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Review Article
Antibiotic resistance of probiotic organisms and safety of probiotic dairy products

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Abstract: Intrinsic resistances to tetracycline, vancomycin and erythromycin are common in *Lactobacillus* species; however, resistance to streptomycin, clindamycin, gentamicin, oxacillin and lincosamide is also reported in these species. Resistant markers *tet*(W), *tet*(M) and *erm*(B) have been frequently detected in the resistant strains while *van*(A), *lnu*(A) and *tet*(L) have also been found in some strains of *Lactobacillus*. Bifidobacteria are commonly resistant to tetracycline, streptomycin, erythromycin, gentamicin and clindamycin. Resistance genes *van*(A), *tet*(L) and *tet*(M) are often detected in *Enterococcus*. Reports suggest enterococci to transfer *tet*(M) to *E. faecalis* or *Listeria* strains and *van*(A) to commercial strain of *Lactobacillus acidophilus*. *Streptococcus* species are highly resistant to tetracycline, ciprofloxacin and aztreonam and *tet*(M) was detected in strains of dairy origin. Clinical cases of endocarditis, septicemia, bacteremia and septic arthritis due to the species of *Lactobacillus*, *Saccharomyces*, *Leuconostoc*, *Pediococcus* and *Bifidobacterium* have been reported in patients with some underlying medical conditions.

Keywords: Antibiotic resistance, probiotics, minimum inhibitory concentration

Introduction

The overwhelming use of antibiotics has played a significant role in the outspread/emergence of antibiotic resistance bacteria. Antibiotics added to animal-feed and given to livestock that are used as human food contribute to additional resistance. Reports suggest that commensal bacteria may act as potential reservoirs for antimicrobial resistance genes, hence bacteria used as probiotics for humans or animals should not carry any transferable antimicrobial resistance genes (von Wright, 2005; European Food Safety Authority-EFSA, 2008; The panel on additives and products or substances used in animal feed-FEEDAP, 2008). According to World Health Organization (WHO) global strategy for the containment of antimicrobial resistance (World Health Organization-WHO, 2001), the rate of emergence of antimicrobial resistance is expected to be increased by misuse of antibacterial substances. The resistant micro-organisms present in food products originating from animal source may cause infections in humans that are difficult to treat. A summary of risk factors for antibiotic resistance particularly relevant to, but not limited to, developing countries is outlined in Table 1.

The European Food Safety Authority (2005) has outlined a scheme based on the qualified presumption of safety (QPS) that involves the individual assessment and evaluation of acquired antibiotic resistance determinants in lactic acid bacteria (LAB). According to the scheme, the members of the *Lactococcus* and *Lactobacillus* are most commonly given "generally regarded as safe" (GRAS) status, whilst members of the genera *Streptococcus* and *Enterococcus* and some other genera of LAB contain some opportunistic pathogens. Microorganisms used in animal feed in the European Union (EU) are mainly strains of *Bacillus* (*B. cereus* var. *toyoi*, *B. licheniformis*, *B. subtilis*), *Enterococcus* (*E. faecium*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus*), *Pediococcus* (*P. acidilactici*), *Streptococcus* (*S. infantarius*), and yeast of *Saccharomyces cerevisiae* and *Kluyveromyces* species (Anadón *et al*., 2006).

Table 1. Human activities that exacerbate resistance (adapted from Okeke *et al*. (2005))

Selective pressure
• Appropriate antimicrobial use in chemotherapy
• Use of a narrow repertoire of antimicrobials on most patients
• Antimicrobial misuse and abuse in human beings
• Agricultural antimicrobial use and misuse
• Use of poor quality antimicrobials
Dissemination of resistant organisms
• Inadequate infection control in health-care institutions
• Shortfalls in hygiene, sanitation, and public health
• Lack of surveillance and consequent late detection

The guidelines updated by the FEEDAP Panel in 2008 are expected to eliminate the possibility of microorganisms from food chain to carry transmissible resistances genes. However, no such guidelines exist

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concerning yeast resistance to antimycotics. As a result, the use of antimicrobial growth promoters such as avoparcin, carbadox and alaquindox has been banned in the EU since 2006. The emergence of vancomycin-resistant enterococci in food-animals is correlated with the use of avoparcin. Avoparcin is a glycopeptide that is used as a feed additive for adding the growth of animals that can cause spread of vancomycin-resistance from animals to humans (Wegener, 2003). Since the resistance in many cases is transmissible, non-pathogenic bacteria added into the food chain could act as a reservoir of resistance and transfer this trait to pathogens.

Types of antibiotic resistance

There are three types of resistance: natural (intrinsic or innate), acquired and mutational. According to FEEDAP (2008), strains carrying the acquired resistance due to acquisition of exogenous resistance genes are unacceptable for use as animal feed additives.

Resistance gene reservoir hypothesis

Colonic bacteria normally residing in colon act as reservoirs for resistance genes that can be acquired from ingested bacteria (Figure 1). According to reservoir hypothesis "commensal bacteria in the colon including those that could act as opportunistic pathogens and those that are truly non-pathogenic, exchange DNA with one another" (Salyers *et al*., 2008).The reservoir hypothesis suggests that antibiotic-resistant bacteria came into existence because of the selective pressures applied by antibiotic drugs (Table 1). 'After antibiotic treatment, there is a decline in the populations of susceptible bacteria, naturally resistant bacteria begin to thrive, creating a reservoir of antibiotic-resistant bacteria' (Salyers *et al*., 2004).

