Review

Antibiotic resistance in food lactic acid bacteria—a review

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Abstract

Antibiotics are a major tool utilized by the health care industry to fight bacterial infections; however, bacteria are highly adaptable creatures and are capable of developing resistance to antibiotics. Consequently, decades of antibiotic use, or rather misuse, have resulted in bacterial resistance to many modern antibiotics. This antibiotic resistance can cause significant danger and suffering for many people with common bacterial infections, those once easily treated with antibiotics. For several decades studies on selection and dissemination of antibiotic resistance have focused mainly on clinically relevant species. However, recently many investigators have speculated that commensal bacteria including lactic acid bacteria (LAB) may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens. The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. Genes conferring resistance to tetracycline, erythromycin and vancomycin have been detected and characterized in Lactococcus lactis, Enterococci and, recently, in Lactobacillus species isolated from fermented meat and milk products. A number of initiatives have been recently launched by various organizations across the globe to address the biosafety concerns of starter cultures and probiotic microorganisms. The studies can lead to better understanding of the role played by the dairy starter microorganisms in horizontal transfer of antibiotic resistance genes to intestinal microorganisms and food-associated pathogenic bacteria.

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Keywords: Antibiotic resistance; Lactic acid bacteria; Antibiotic resistance genes

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1. Introduction

About 50 years ago, antibiotics were introduced for the treatment of microbial diseases. Since then, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria. Antibiotic resistance has been shown to have occurred rarely in bacteria collected before the antibiotic era (Hughes and Datta, 1983). Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997). The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria (Levy, 1992). Scott (2002) concluded that gene transfer occurs widely in vivo between gastrointestinal tract bacteria, and between gastrointestinal tract bacteria and pathogenic bacteria, as identical resistance genes are present in diverse bacterial species from different hosts. In fact we face the frightening probability that most pathogenic bacteria that threaten human health will soon be resistant to all known antibiotics.

However, for several decades studies on the selection and dissemination of antibiotic resistance have focused mainly on clinically relevant bacterial species. The evolution of antibiotic resistant food borne pathogens has been amply documented in recent years (Witte, 1998; Ridley and Threlfall, 1998; Teuber, 1999; Teuber and Perreten, 2000; Threlfall et al., 2000; White et al., 2002). Recently many investigators have speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (Perreten et al., 1997a; Levy and Salyers, 2002) and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations (Levy and Miller, 1989). The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. Such reservoir organisms could possibly be found in various foods and food products containing high densities of non-pathogenic bacteria as a result of their natural production process.

The food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population (Witte, 1997). More specifically, fermented dairy products and fermented meats that are not heat-treated before consumption provide a vehicle for antibiotic resistant bacteria with a direct link between the animal indigenous microflora and the human gastrointestinal tract. In 1998, the Reservoirs of Antibiotic Resistance (ROAR) network project [http://www.apua.org] was set up in order to promote the studies on the selection and dissemination of non-pathogenic antibiotic resistant bacteria in humans, during food production and agricultural processes, and in the environment. Although most food-associated lactic acid bacteria (LAB) have acquired the ‘Generally Regarded As Safe’ (GRAS) status, the potential health risk, due to the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to pathogenic bacteria, has not been fully addressed.

Fermented milk products use lactic starter cultures, and these bacteria enter into our intestines in large numbers where they interact with the intestinal microflora. Commercial introduction of probiotics containing antibiotic resistance strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens. There is little information regarding the presence of antibiotic resistance genes on starter strains and their potential to transfer the resistance genes to pathogens. A number of initiatives have been recently launched across the globe to
address the biosafety concerns of starter cultures and probiotic microorganisms.

In the current report, extensive study is presented to review the distribution and origin of antibiotic resistance in LAB, the potential mechanisms of transfer and their role in the dissemination of antibiotic resistances in animal and human population.

2. Emergence and spread of antibiotic resistance

The greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens. Extensive recent reviews of the application of antibiotics in human and veterinary medicine (Levy, 1997; WHO, 1997), agriculture (National Research Council and Institute of Medicine US, 1998; MAFF, 1998; Falkiner, 1998) and aquaculture (Reilly and Kaferstein, 1997) have documented the evolution and enrichment of antibiotic resistant bacteria: the phenomenon is regularly observed upon the introduction of a new antibiotic (Levy, 1997). Development of antibiotic resistance in bacteria is mainly based on two factors, the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 1992).

