially transferable resistance (Ammor *et al.*, [54]). Microbiological breakpoints are set by studying the distribution of minimum inhibitory concentrations of a certain antimicrobial for a bacterial population (Olsson-Liljequist *et al.*, [80]). According to the concept, the part of the population that clearly deviates from the susceptible majority is considered resistant i.e. as having acquired and potentially transferable resistance. In Table 3, breakpoint values are given for some LAB strains, and strains with MICs higher than the breakpoints are considered as resistant (EFSA, [50]). Breakpoints used for antibiotic susceptibility profiling are harmonized in Europe by the EUCAST but unfortunately no harmonized guidelines regarding the resistance-susceptibility breakpoints for non-enterococcus LAB are available, and therefore results are not well comparable (Flórez *et al.*, [67]; Patel *et al.*, [81]).

2.5 Genotypic antibiotic resistance of LAB

Phenotypic assays have now been complemented by molecular methods in which bacterial strains are directly screened for the presence of antibiotic resistance determinants. Generally, these methods include amplification by PCR with specific primers for single or multiplex antibiotic resistance genes (Strommenger *et al.*, [82]), real time PCR (Volkmann *et al.*, [83]) or the use of DNA microarrays containing large collections of antibiotic resistance genes (Perreten *et al.*, [84]). The existing genetic studies used to confirm the transmission of known resistance determinants are hampered by many experimental factors and thus show variable results. Moreover, positive control strains for conjugation and/or transposition experiments are not available, and standard protocols for gene transfer demonstration are lacking. Two of the most commonly observed resistance genes in LAB found so far are tet(M) for tetracycline resistance and erm(B) for erythromycin, followed with cat genes coding for chloramphenicol resistance (Danielsen, [60]); Gevers *et al.*, [75]); Cataloluk and Gogebakan, [85]).

3. Conclusions

- The complex issue of antimicrobial resistance requires a wide multidisciplinary approach to predict and avoid the undesirable publichealth consequences along the whole food-producing chain.

- There have been very few systematic studies to investigate acquired antibiotic resistance in LAB of food origin. Most data exist on opportunistic pathogenic enterococci, while the number of reports on lactococci and lactobacilli is limited. However, it is recently expanding due to increased interest in probiotic lactic acid bacteria and genetic modification of LAB for different purposes.

- Nevertheless, their significance in human nutrition (probiotics) and food technology (starter cultures) should not be questioned. Strategies for reduction of AMR in fermented food microbiota should be based on prudent use of antimicrobials in food animals and application of competitive starter cultures in food fermentation.

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