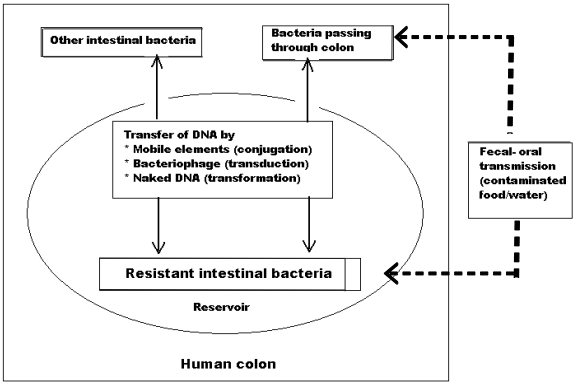

Figure1. The reservoir gene hypothesis. Bacteria residing in human colon can act as reservoir of resistant genes that can be acquired from ingested bacteria (adapted from Salyers *et al*. (2008)

Methods for determining antibiotic resistance

Methods that are routinely used for testing antibiotic susceptibility of bacteria include Kirby-Bauer (disc diffusion) method, Stokes method, E-test (based on antibiotic diffusion), agar and broth dilution or agar dilution methods for the determination of minimum inhibitory concentration (MIC). The E-test (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*., 2005). The technique is accurate and reproducible because of the stability of the antibiotics (Sader *et al*., 1994).

These methods have been tested and compared for different LAB and bifidobacteria. MICs can be determined by agar or broth dilution techniques by following the reference standards established by various authorities such as the Clinical and Laboratory Standards Institute (CLSI, USA), British Society for Antimicrobial Chemotherapy (BSAC, UK), Agence Francaise de Securite Sanitaire des Produits de Sante (AFFSAPS, France), Deutsches Institut für Normung e.V. (DIN, Germany) & ISC/WHO. FEEDAP has published guidelines regarding the testing procedures since 2001. FEEDAP requires the determination of the MICs of the most relevant antimicrobials for each bacterial strain that is used as a feed additive in order to eliminate the possibility of transmissible resistances.

Mayrhofer *et al*. (2008) tested 104 strains of *L. acidophilus* using broth microdilution, disk diffusion, and E-test. A good agreement was found between MICs from the broth microdilution method and the E- test method. Agar based methods such as E- test and agar disk diffusion were suggested as valid methods compared to the broth microdilution method. Blandino *et al*. (2008) found MICs as identical to those obtained with the E-test. Danielsen and Wind (2003) suggested that MICs can be used as a microbiological breakpoint when screening *Lactobacillus* strains for transferable resistance genes. For antimicrobial susceptibility testing of bifidobacteria, Mättö *et al*. (2007) suggested that the E-test on LAB susceptibility test medium supplemented with cysteine was useful. The swab and agar overlay gradient diffusion method was found to be reliable by Charteris *et al*. (2001) for antibiotic susceptibility testing of rapidly growing, facultative anaerobic lactobacilli, using MRS agar as test medium.

Egervärn *et al*. (2007) found that results obtained with the E-test or the broth microdilution method for the assessment of antibiotic susceptibility of *L. reuteri* and *L. fermentum* strains (56 each) corresponded well with each other. This is supported by the study of Brown and Brown (1991) that showed a good correlation between MICs by the agar dilution and E-test methods. Turnidge and Paterson (2007) found that the distribution of MICs for wildtype strains of a single species was log-normal.

Acquisition and spread of resistances

The antibiotic resistance gene can be transferred by conjugation, transduction or transformation (Figure 1). At present, reports regarding the spread of antibiotic resistance among LAB and bifidobacteria suggest that resistant strains from human and animal colons are rather common, that confirms the transfer of resistances between commensal organisms in the complex ecosystem of gastro-intestinal tract (GIT) (Ammor *et al*., 2007). There is a general concern that such microbes may harbor genes that may contribute to opportunistic infections (Tompkins *et al*., 2008). Theoretical risks that have been raised with respect to the use of probiotics in humans include the potential for transmigration and colonization and an adverse immunological effect. There is also a potential for antibiotic resistance transfer within the gastrointestinal tract from commensal or probiotic bacteria to other bacteria or potential pathogen (Snydman, 2008).

Starter cultures used in food products could also be a source of spread of antibiotic resistance. Hence, strains intended for use in feed and food systems should be systematically monitored for resistance in order to avoid their inclusion in starters and probiotic preparations (Ammor *et al*., 2007). Two genes namely, transposon-associated *tet*(M) gene and plasmid-carried *tet*(L) gene that mediate 2 different tetracycline resistance mechanisms have been described in *L. sakei* Rits 9 strain isolated from Italian Sola cheese made from raw milk (Ammor *et al*., 2008). Tetracycline resistance gene *tet*(K) in 5 *Staphylococcus* isolates used as meat starter cultures were detected by Kastner *et al*. (2006). In a recent report where the gene *tet*(M) of *L. plantarum* isolated from pork abattoir was transferred to *Lc. lactis* BU-2-60 and to *E. faecalis* JH2-2 (Toomey *et al*., 2010).

Antibiotic resistance in LAB, *Bifidobacterium* and *Bacillus* spp.

In the EFSA guidelines (The panel on additives and products or substances used in animal feed-FEEDAP, 2008), the MICs for relevant antimicrobials have been set for the following genera (and in some cases individual species): *Lactobacillus*, *Lactococcus*, *Streptococcus thermophilus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Propionibacterium*, *Bifidobacterium* and *Bacillus*. These genera also cover the recent QPS lists for bacteria, and consequently the FEEDAP approach can be directly applied.

LAB are intrinsically resistant to many antibiotics. In many cases, resistance is not always transmissible, and the species are also sensitive to many clinically used antibiotics in the case of a LAB-associated opportunistic infection. Therefore no particular safety concern is associated with intrinsic type of resistance. Plasmid-associated antibiotic resistance, which occasionally occurs, may spread resistance to other more harmful species and genera.

Using the disc diffusion method, antibiotic resistance among 187 isolates from 55 European probiotic products showed that 79% of the isolates were resistant against kanamycin and 65% of the isolates were vancomycin resistant. Remaining resistances were in the order of tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%). Overall, 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistance (Temmerman *et al*., 2003). In a study by Toomey *et al*. (2010), intrinsic streptomycin resistance was observed in lactobacilli, streptococci, lactococci and *Leuconostoc* spp.