Prior to discussing these two factors, a distinction between intrinsic and acquired resistance has to be made. Resistance to a given antibiotic can be intrinsic to a bacterial species or genus (inherent or natural resistance) that results in an organism’s ability to thrive in the presence of an antimicrobial agent due to an inherent characteristic of the organism. Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria. In contrast, acquired resistance is present in some strains within a species usually susceptible to the antibiotic under consideration, and might be horizontally spread among bacteria. Acquired resistance to antimicrobial agents can arise either from mutations in the bacterial genome or through the acquisition of additional genes coding for a resistance mechanism. These genetic changes alter the defensive functions of the bacteria by changing the target of the drug by changing the membrane permeability, by enzymatic inactivation of antibiotic (e.g. by β-lactamases, aminoglycoside acetyl-, nucleotidyl- and phosphoryl-transferases), by active transport of antibiotics (e.g. by membrane inserted ATP-dependent efflux systems), by target modification (e.g. methylation of 23S rRNA, mutation of aminoacid sequence of topoisomerase) (Davies, 1997), or by routing metabolic pathways around the disrupted point (Poole, 2002). Resistances are likely to have developed long before the clinical use of antibiotics. Such resistance genes may originate from the antimicrobial producers that carry resistance genes for protecting themselves from their antimicrobial products (Davies, 1997). Potentially, another origin of resistance genes may be genes of which the products play a role in the bacterial metabolism. Such genes may undergo stepwise mutations, which change the substrate spectrum from substrates of biosynthetic or biodegradative pathways to antibiotics (Davies, 1994).

Antibiotic resistance determinants may be vertically or horizontally spread in natural microbial communities. A vertical dissemination is mediated by the clonal spread of a particular resistant strain. For horizontal gene transfer in bacteria three mechanisms have been identified (Davison, 1999): natural transformation, involving the uptake and incorporation of free DNA from the extracellular medium; conjugation, a cell contact dependent DNA transfer mechanism found to occur in most bacterial genera; and transduction, a transfer mediated by bacteriophages. The evolution of antibiotic resistance in microbial communities is enhanced by the horizontal transfer of resistance genes over species and genus border by conjugative plasmids, transposons, the possession of integrons and insertion elements, as well as lytic and temperate bacteriophages (Davies, 1994). The relative contribution of these different mechanisms is unknown, but conjugation is thought to be the main mode of antibiotic resistance gene transfer (Salyers, 1995). One reason for thinking this is that many antibiotic resistance genes have been found on mobile elements like plasmids and conjugative transposons. A second reason is that conjugation allows DNA to move across genus and species lines, whereas transformation and transduction are usually restricted to within the same species.

The selective pressure imposed by the use of antimicrobial agents plays a key role in the emergence of resistant bacteria. Whenever a mixed bacterial population is exposed to antimicrobial agents, it is likely that there will be bacteria that are resistant to the respective drugs at the concentration applied. Under
selective pressure, the numbers of these will increase and some may pass their resistance genes to other members of the population (Aarestrup, 1999).

A single antibiotic may not only select for resistance to that particular drug. It can also include resistance to other structurally related compounds of the same class; e.g. resistance to tetracycline by tet (M) includes also resistance to oxytetracycline, chlorotetracycline, doxycycline and minocycline (Chopra and Roberts, 2001; Chopra, 1994). When antibiotics of different classes share the same target site, and this target site is modified by the product of a resistance gene, cross-resistance between structurally unrelated antibiotics is observed; e.g. combined resistance to macrolides, lincosamides and streptogramin B by the erm genes (Roberts et al., 1999). In addition, a number of plasmids have been identified which carry multiple resistance genes, resulting in co-transfer (Levy, 1992).

3. Antibiotic resistance in lactic acid bacteria isolated from foods

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, which excrete lactic acid as a main fermentation product into the medium. This biochemical definition associates lactic acid bacteria of different phylogenetic branches of bacterial evolution: the “low GC” taxa, e.g. Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus and the “high GC” genus Bifidobacterium (Schleifer and Ludwig, 1995). Some LAB strains from animal and human intestinal microflora have been adopted as ‘probiotic’ food supplements including Enterococcus faecium, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei subsp. rhamnosus, and several Bifidobacterium and Propionibacterium species (Tannock, 1998). Lactic acid bacteria widely used as probiotics or in starter cultures have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes in many lactic acid bacteria and other pathogenic bacteria.