Several studies have been carried out to test the antimicrobial susceptibility of different probiotic and LAB in different food products but only some of these have demonstrated the genetic basis of these resistances. Also, the data is available regarding antimicrobial resistance pattern in food-associated LAB such as lactobacilli but it is mostly based on non-standardized methodologies and/or has been obtained for only a limited number of strains (Huys *et al*., 2008). Studies regarding antimicrobial testing of different LAB, bifidobacteria and *Bacillus* strains have been summarized in Table 2 and discussed below.

Lactobacillus

Lactobacilli display a wide range of antibiotic resistance naturally, but in most cases antibiotic resistance is not of the transmissible type. *Lactobacillus* strains with non-transmissible antibiotic resistance do not form a safety concern. In a study by Danielsen and Wind (2003), out of 62 strains tested for antibiotic susceptibility, 6 strains

Table 2. Antibiotic resistance and safety implication of different LAB, bifidobacteria and *Bacillus* spp.

Probiotic studied	Antibiotics found to be resistant*	Resistance gene	Acquisition or spread of resistance/	Origin/ source of probiotic (Country of study conducted)	Method used for antibiotic resistance analysis	References	Implication (Safety)
L. farciminis BFE 7438	Cip, Gen, Str	-	Intrinsic	3 strains were newly developed and 2 from commercial probiotic product. (Germany)	E-test, PCR, Southern hybridization, filter mating experiment	Hummel *et al.* (2007)	Incidences of erythromycin, chloramphenicol, tetracycline, or β-lactam resistance in this study were low (<7%). In contrast, aminoglycoside (gentamicin and streptomycin) and ciprofloxacin resistances were higher than 70%, indicating that these may constitute intrinsic resistance, which could not be transferred in filter-mating experiments.
L. salivarius BFE 7441	Cip, Ery, Gen, Str	*ermB*	Chromosomal				
L. rhamnosus BFE 7442	Cip, Gen, Str	-	Intrinsic				
L. acidophilus BFE 7444	Cip, Gen, Str	-	Intrinsic, inactive *cat* genes				
L. casei BFE 7445	Cip, Gen, Str	-	Intrinsic				
L. paracasei	Gen, Van	-	Intrinsic (Van)	Probiotic product -dried food supplements and fermented functional foods (Italy)	E-test, broth microdilution test	Blandino *et al.* (2008)	The observed resistance in tested strains seemed to be intrinsic, except for *L. salivarius* strain. Vancomycin and erythromycin resitance was present. Antibiotic resistance determinants are likely to be present or can be transferred.
L. plantarum	Ery, Gen, Van	-	Intrinsic (Van)				
L. salivarius	Ery, Van	-	Intrinsic (Van), Atypical (Ery)				
L. acidophilus	Gen	-	Intrinsic				
S. thermophilus	Cip	-	Intrinsic				
L. rhamnosus HN001 (DR20™)	Fus, Gen, Kan, Nal, Neo, Pol, Str, Van	-	Intrinsic (contain plasmids but antibiotic resistance is not linked)	Fonterra Research Centre Culture Collection (New Zealand, USA)	Disk diffusion method	Zhou *et al.* (2005)	None of the 4 new probiotic strains (*L. rhamnosus* HN001 and HN067, *L. acidophilus* HN017 and *B. lactis* HN019) tested in this study contained clinically significant transmissible antibiotic resistance genes. While some of the strains were intrinsically vancomycin resistant, all strains were sensitive to a number of clinically effective antibiotics. None of these new probiotic strains were any more resistant than the present-day commercial probiotic strains GG and LA-1.
L. rhamnosus HN067	Fus, Kan, Nal, Neo, Pol, Van,	-	Intrinsic				
B. lactis HN019 (DR10™)	Clo, Gen, Kan, Nal, Neo, Pol, Str	-	Intrinsic				
L. acidophilus HN017	Fus, Kan, Nal, Pol, Str	-	Intrinsic				
L. plantarum HN045	Clo, Fus, Gen, Kan, Nal, Neo, Pol, Str, Van,	-	Intrinsic				
L. rhamnosus GG	Clo, Fus, Gen, Kan, Nal, Neo, Pol, Str, Van	-	Intrinsic	Commercial strain, Fonterra Research Centre Culture Collection (New Zealand, USA)			
L. acidophilus LA-1	Fus, Gen, Kan, Nal, Neo, Pol, Str	-	Intrinsic				
B. lactis Bb12	Clo, Fus, Gen, Kan, Nal, Neo, Pol, Str, Van	-	Intrinsic				
B. lactis HN049	Clo, Gen, Kan, Nal, Neo, Pol, Str	-	Intrinsic				
B. lactis HN098	Clo, Fus, Gen, Kan, Nal, Neo, Pol, Str	-	Intrinsic				
B. breve (Yakult)	Str	*rpsL* gene	Atypical	Culture Collection Research Laboratory of Yakult Central Institute for Microbiological Research (Tokyo, Japan)	Broth microdilution method, PCR and sequencing experiment	Kiwaki and Sato (2009)	Chromosomal mutation of the *rpsL* gene for ribosomal protein S12 cause resistance so it is unlikely to be transferred to other microorganisms.

B. lactis DSM 10140	Gen, Kan, Nal, Ofl, Str, Tet, Tob (confirmed by E-test)	*tet*(W)	Plasmid borne	French yogurt (Switzerland)	Disk diffusion, E-test, microarray and membrane hybridization techniques, PCR, partial sequencing methods and filter mating experiments	Kastner *et al.* (2006)	Antibiotic resistance determinants are likely to be transferred or spread, so these strains should be tested for the presence of transferable resistance gene before being used as probiotics.
L. reuteri SD 2112 (ATCC 55730)	Cli, Ctx, Fus, Kan, Lin, Met, Nal, Nit, Ofl, Oxa, Pen, Str, Tet, Tob, Van	*tet*(W), *Inu*(A)	Plasmid borne	Human origin. One strain obtained from ATCC and other from commercial tablet (Switzerland)			
L. rhamnosus strain GG (ATCC 53103)	Fus, Kan, Nal, Nit, Tob, Oxa, Str, Van	*vanE*^{c}, *vatE*^{c}, *mef*^{c}(detected by microchip hybridization)	Phenotypic	Human (Switzerland)			
Bacillus subtilis VKPM B2335	Oxa	n/a	n/a	Ukrainian Collection of Microorganisms (France, Russia, UK)	Disk diffusion method, serial antibiotic dilution procedure	Sorokulova *et al.* (2008)	Both strains did not harbour plasmid. Certain risks may exist for the *B. licheniformis* strain considering antibiotic resistance, complementary studies are necessary in order to exclude that chloramphenicol and clindamycin resistances are harboured in a transposable element. *B. subtilis* strain may be considered as non-pathogenic and safe for human consumption
Bacillus licheniformis VKPM B2336	Amp, Cet, Cex, Chl, Cli, Ctx, Met, Oxa,	n/a	Intrinsic (Cli)				
L. acidophilus	Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Sul, Tri	*gyrA* (Cip)	Intrinsic	Human, adult biopsy, Europe (Denmark)	Micro-broth and agar dilution, PCR and conjugation experiments	Ouoba *et al.* (2008)	Positive amplicons were obtained for resistance genes encoding aminoglycoside (aph(3′)-III, aadA, aadE) and tetracycline (tet(S). Higher prevalence of phenotypic resistance for aminoglycoside was found. The reduce susceptibility to the cephalosporin and cefpodoxime can be attributed to intrinsic resistance of the different species since none of these bacteria were resistant to penicillin (first generation β- lactam). These organisms could act as reservoir of antimicrobial resistant genes and can transfer the resistances to other bacteria.
L. rhamnosus	Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Spe, Sul, Tri, Van	-	Intrinsic				
L. plantarum	Amp, Cip, Col, Ery, Gen, Kan, Nan, Neo, Spe, Str, Sul, Tet, Tri, Van	*gyrA* (Cip), *aadE* (Str)	Intrinsic				
L. casei	Amp, Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Sul, Tet, Tri, Van	-	Intrinsic				
L. paraplantarum	Amp, Apr, Cip, Nan, Neo, Spe, Sul, Tet, Tri, Van	*gyrA* (Cip), *tet*(S)	Intrinsic				
B. longum	Apr, Cip, Col, Gen, Kan, Nan, Neo	*aadE* (Str)	Intrinsic				
B. bifidum	Apr, Cip, Col, Gen, Kan, Nan, Neo, Tri	*gyrA* (Cip)	Intrinsic				
L. rhamnosus	Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Spe, Sul, Tri, Van	-	Intrinsic				
L. rhamnosus	Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Sul, Tri, Van	-	Intrinsic	Human, child feces, Europe (Denmark)			
L. paracasei	Amp, Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Str, Sul, Tri, Van	*aph(3')-III* (Kanamycin, Neo) *aadA* (Str)	Intrinsic	Human, adult feces, Europe (Denmark)			
L. casei	Amp, Apr, Cep, Cip, Col, Gen, Kan, Nan, Neo, Spe, Str, Sul, Tri, Van	*aph(3')-III* (Kan, Neo) *aadA, aadE* (Str), *aadA* (Spe)	Intrinsic	Semi-hard cheese, Europe (Denmark)			
Bifidobacterium sp	Apr, Cip, Col, Gen, Kan, Nan, Neo, Tri	-	Intrinsic	Human, child biopsy, Europe (Denmark)			

E. faecium	Chl, Ery, Gen, Str,	*erm*(B), *cat*(pC194), *cat*(piP501), *aad*(E), *aad*(E)-*aph*(A)	Phenotypic and genetic resistances	6 strains from Probiotic products, 2 strains as probiotics for human and animal consumption comes from fecal flora (Sweden; 1968) and dairy product, cheese (Italy), 2 strains used commercially as a probiotic for human consumption. (Belgium, Germany)	Broth microdilution, filter mating experiments, PCR-based detection of resistant genes, PFGE and $(GTG)_5$-PCR, multiplex PCR	Vankerckhoven *et al.* (2008)	Resistant to fusidic acid was demonstrated in a high percentage (92%) of isolates. Two probiotic isolates were phenotypically resistant to erythromycin, one of which contained an *erm*(B) gene that was not transferable to enterococcal recipients.
L. reuteri DSM 20016	Cip, Gen, Oxa, Sul/Tri, Van,	n/a		Strain collection of BioGaia Biologics, Inc., Raleigh, NC, USA (USA)	Microdilution, PCR for *van*A- and *van*B-genes, Southern hybridization, probe and DNA/DNA hybridization for the detection of the *van* genes, plasmid analysis	Klein *et al.* (2000)	Neither none of the strains possessed *vanA*, *vanH*, *vanR*, *vanS*, *vanX*, *vanY*, *vanZ* or *vanB* genes nor they hybridized with *vanA*, *vanB* or *vanC* specific probes. Plasmids could be found in only two of the six strains. Six plasmid bands in *L. reuteri* SD2112 and 5 bands in *L. reuteri* 11284 were identified. Safety of these strains as probiotic with regard to vancomycin resistance has been reassured.
L. reuteri T-1 (ATCC 55149)	Cip, Gen, Oxa, Sul/ Tri, Van	n/a					
L. reuteri 1063 (ATCC 53608)	Cip, Gen, Oxa, Sul/Tri, Van	n/a					
L. reuteri SD2112 (ATCC55730)	Amp, Cep, Cip, Gen, Oxa, Sul/ Tri, Tet, Van	n/a	Plasmid borne				
L. reuteri 11284 (ATCC 55148)	Cip, Gen, Oxa, Sul/Tri, Tet, Van	n/a	Plasmid borne				
L. rhamnosus GG (ATCC 53103)	Cip, Gen, Oxa, Sul/Tri, Van	n/a					
L. reuteri ATCC 55730 (SD2112)	Amp, Lin, Tet	*tet*(W), *lnu*(A)	Plasmid borne and intrinsic resistances	Biogaia AB, Human milk. (Sweden)	Broth microdilution, E-test, draft genome sequence analysis, BLAST, PCR based methods	Rosander *et al.* (2008)	No known β-lactam resistance genes were found. The β-lactam resistance is probably caused by a number of alterations in the corresponding genes and can be regarded as not transferable. It harbor two plasmids carrying *tet*(W) and *lnu*(A) resistance genes which were cured and daughter strains retained probiotic properties, confirmed after series of in vitro properties and human clinical trials.
Lactobacillus strain *GG*	Gen, Mez, Tri/Sul, Str, Van	n/a	n/a	Adult human feces. Prof. Range Fondén (Panove Partner AB, Arla group, Stockholm Sweden). (Ireland)	Agar overlay disc diffusion test, gradient diffusion test (E-test),	Charteris *et al.* (2001)	All strains were resistant to vancomycin but the nature of resistance was not determined. The swab and agar overlay gradient diffusion was proposed to be a reliable method for antibiotic susceptibility testing.
L. rhamnosus	Gen, Mez, Tri/Sul, Str, Van	n/a	n/a	Dairy product, NCFB(NCIMB Ltd., Aberdeen, Scotland). (Ireland)			