In recent years, increased focus has been given to food as vehicles of antibiotic resistance genes (Perreten et al., 1997a; Franz et al., 1999; Klein et al., 2000). However, there have been very few systematic studies to investigate acquired antibiotic resistance in LAB from food. Most data exist on opportunistic pathogenic enterococci, while the number of reports on lactococci and lactobacilli is limited. Vancomycin resistant enterococci (VRE) have emerged in the last decade as a frequent cause of nosocomial infections. Of considerable concern is the possibility that VRE, selected and enriched by the use of avoparcin (with cross resistance to vancomycin) as a growth promoter in animal husbandry, are spread via the food chain (Wegener et al., 1997; Klein et al., 1998; Pavia et al., 2000; Van Den Braak et al., 1998; Giraffa and Sisto, 1997). Enterococcal food isolates (mainly Enterococcus faecalis and E. faecium) were analysed for resistances to a broader range of different antibiotics using phenotypic susceptibility testing, both in raw meat (Klein et al., 1998; Quednau et al., 1998; Knudtson and Hartman, 1993; Robrido et al., 2000; Davies and Roberts, 1999) and fermented milk and meat products (Teuber and Perreten, 2000; Franz et al., 2001; Batish and Ranganathan, 1986; Giraffa, 2002). Their data suggest a high prevalence of (multiple) antibiotic resistant enterococci in foods, which nevertheless were mostly susceptible to the clinically relevant antibiotics ampicillin and vancomycin. Butaye et al. (2000) studied the in vitro susceptibility and resistance of E. faecium strains isolated from raw poultry meat, cheese, raw pork, and preparations of cheese and raw pork to growth promoting antibacterials used in animals and antibiotics used therapeutically in humans. Resistance against bacitracin, virginiamycin, narasin and tylosin, a macrolide antibiotic, was found to be high among strains from poultry meat. Only one poultry strain showed high resistance to ampicillin. In another study enterococci isolated from Portuguese dairy products (milk and cheese) were screened for gentamicin resistance (Lopes et al., 2003). Although enterococci are generally regarded as being intrinsically resistant to low levels of gentamicin, a high-level gentamicin resistance was detected in many dairy isolates. Donabedian et al. (2003) evaluated the molecular mechanisms of gentamicin resistance in Enterococcus isolates from animals, foods and humans. It was suggested that there are similarities in gentamicin resistance in enterococci isolated from humans, retail food, and farm animals from geographically diverse areas and there is evidence of spread of gentamicin resistant enterococci from animals to humans through the food supply. Recently, Huys and coworkers (2004) have reported the prevalence of tetracycline resistance in Enterococcus isolates.
from European cheeses and studied the phenotypic and genotypic assessment of tetracycline resistance. The tetracycline resistance could be linked to the presence of tet (M) genes in Enterococcal isolates, all of which also harbored a member of the Tn916–Tn1545 conjugative transposon family. Cataloluk and Gogebaken (2004) reported the prevalence of tet (M) and erm (B) genes in majority (61.961%) of lactobacilli of human and dairy origin isolated from Turkey. The resistant strains belonged to Lb. acidophilus, Lactobacillus crispatus, Lactobacillus gasseri and Lb. plantarum. It shows that drug resistance may be acquired in the intestinal tract during passage and spread to dairy products by the hands of workers during production. An overview of antibiotic resistances reported in the other food-associated LAB is given in Table 1, which can be summarized by stating that only a limited number of papers have reported the prevalence of antibiotic resistance in mainly Lactobacillus spp. isolated from raw meat and fermented food products. A few studies have reported an overall susceptibility to antimicrobial agents (with the exception of intrinsic resistances) in strains used as meat starter cultures (Raccach et al., 1985; Holley and Blaszyk, 1997) or dairy starter cultures (Katla et al., 2001; Reinbold and Reddy, 1974).

3.1. Antibiotic resistance profiles of LAB

The knowledge of intrinsically coded resistance of LAB to common antibiotics is necessary to recognize acquired resistance traits. Enterococci are intrinsically resistant to cephalosporins and low levels of amino-