* Amp, Ampicillin; Apr, Apramycin; Ctx, Cefotaxime; Cex, Cefoxitin; Cep, Cefpodoxime; Cet, Ceftriaxon; Cep, Cephalthoin; Chl, Chloramphenicol; Cip, Ciprofloxacin; Cli, Clindamycin; Clo, Cloxacillin; Col, Colistin; Ery, Erythromycin; Fus, Fusidic acid; Gen, Gentamicin; Kan, Kanamycin; Lin, Lincomycin; Met, Methicillin; Mez, Metronidazole; Nan, Nalidixan; Nal, Nalidixic acid; Neo, Neomycin; Nit, Nitrofurantoin; Ofl, Ofloxacin; Oxa, Oxacillin; Pen, Penicillin; Pol, Polymyxin B; Spe, Spectinomycin; Str, Streptomycin; Sul, Sulphamethoxazole; Tet, Tetracycline; Tob, Tobramycin; Tri, Trimethoprim; Van, Vancomycin

of lactobacilli showed transferable resistance genes on the basis of their resistance to chloramphenicol, erythromycin/clindamycin, and tetracycline. One strain of *L. rhamnosus* exhibited an elevated MIC for oxacillin. The genetic basis of this kind of resistance was proposed to be either due to mutations in the penicillin-binding proteins or due to the presence of a β-lactamase.

In the study of D'Aimmo *et al*. (2007), lactobacilli were found resistant to nalidixic acid, aztreonam, cycloserin, kanamycin, metronidazole, polymyxin B, spectinomycin and susceptible to rifampicin, bacitracin, clindamycin, erythromycin, novobiocin and penicillin. High resistance to nalidixic acid was found among all strains of *L. acidophilus* and *L. casei* whereas *L. casei* also demonstrated high resistance to aztreonam, cycloserine, polymyxin B and vancomycin.

MICs of 16 antimicrobials for 473 isolates of LAB comprising of the genera *Lactobacillus*, *Pediococcus* and *Lactococcus* were determined by Klare *et al*. (2007). The results suggested that majority of LAB were susceptible to penicillin, ampicillin, ampicillin/sulbactam, quinupristin/dalfopristin, chloramphenicol and linezolid. LAB exhibited a broad or partly species-dependent MIC profile of trimethoprim, trimethoprim/sulfamthoxazole, vancomycin, teicoplanin and fusidic acid. Noticeably, 3 probiotic *Lactobacillus* strains were highly resistant to streptomycin. Although erythromycin, clindamycin, and oxytetracycline possessed high antimicrobial activities, 17 *Lactobacillus* isolates were resistant to one or more of these antibiotics. Eight of them, including 6 probiotic and nutritional cultures possessed *erm*(B) and/or *tet*(W), *tet*(M) or unidentified members of the *tet*(M) group. High resistance against streptomycin has also been reported in 1 strain of *Lactobacillus* isolated from Norwegian dairy product (Katla *et al*., 2001).

In the study of Huys *et al*. (2008), genotypically unique 65 strains of *L. paracasei* and *L. casei* were assayed for antibiotic resistance with broth microdilution and E-test assays using the LAB susceptibility test medium. In both methodologies, strains appeared uniformly susceptible to ampicillin and clindamycin but exhibited natural resistance to streptomycin and gentamicin. Three *L. paracasei* strains from cheese displayed acquired resistance to tetracycline (MIC ≥ 32 μg per mL) and/or erythromycin (MIC >16 μg per mL), which were linked to the presence of a *tet*(M) or *tet*(W) gene and/or an *erm*(B) gene, respectively. In the study of Kastner *et al*. (2006), *L. reuteri* SD 2112 has been shown to harbor tetracycline resistance gene *tet*(W) (residing on a plasmid) and the lincosamide resistance gene *lnu*(A). Two plasmids carrying *tet*(W) tetracycline, and *lnu*(A) lincosamide resistance genes were also identified by Rosander *et al*. (2008) in a commercial strain of *L. reuteri* ATCC55730.

Both a transposon-associated *tet*(M) gene, and plasmid-carried *tet*(L) gene presenting 2 different tetracycline resistance mechanisms have been characterized in *L. sakei* Rits 9 strain isolated from Italian Sola cheese made from raw milk (Ammor *et al*., 2008). The 2 resistance determinants conferred different levels of resistance and their expression is induced by different tetracycline concentrations.

In a recent double blind clinical study by Egervärn *et al*. (2010), the transferability of tetracycline resistance gene *tet*(W) from *L. reuteri* to human gut flora was investigated particularly to fecal enterococci, bifidobacteria and lactobacilli. *L. reuteri* ATCC 55730 harboring a plasmid-encoded *tet*(W) gene was consumed by 7 subjects and an equal number of subjects consumed *L. reuteri* DSM 17938. No *tet*(W)-reuteri signal was produced from any of the DNA samples and thus evidence of gene transfer to entrococci, bifidobacteria and lactobacilli during intestinal passage of the probiotic strain was not found under the conditions tested.

In the study of Gfeller *et al*. (2003), *L. fermentum* ROT1 isolated from a raw milk dairy product was found resistant to novobiocin, tetracycline, erythromycin and dalfopristin. A chromosomal tetracycline-resistance determinant *tet*(M) was identified in the strain and a 19,398-bp plasmid (pLME300), present in several erythromycin-resistant strains of *L. fermentum*, was isolated and completely sequenced.

Several species of *Lactobacillus* including *L. rhamnosus* and *L. casei* are intrinsically resistant to vancomycin. There is an underlying possibility that vancomycin resistance could be transferred to other bacteria but there are no such reports to date. However, the transfer of vancomycin resistance (*vanA*) from enterococci to a commercial *L. acidophilus* strain was observed *in vitro* and *in vivo* in mice (Mater *et al*., 2008). In a study by Klein *et al*. (2000), all *Lactobacillus* strains namely 6 *L. reuteri* strains (ATCC 55730, ATCC 55149, ATCC 55148, ATCC 53608 and DSM 20016^T) and 1 *L. rhamnosus* strain GG (ATCC 53103) were found resistant to vancomycin but susceptible to a broad range of antibiotics. Four of the *Lactobacillus* strains (including *L. rhamnosus* strains) did not harbor any plasmid but 2 of them showed 5 and 6 plasmid bands, respectively. None of the strains possessed the *vanA*, *vanB* or *vanC* gene. The findings established the safety of the *Lactobacillus* strains for use as probiotics concerning their vancomycin resistance (Klein *et al*., 2000). Zhou

et al. (2005) found 3 *L. rhamnosus* strains (HN001, HN067 and GG) resistant to vancomycin and of the 4 new probiotic strains namely, *L. rhamnosus* HN001, HN067, *L. acidophilus* HN017 and *B. lactis* HN019, only *L. rhamnosus* HN001 contained plasmids. A plasmid-free derivative of the strain had the same antibiotic susceptibility profile as the parent strain.

Charteris *et al*. (2001) found vancomycin resistance in all tested strains of *Lactobacillus* strain GG and 11 closely related, rapidly growing, facultatively anaerobic, potentially probiotic *L. rhamnosus* strains. Moreover, these strains were also resistant to co-trimoxazole, metronidazole, gentamicin, and streptomycin but sensitive to pencillin G, ampicillin, rifampicin, tetracycline, chloramphenicol, and erythromycin. Antibiotic susceptibility pattern of the strains derived from 10 Italian probiotic products was determined by Blandino *et al*. (2008). Intrinsic resistance to vancomycin was confirmed for *L. paracasei*, *L. salivarius* and *L. plantarum*, and atypical resistance to erythromycin was detected in 1 strain of *L. salivarius* according to FEEDAP and CLSI breakpoints (MIC ≥8 mg per L) (Blandino *et al*., 2008).

In the study of Toomey *et al*. (2010), all strains of *Lactobacillus* spp. including *L. paracasei*, *L. reuteri* and *L. curvatus*, except *L. plantarum* were resistant to erythromycin containing *erm*(B) and *msr*A/B genes. Tetracycline resistance was demonstrated by only *L. plantarum* determined by *tet*(M) gene and *Leuconostoc mesenteroides* spp. containing *tet*(S) gene, respectively. *L. plantarum* was also intrinsically resistant to vancomycin, however no vancomycin gene markers were found in *Lactobacillus* species. Intrinsic streptomycin resistance was observed in lactobacilli besides streptococci, lactococci and *Leuconostoc* species. In another report, *L. reuteri* 12002 of African origin, isolated from pig feces and used as probiotic intervention studies was found to harbor the *erm*(B) gene that could be transferred *in vitro* to enterococci. Twelve probiotic isolates of European origin demonstrated high prevalence of phenotypic resistance for aminoglycosides (Ouoba *et al*., 2008).

In a study by Egervärn *et al*. (2007), *L. reuteri* and *L. fermentum* (56 strains of each) were assessed for antibiotic susceptibility using an E-test kit and a broth microdilution method. *L. fermentum* has shown an uniform distribution for tested antibiotics including ampicillin, tetracycline, erythromycin, clindamycin, streptomycin, and gentamicin, whereas *L. reuteri* strains displayed bimodal distribution of MICs or above the test range for erythromycin, clindamycin, kanamycin, vancomycin, tetracycline, and trimethoprim. *L. reuteri* strains with high MICs for both ampicillin, and tetracycline exhibited genetic relatedness and 6 strains with high MICs for both erythromycin and clindamycin were also closely related.

Bifidobacterium

In the study of Mättö *et al*. (2007), human or probiotic associated *Bifidobacterium* species (203 strains) showed high MIC for tetracycline i.e. ≥16 mg per mL (prevalence of 4-18%) that was attributed to the presence of tet gene, where *tet*(W), and *tet*(O) were detected. Occasional erythromycin (2%) and/or clindamycin (5%) resistant strains were found, while the strains were uniformly susceptible to ampicillin and vancomycin. MICs of tetracyclines were determined for 86 human *Bifidobacterium* isolates and 3 environmental strains. The *tet*(O) gene was absent in these isolates. *tet*(W), and *tet*(M) were found in 26, and 7%, respectively, of the *Bifidobacterium* isolates, and one isolate contained both genes. Chromosomal DNA hybridization showed that there was one chromosomal copy of *tet*(W), and/or *tet*(M) (Aires *et al*., 2007). The tetracycline resistance gene *tet*(W) in the probiotic culture of *B. lactis* DSM 10140 was detected by Kastner *et al*. (2006).