Table 1
Overview of antibiotic resistances reported in the food-associated LAB

<table>
<thead>
<tr>
<th>Foods</th>
<th>Species</th>
<th>Resistance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw meat products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poultry</td>
<td>Lb. reuteri G4</td>
<td>cat</td>
<td>Lin et al., 1996</td>
</tr>
<tr>
<td>Raw ground pork</td>
<td>Lb. reuteri 100-63</td>
<td>erm(T)</td>
<td>Tannock et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Lb. plantarum catC2R</td>
<td>Cm</td>
<td>Ahn et al., 1992</td>
</tr>
<tr>
<td>Raw ground pork and beef</td>
<td>Lb. sakei, Lb. curvatus,</td>
<td>Tetracycline (69%);</td>
<td>Vidal and Collins-Thompson,</td>
</tr>
<tr>
<td></td>
<td>Lb. plantarum, Lb. brevis,</td>
<td>chloramphenicol (3%);</td>
<td>1987</td>
</tr>
<tr>
<td></td>
<td>Leuco. mesenteroides</td>
<td>methicillin (85%)</td>
<td></td>
</tr>
<tr>
<td>Fermented products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw milk soft cheese</td>
<td>Lc. lactis strain K214</td>
<td>Str–tet (S)–cat</td>
<td>Perreten et al., 1997b</td>
</tr>
<tr>
<td>Greek cheese</td>
<td>Lb. acidophilus ACA-DC 243</td>
<td>Penicillin</td>
<td>Charteris et al., 1998</td>
</tr>
<tr>
<td>Yoghurt starter cultures</td>
<td>S. thermophilus and</td>
<td>Neomycin, polymyxin B</td>
<td>Sozzi and Smiley, 1980</td>
</tr>
<tr>
<td></td>
<td>Lb. delbrueki spp. bulgaricus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nigerian fermented foods</td>
<td>Lb. pentosus, Lb. acidophilus,</td>
<td>Tetracycline (42.5%)</td>
<td>Olukoya et al., 1993</td>
</tr>
<tr>
<td>and beverages</td>
<td>Lb. casei, Lb. brevis, Lb.</td>
<td>Erythromycin (17.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>plantarum, Lb. jensenii</td>
<td>Ampicillin (47.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cloxacillin (80%);</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>penicillin (77.5%);</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
<td>Gevers et al., 2003</td>
</tr>
<tr>
<td>Fermented dry sausages</td>
<td>Lactobacillus species</td>
<td>Gentamicin (79%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillin G (64%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kanamycin (79%)</td>
<td></td>
</tr>
<tr>
<td>Turkish yoghurts</td>
<td>S. thermophilus</td>
<td>Vancomycin (65%)</td>
<td>Aslim and Beyatli, 2004</td>
</tr>
<tr>
<td>European probiotic products</td>
<td>Lb. acidophilus, Lb. rhamnosus,</td>
<td>Tetracycline (26%)</td>
<td>Temmerman et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Lb. casei, Lb. johnsonnii,</td>
<td>Penicillin G (23%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lb. plantarum, Lb. reuteri,</td>
<td>Erythromycin (16%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lb. delbreukii spp. bulgaricus</td>
<td>Chloramphenicol (11%)</td>
<td></td>
</tr>
</tbody>
</table>

Others

Maize silage Lb. plantarum 5057 tet (M) Danielsen, 2002

glycoside and clindamycin (Teuber et al., 1999; Knudtson and Hartman, 1993). Lactobacilli, pediococci and Leuconostoc spp. have been reported to have a high natural resistance to vancomycin, a property that is useful to separate them from other Gram-positive bacteria (Hamilton-Miller and Shah, 1998; Simpson et al., 1988). Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoïn, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen and Wind, 2003). For a number of lactobacilli a very high frequency of spontaneous mutation to nitrofurazone (10-5), kanamycin and streptomycin was found (Curragh and Collins, 1992). From these data it is clear that intergenus and interspecies differences exist, and consequently identification at species level is required in order to interpret phenotypic susceptibility data. Another study was undertaken to establish the levels of susceptibility of Lactobacillus spp. to various antimicrobial agents (Danielsen and Wind, 2003) and it was shown to be species-dependant. For the following antimicrobial agents, susceptibility varied several folds between species: vancomycin, teicoplanin, tetracycline, norfloxacin, ciprofloxacin, fusidic acid, and clindamycin. The differences between the species were more subtle for the rest of the tested antimicrobials.

In a study undertaken by Temmerman et al. (2002), a total of 55 European probiotic products were evaluated with regard to the identity and the antibiotic resistance of the bacterial isolates recovered from these products. Using the disc diffusion method, antibiotic resistance among 187 isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%). Overall 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistances.

The resistance spectrum of Bifidobacterium was described by Charteris et al. (1998). The investigated (probiotics) bifidobacteria are susceptible to ampicillin, penicillin G, cephalosporin, bacitracin, chloramphenicol, erythromycin, clindamycin, nitrofurantoïn and tetracycline. 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.

The resistance profiles of enterococci (E. faecium) isolated from food or patients are widely varying, containing many acquired traits. A possible baseline is provided by E. faecium strain 68, which is used as a probiotic for man as well as for animal, and as a silage inoculant. This strain was isolated around 1920 in the pre-antibiotic area. It is susceptible to erythromycin (15 µg disc), framycitin (100 µg), streptomycin/penicillin (streptopen 35 µg), gentamicin (10 µg), penicillin, tetracycline (30 µg) and chloramphenicol (30 µg). It is intrinsically resistant to kanamycin (30 µg), streptomycin (10 µg), and oxacillin (5 µg).

Investigated strains of Lactococcus lactis were sensitive to amikacin, ampicillin, 1st generation cephalosporin, chloramphenicol, erythromycin, gentamicin, imipenem, oxacinil, penicillin, piperlicillin, sulphonamide, tetracycline, trimethoprim/sulphamethoxazole, and vancomycin (de Fabrizio et al., 1994). A slightly lowered susceptibility was observed towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin. Intrinsic resistances were recorded towards colistin, fosomycin, pipemide acid and rifamycin. Recently, multiple drug efflux proteins were discovered in Lc. lactis subsp. lactis MG1363 (van Veen and Konings, 1998), one being an ABC transporter (lmr A), and the other proton motive force dependent drug transporter (lmr P). Both are responsible for a resistance to high concentrations of ethidium bromide. The natural substrates are not known. Twenty-six strains of Lc. lactis subsp. cremoris and subsp. lactis were all resistant to trimethoprim and almost all to sulfathiazole. Resistances to gentamicin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifampin and streptomycin varied (Orberg and Sandine, 1985).