Kiwaki and Sato (2009) determined the MICs of 17 antimicrobials for 26 *Bifidobacterium breve* strains of various origins by broth microdilution. MIC distributions for 17 antimicrobials were unimodal except streptomycin and tetracycline, in which it was bimodal. The probiotic *B. breve* strain Yakult showed intrinsic susceptibility to all antimicrobials except streptomycin to which the strain showed an atypically higher MIC of >256 μg per mL. The resistance of *B. breve* strain Yakult to streptomycin was caused by a chromosomal mutation of the *rps*(L) gene for ribosomal protein S12, and thus unlikely to be transferred to other microorganisms.

In another study by Blandino *et al*. (2008), the strains of *Bifidobacterium* were found susceptible to ampicillin, cefotaxime and erythromycin. In the study of Mättö *et al*. (2007), *Bifidobacterium* strains displayed generally high MICs for streptomycin and gentamicin suggesting intrinsic resistance. D'Aimmo *et al*. (2007) found that bifidobacteria were resistant to aminoglycosides, cycloserine, nalidixic acid and strongly resistant to kanamycin, polymixin B, and aztreonam (MIC90 = 1000 μg per mL).

Enterococcus

Members of *Enterococcus* contain some

opportunistic pathogens, hence, it is debated as to whether these organisms could be used as probiotics. Several studies have examined the antibiotic resistance profile, and evaluated the transferability of the resistance determinants to other microorganisms. Rizzotti *et al*. (2009) studied the diversity and transferability of tetracycline gene *tet*(M) of 20 enterococci belonging to species of *E. faecalis* (12 strains), *E. faecium* (4), *E. durans* (2), *E. hirae* (1), and *E. mundtii* (1) originating from swine meat. The gene *tet*(L) was observed in the 50% of the strains and *tet*(M) was found correlated with a transposon of the Tn*916-1545* family. Moreover 50% of enterococcal strains showed the ability to transfer *tet*(M) gene to *E. faecalis* or *Listeria innocua* strains, which affirms the spread of tetracycline resistance in enterococci to potentially pathogenic bacteria occurring in food chain.

Mater *et al*. (2008) observed the transfer of vancomycin resistance (*vanA*) from enterococci to a commercial strain of *L. acidophilus in vitro* and *in vivo* in mice. The transconjugants were obtained in high ferquency and were capable of persisting in the digestive environment of mice. Since the same transfer is expected to occur in human digestive tract, it raises a safety concern regarding the use of probiotics comprising lactobacilli in either immunocompromised individuals or during antibiotic therapy. In vancomycin resistant *E. faecium* isolates collected from Michigan hospitals, the location of *vanA* genes was found on both plasmid and chromosome that suggests the possibility of transposon dissemination among these isolates (Thal *et al*., 1998).

Regarding the prevalence of antimicrobial resistance of enterococcal strains in different environments, the frequency of various antimicrobial resistances was much lower in food isolates in comparison to clinical strains (Abriouel *et al*., 2008). Similar findings were reported by Blandino *et al*. (2008) where *E. faecium* derived from probiotic product from Italy was susceptible to all the tested antibiotics including vancomycin, ampicillin, cefaclor, cefotaxime, erythromycin, ciprofloxacin and gentamicin. However, in the Moroccan food isolates studied by Valenzuela *et al*. (2008), the frequency of antimicrobial resistance was remarkably high. The resistance profiles of *E. faecalis* were different from those of *E. faecium*, tetracycline resistance being typical to the former and erythromycin resistance to the latter. Similarly, in the study of (Devirgiliis *et al*., 2010), high MIC values for tetracycline were found among 16 strains of *E. faecalis* isolated from Italian fermented dairy products. The presence of *tet*(M) was demonstrated by the resistant strains that pose a potential risk of horizontal transfer of the resistant gene among other food borne commensal bacteria.

E. faecalis strains isolated from Irish pork and beef abattoirs were susceptible to vancomycin, however, 4 of 10 strains of *E. faecium* were resistant to vancomycin but no corresponding genetic determinants for this phenotype were detected (Toomey *et al*., 2010). *E. faecium* isolated from an European probiotic product was found resistant to vancomycin using disc diffusion method but later it was confirmed by broth dilution and PCR that the isolates were vancomycin sensitive (Temmerman *et al*., 2003). Susceptibility of 128 isolates of *E. faecium* used as probiotic cultures was tested for 16 antimicrobial agents using broth microdilution. Two isolates were phenotypically resistant to erythromycin, 1 of which contained an *erm*(B) gene that was not transferable to enterococcal recipients (Vankerckhoven *et al*., 2008). In the study of Tompkins *et al.* (2008), MIC values for *E. faecium* R0026 for 17 antimicrobials were below the break-point values published by EFSA. The strain used in different commercial probiotic products was susceptible to gentamicin, streptomycin and vancomycin.

Use of growth promoters creates a major food animal reservoir of resistant bacteria, with a potential for spread to humans through food intake or by contact with animal (Wegener, 2003). Butaye *et al*. (2000) tested 76 *E. faecium* strains originated from poultry meat, cheese and raw pork for their susceptibility and resistance to growth-promoting antibacterials used in animals and antibiotics used therapeutically in humans. High-level of streptomycin resistance was observed in strains of all origins, though infrequently but the strains isolated from poultry meat showed more resistances against bacitracin, virginiamycin, narasin, tylosin (a macrolide antibiotic), ampicillin, glycopeptides avoparcin and vancomycin.