Thirty-one strains of Lactobacillus delbrueckii subsp. bulgaricus as components of yoghurt cultures showed intrinsic resistance towards mycostatin, nalidixic acid, neomycin, polymyxin B, trimethoprim, colimycin, sulfamethoxazol and sulphonamides. Susceptibilities to cloxacillin, dihydrostreptomycin, doxycycline, furadantin, novobiocin, oleandomycin, oxacillin and streptomycin were prominent while kanamycin and streptomycin susceptibilities varied (Sozzi and Smiley, 1980). Many strains of Lb. plantarum, Lb. casei, Lactobacillus salivarius, Lactobacillus leishmannii, Lb. acidophilus carry intrinsic
resistance towards vancomycin which is due to the presence of D-alanine: D-alanine ligase-related enzymes (Elisha and Courvalin, 1995). Fifteen strains of *Streptococcus thermophilus* from yoghurt cultures showed varying resistance levels to colimycin, gentamicin, kanamycin, mycostatin, nalidixic acid, neomycin, polymyxin b, trimethoprim/sulfamethoxazol, streptomycin and sulphonamides (Sozzi and Smiley, 1980). In a study by Aslim and Beyatli (2004) 34 *S. thermophilus* strains isolated from Turkish yoghurts were examined for their antibiotic resistance patterns and plasmid carriage. Most strains of *S. thermophilus* were found to be resistant to gentamicin (79%) and penicillin G (64%) and susceptible to chloramphenicol (94%) and tetracycline (88%); however, no correlation was observed between the resistance to antibiotics and the occurrence of plasmids in some strains. Recently Citak and coworkers (2004) studied the antibiotic resistance and incidence of *Enterococcus* species in Turkish white cheese. Resistance to streptomycin, erythromycin, oxacillin and vancomycin was frequently found in these enterococci, and resistance to vancomycin was found in 96.8% of *E. faecalis* isolates and 76% *E. faecium*. This examination indicated poor sanitary conditions during production and processing and a significant health risk for consumers. Furthermore, Maskell and Pead (1992) reported an increasing incidence of isolation of lactobacilli from patients in England and Wales and the detection of oflaxacin resistance in these isolates. In another study seven *Lb. acidophilus* strains were examined for their antibiotic sensitivity against various chemotherapeutic agents and all were found to be sensitive to novobiocin, chloramphenicol, ampicillin, erythromycin, oxytetracycline and chlorotetraycine, whereas all were resistant to norfloxacain and nalidixic acid (Gupta and Mittal, 1995). The authors recommended that cultures should be tested for their sensitivity toward commonly used chemotherapeutic agents to be able to survive in the gut even during antibiotic treatment.

LAB are also used as additives in animal nutrition. The safety issues concerning the use of these organisms have been addressed by various regulatory bodies in different countries. The presence of acquired antibiotic resistance factors is considered highly undesirable in Europe but of lesser issue in the USA. In the United States of America, the Food and Drug Administration (FDA) classifies certain microorganisms as Generally Recognised as Safe (GRAS). An organism or a product with a GRAS status is exempt from the statutory premarket approval requirements. An organism can be in the GRAS list either on the basis of a history of safe use. However, the European viewpoint is more stringent as the Scientific Committee on Animal Nutrition (SCAN) proposed that any listing should be qualified, allowing the general safety of the organism/group of organisms to be concluded provided that certain specifics are met. In the case of many of the live organisms currently used in the manufacture of, or added to, dairy products, this may simply be a requirement to demonstrate the absence of acquired resistance factors. It is recommended to develop a system that would allow a qualified presumption of safety (QPS). In a QPS system the safety assessment of food LAB could be limited to the presence of transmissible antibiotic resistance markers as other tests are not relevant for lactic bacteria. (European Commission, 2004) Opinion of the Scientific Committee on Animal Nutrition (SCAN) on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance was adopted on 3 July 2001 and later revised on 18 April 2002 (European Commission, 2002). According to SCAN all bacterial products intended for use as feed additives must be examined to establish the susceptibility of the component strain(s) to a relevant range of antibiotics. Such tests must be made in a consistent manner using internationally recognized and standardised methods. Determination of the minimum inhibitory concentration (MIC) of the antibiotic expressed as mg/l or μg/ml has been suggested along with breakpoints categorizing bacterial species as resistant. It has been mentioned that determination of MIC is not necessary for species designated as inherently resistant to the antibiotic. A major obstacle in proposing breakpoints for lactic acid bacteria is the general lack of relevant data. For some genera of lactic acid bacteria, such as *Lactobacillus*, there are no generally accepted standard procedures for MIC determination and information on MIC ranges is rather limited. Accordingly the breakpoints suggested by SCAN may be seen as a pragmatic response to introduce consistency in the separation of strains with acquired transferable resistance form susceptible strains. In view of the SCAN, in each case identification of an MIC value at or above...
the given breakpoints should require further investigation. The European Commission SCAN documents (EC Health and Consumer Protection) are available on the Internet site of EC.

3.2. Mobile genetic elements in LAB

A prerequisite to acquire antibiotic resistance genes from other bacteria is the potential of LAB to communicate actively and passively with these bacteria with the aid of conjugative plasmids and transposons. Plasmids are common in LAB, and differences are found in size, function and distribution (Davidson et al., 1996; Wang and Lee, 1997). The functions found on plasmids include hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins and exopolysaccharides, and resistance to antibiotics, heavy metals and phages. At least 25 species of lactobacilli contain native plasmids (Wang and Lee, 1997), and often appear to contain multiple (from 1 to 16) different plasmids in a single strain. Plasmids are common in enterococci, lactococci, leuconostocs, pediococci, and present in some strains of lactobacilli and bifidobacteria (Dellaglio et al., 1995; Severin and Pot, 1995; Sgorbati et al., 1995; Simpson and Taguchi, 1995; Teuber, 1995). Although several S. thermophilus plasmids have been sequenced (Mercenier, 1990; Janzen et al., 1992; Somkuti et al., 1998; Geis et al., 2003), only recently, a few genes other than those necessary for plasmid replication have been identified (Geis et al., 1999; O’Sullivan et al., 1999; Somkuti et al., 1998). Certain conjugative antibiotic resistance plasmids, such as pAMβ1 and PIP501, are capable of both mobilization and intergeneric conjugation (Tannock, 1987; Langella et al., 1993). Conjugative transposons (broad and narrow host range) have been described in enterococci, lactococci and streptococci (Clewell, 1993; Salyers et al., 1995).

3.3. Plasmids encoding AR genes in LAB

R-plasmids encoding tetracycline, erythromycin, chloramphenicol, or macrolide–lincosycin–streptogramin resistance have been reported in Lb. reuteri (Vescovo et al., 1982; Axelsson et al., 1988; Lin et al., 1996; Tannock et al., 1994), Lactobacillus fermentum (Ishiwa and Iwata, 1980; Fons et al., 1997), Lb. acidophilus (Vescovo et al., 1982), and Lb. plantarum (Ahn et al., 1992; Danielsen, 2002), isolated from raw meat, silage and faeces. Most of these R-plasmids had a size smaller than 10 kb (5.7–18 kb). Lb. fermentum isolated from pig faeces carried a 5.7 kb plasmid with an erm gene conferring high level erythromycin resistance which was 98.2% identical to the gene of the enterococcal conjugative transposon Tn1545 (Fons et al., 1997). The reported prevalence of antibiotic resistance genes such as erythromycin, vancomycin, tetracycline, chloramphenicol, and gentamicin resistance genes, on transferable genetic elements in enterococci is more extensive, both on plasmids (Christie et al., 1987; Rice et al., 1998; West and Warner, 1985; Clewell et al., 1974; Murray et al., 1988) and transposons (Perreten et al., 1997b; Clewell et al., 1995; Rice and Marshall, 1994).

A multiple antibiotic resistance plasmid pK214 was reported in a Lc. lactis strain K214 isolated from raw milk soft cheese (Perreten et al., 1997a), encoding streptomycin, tetracycline and chloramphenicol, and drug efflux gene mef 214. The tetracycline-resistant gene (providing ribosome protection) is 99.8% homologous to tet (S) from Listeria monocytogenes. Characterization of mef 214 demonstrated that it mediated multiple antibiotic resistance hence recently it has been renamed mdt (A) for multiple drug transporter. In Lc. lactis the gene mediated increased resistance to erythromycin and tetracycline (Perreten et al., 2001). Two other multidrug transporters have been described in Lc. lactis. The first (LmrA) is a member of the ABC superfamily (Bolhuis et al., 1994); the second (LmrP) is a proton motive force dependent transporter (Bolhuis et al., 1995). Recent work shows resistance to macrolides, lincosamides and streptogramins, and tetracycline is associated with LmrP expression (van Veen et al., 1999). Interestingly, multidrug transporter LmrA in Lc. lactis is a homologue of the human multidrug-resistance P-glycoprotein, another drug pump involved in drug resistance of human cancer cells encoded by the MDR1 gene. It was demonstrated that they are functionally interchangeable and that this type of multidrug-resistance efflux pump is conserved from bacteria to man (van Veen et al., 1998).

Plasmid encoding tetracycline resistance gene tet (M) was detected in Lactobacillus isolates from fermented dry sausages (Gevers et al., 2002). Most of
these R-plasmids had a size of approximately 10 kb, and in few cases the R-plasmid was larger than 25 kb. The two allele types of the plasmid encoded tet (M) gene displayed high sequence similarities (>99.6%) with the tet (M) gene previously reported in *Staphylococcus aureus* MRSA101 and in *Neisseria meningitides*, respectively. In another study by Danielsen (2002), the 10877 bp tetracycline resistance plasmid pMD5057 from *Lb. plantarum* 5057 was completely sequenced. The sequence revealed a composite structure containing DNA from up to four different sources. The tetracycline resistant region containing a tet (M) gene had high homology to sequences from *Clostridium perfringens* and *S. aureus*. Recently a 19.3 kb plasmid, encoding a new erythromycin determinant erm (LF) in erythromycin resistant *Lb. fermentum* strains, was reported; however, in the same study tet (M) gene for ribosomal protection protein Tet (M) found in six different *Lactobacillus* strains were not encoded by plasmids, but probably located on the chromosome (Gfeller, 2003). The above-reported tet (M) gene showed high homology to the nucleotide sequence of the tet (M) gene from the *E. faecalis* transposon Tn916 (accession no. M85225; Su et al., 1992).

### 3.4. Conjugative transposons encoding AR genes in LAB

Conjugative transposons are a main type of vehicle regarding antibiotic resistance transport in Gram-positive bacteria. They have been discovered in *E. faecalis* (Tn916, Tn918, Tn920, Tn925, Tn2702), *E. faecium* (Tn5233), *Streptococcus pyogenes* (Tn3701), *Streptococcus agalactiae* (Tn93951) and *Lc. lactis* (Tn5276, Tn5301). In enterococci and streptococci, they determine resistances to tetracycline (tet (M)), erythromycin (ermAM, erm), chloramphenicol (cat) and kanamycin (aphA-3). In lactococci, they code for nisin (nis) production and sucrose fermentation ( sac ). These transposons vary in size between 16 and 70 kb and may be inserted into plasmids or the chromosome in one or multiple copies. They may mobilize plasmids or chromosomal genes. The most remarkable observation, however, is the extreme host range, which is the property of the Tn916/Tn1545 family. Resistance transfer rates are in the order of $10^{-9}$ to $10^{-6}$ per donor filter matings.

### 3.5. Conjugative transfer among LAB

Indigenous conjugation systems in lactococci are very common, reflecting the abundance of plasmid DNA (Neve et al., 1987; Gasson and Fitzgard, 1994). In contrast reports of the literature on the conjugal transfer of native *Lactobacillus* plasmids is limited: one plasmid-associated transfer of lactose fermenting ability in *Lb. casei* and bacteriocin production and immunity in *Lb. johnsonni*. A similar situation is evident in *Leuconostoc* and *Pediococcus* which, however, can accept broad host range antibiotic resistant plasmids like pAMβ1, pVA797::Tn917, and pIP501 by high frequency conjugation from *Lactococcus* harbouring these plasmids (Dessart and Steenson, 1991).

Some of the above-listed R-plasmids and transposons have been shown to be transferable to other LAB, Gram-positive bacteria and even Gram-negative bacteria. Enterococci are known to be very well receptive for conjugation (Clewell and Weaver, 1989), but are also successful donor organisms for the transfer of antibiotic resistance genes to unrelated enterococci (Rice et al., 1998), lactobacilli (Shrago and Dobrogosz, 1988), other Gram-positives including *Bacillus subtilis* (Christie et al., 1987), *Staphylococcus* and *Listeria* spp. (Perret et al., 1997b), and even Gram-negative bacteria (Courvalin, 1994; Brisson-Noel et al., 1988; Trieu-Cuot et al., 1988). Moreover, the transfer of conjugative elements, including a plasmid-encoded kanamycin resistance (Doucet-Populaire et al., 1992) and a transposon-encoded tetracycline and erythromycin resistance (Doucet-Populaire et al., 1991), were shown to be transferable from *E. faecalis* to *Escherichia coli* and *L. monocytogenes*, respectively, in the digestive tract of mice. In contrast, reports of conjugative transfer of antibiotic resistance genes in other LAB are rare. Two in vivo studies were performed, to examine the possibility of conjugative transfer between native Gram-positive members of the gut. Therefore, the broad host range conjugative plasmid pAMβ1 was transferred in vitro to *Lb. reuteri* (Morelli et al., 1988) and *Lc. lactis* (Igimi et al., 1996) and administered orally or using gastric intubation to mice. By analysis of faecal content, plasmid transfer to *E. faecalis* was observed in both studies. To improve existing properties or add new properties (e.g. bacteriocin production or lactose fermentation) to strains with industrial applications, the transfer of plasmids
between different lactococci was studied (Gasson and de Vos, 1994; Neve et al., 1984, 1987).

A well-characterized broad host range conjugative plasmid pAMβ1 isolated from *E. faecalis* carries a constitutive MLS resistance. Self-transfer at frequencies of about $10^{-4}$ per donor has been observed in filter mating experiments with *Enterococcus, Staphylococcus, Clostridium, Lactobacillus* and *Bacillus* species (Macrina and Archer, 1993). Elaborate studies have shown that pAMβ1 could move from *E. faecalis* into the plasmid free strain Bu2-60 of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* during filter mating. From there, it conjugated to 8 out of 18 commercial isolates of *S. thermophilus* with frequencies of $3.5 \times 10^{-5}$ to $7.6 \times 10^{-9}$ per recipient. Back transfer into *Lactococcus* Bu2-60 occurred at high frequencies of about $10^{-4}$ (Kleinshmidt et al., 1993). pAMβ1 was quite stably maintained in *S. thermophilus* at 37 °C, but lost within 90 generations of growth at 42 °C. Out of 13 *Lb. delbrueckii* subsp. *lactis, 44 *Lb. acidophilus, 1 Lactobacillus helveticus, 1 Lactobacillus brevis, 5 Lb. plantarum, 6 Lb. casei* subsp. *rhamnosus, 1 Lb. fermentum* and *1 Lb. salivarius* subsp. *salicinius* strains only two strains, i.e. *Lb. brevis* 3030/62 and *Lb. helveticus* 3048b, accepted pAMβ1 during filter mating experiment at about $10^{-7}$ frequencies: (Soedings et al., 1993).

The chloramphenicol and erythromycin resistances in *E. faecalis* RE25 are encoded on 49-kb conjugative plasmid pRE25 which transfers these resistances to *Listeria innocua, E. faecalis* JH-2, and *Lc. lactis* BU2-60 at frequencies of $10^{5}$ to $10^{6}$. The nucleotide sequence of the cloned chloramphenicol acetyltransferase is 100% identical with that of the *S. agalactiae* pIP501 gene (Perreten, Moschetti and Teuber Gen-Bank accession no. X92945). The erythromycin resistance region of this plasmid hybridized with the specific *erm* nucleotide probe from pAMβ1. The complete nucleotide sequence of this plasmid is currently being investigated.

Transfer of the tetracycline resistance gene (at the nucleotide level 99.8% identical to *tet* (M) of transposon Tn916) from *E. faecalis* FO1 isolated from a raw milk mountain cheese, to *S. aureus* ($10^{-9}$), *E. faecalis* JH2-2, *L. innocua*, *Leuconostoc mesenteroides* ($10^{-7}$), and *Lc. lactis* Bu2-60 ($10^{-6}$) was achieved by filter mating. The mobile genetic element was identified as the Tn916-type transposon TnFO1. It resides on a 30 kb plasmid in this strain (Perreten et al., 1997b).

Gevers et al. (2003) isolated Te<sup>+</sup> *Lactobacillus* isolates from fermented dry sausages and studied their conjugative transfer to other Gram-positive bacteria. Seven out of 14 tetracycline resistant *Lactobacillus* isolates were able to transfer in vitro this resistance to *E. faecalis* at frequencies ranging from $10^{-4}$ to $10^{-6}$ transconjugants per recipient. Two of these strains could also transfer their resistance to *Lc. lactis* subsp. *lactis*, whereas no conjugal transfer to a *S. aureus* recipient was found. Recently, Huys and coworkers (2004) studied the phenotypic and genotypic assessment of tetracycline resistance in *Enterococcus* isolates from European cheeses. The tetracycline resistance could be linked to the presence of *tet* (M) genes in enterococcal isolates, all of which also harbored a member of the Tn916–Tn1545 conjugative transposon family.

4. Conclusions

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. The following general observations can be made after examination of these data:

There may be intrinsic resistance traits, e.g. vancomycin resistance in *Leuconostoc*, certain lactobacilli and others, or resistance to nalidixic acid. Distinction between intrinsic and acquired resistance is difficult as it is not possible to trace an investigated strain into the preantibiotic era. If LAB live in a biotope challenged regularly with antibiotics (human intestine, animal intestine, bovine udder) acquired antibiotic resistance is found in LAB from such habitats including *Enterococcus, Lactococcus* and *Lactobacillus* species.

There is no barrier between pathogenic (e.g. streptococci), potentially pathogenic (e.g. enterococci) and commensal (e.g. enteric lactobacilli, lactococci) LAB regarding acquired resistances. Identical genes responsible for resistances are found, e.g. for tetracycline (e.g. *tet* (M)), erythromycin (*erm*AM), chlorampheni-
of the probiotic lactic acid bacteria (e.g. lactobacilli, lactococci, enterococci, bifidobacteria) for human consumption must be assessed by proposing criteria, standards, guidelines and regulations on the one hand, and standardizing methodologies of premarketing biosafety testing and post marketing surveillance on the other hand.

**References**