Enterococcus species can be found in the same habitat as of the *Listeria* species. Hence, these can be important sources of transferring antibiotic resistance through mobile genetic elements such as transposons to *Listeria*. A horizontal spread of resistance to *Listeria* spp. could be possible in some steps of the food production (Rizzotti *et al*., 2009).

Streptococcus

A strain of *S. thermophilus* isolated from a probiotic product available in Italy was found resistant only to ciprofloxacin among the tested antibiotics (Blandino *et al.*, 2008). D'Aimmo *et al.* (2007) reported that *S. thermophilus* was resistant

to cycloserine, kanamycin, metronidazole, nalidixic acid, neomycin, paromomycin, polymyxin B, spectinomycin, and streptomycin (MIC_{90} ranging from 64 to 500 µg per mL). It was found highly resistant to aztreonam having a MIC_{90} of 1000 µg per mL.

Antibiotic resistance of 39 srains of *S. bovis* representing the microflora of a typical Italian dairy product was found. It displayed high MIC values for tetracycline and the presence of *tet*(M) was detected in these strains. This poses a potential risk of horizontal transfer of antibiotic-resistance genes among foodborne commensal bacteria (Devirgiliis *et al*., 2010).

Bacillus

Bacillus strains have been increasingly proposed for prophylactic and therapeutic use against several gastro-intestinal diseases (Sorokulova *et al*., 2008). Reports suggest higher MIC for *Bacillus* strains. In the study of Luna *et al*. (2007), all *B. anthracis* isolates (18) were found resistant to trimethoprim/sulfamethoxazole. Only *B. thuringinesis* (19) was resistant to β-lactams, 3 of 42 isolate of *B. cereus*, 1 of 5 isolates of *B. mycoides* and all species of *B. pseudomycoides* (6 isolates) were resistant to clindamycin. Of 7 erythromycin resistant/intermediate *B. cereus* species, 3 were clindamycin resistant and 1 was both clarithromycin and clindamycin resistant. Vancomycin-resistant *B. cereus* was isolated from respiratory samples from patients in a paediatric intensive care unit of a hospital Kalpoe *et al*. (2008). *B. licheniformis* strain was reported to be resistant to chloramphenicol and clindamycin (Sorokulova *et al*., 2008).

Presence of mobile plasmid-encoded tetracycline resistance in the *B. cereus* group was mentioned in the EFSA opinion on QPS (European Food Safety Authority-EFSA, 2007). *B. brevis* and *B. firmus* intended to be used as biomass for animal feed were inappropriate for QPS (European Food Safety Authority-EFSA, 2008).

Lactococcus

Some potential risks are involved regarding the use of fermented foods that could act as potential vehicles for the spread of antibiotic resistance to consumers through the food chain. Tetracycline and erythromycin-resistance genes were found among the strains of *Lc. lactis*, representing the fermenting microflora of typical Italian traditional cheese Mozzarella di Bufala Campana. High MIC values for tetracycline were found for 26 strains while 17 strains showed high MIC values for both tetracycline and erythromycin (Devirgiliis *et al*., 2010).

Safety of probiotic foods

Lactobacillus, *Bifidobacterium*, *Pediococcus*, and *Lactococcus* have long history of use in food and extensively been used as probiotics (Shah, 2007). It is estimated that per capita consumption of fermented milk in Europe is 22 kg; this amounts to approximately 8.5 billion kg per year, a total of 8.5 x 10^{20} LAB (assuming 10^8 cfu per g), and 3400 tones of LAB cells (assuming each cell weighs 4 x 10^{-12} g) (Shah, 2010). US sales of probiotics were estimated to be worth $764 million in 2005 and were projected to be worth $1.1 billion in 2010. Sales of probiotics used in the manufacture of food supplements were projected to reach at $291.4 million in 2010, and food applications are expected to dominate the market, with sales estimated at $700 million in 2010 which include yogurts, kefir, and cultured drinks as major categories (Vanderhoof *et al*., 2008).

The most common microorganisms used in fermented products belong to the genera *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus*. Lactobacilli and bifidobacteria are important indigenous microbiotia of man and animals, rarely being implicated as cause of infection with quite few exceptions and generally recognized as safe (GRAS). However *B. dentium*, a causative agent of dental caries, was found to be pathogenic. Similarly, *B. animalis* naturally colonizes animal habitats, so its use in humans appears to be inappropriate because the criteria for a probiotic product consumed by humans must contain bacteria from human origin (D'Aimmo *et al*., 2007).

Based on safety records, microorganisms can be placed in 3 groups: safe strains (*Lactococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, *Oenococcus*, *S. thermophilus*, *Bifidobacterium*, *Carnobacterium*, *E. saccharolyticus*, and *E. faecium*), doubtful strains (*Enterococcus*, *L. rhamnosus*, *L. catenaforme*, *Vagococcus*, and *B. dentium*) and risky strains (*Peptostreptococcus*, and *Streptococcus*) (Mogensen, 2003). There are 3 theoretical concerns regarding the safety of probiotic organisms: (1) the occurrence of disease, such as bacteremia or endocarditis; (2) toxic or metabolic effects on the gastrointestinal tract; and (3) the transfer of antibiotic resistance in the gastrointestinal flora (Snydman, 2008).

According to Food and Agriculture Organisation (FAO)/WHO guidelines for the evaluation of

probiotics in food (2002), it is suggested that probiotic organisms may theoretically be responsible for side-effects including systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer. Regarding the safety assurance of probiotic organisms in food, FAO/ WHO guidelines (2002) suggest testing probiotic strains for antibiotic resistance patterns, certain metabolic (e.g., D-lactate production, bile salt deconjugation) and hemolytic potential, toxin production, side-effects, and epidemiological surveillance of adverse incidents during human studies and infectivity deficit in immunocompromised animals.

Animal studies

The safety concerning the use of these bacteria has not been doubted for many years. However, some of the members of genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* have been frequently reported to be the cause of various infections in patients with clinical conditions such as endocarditis and bloodstream infections (Gasser, 1994). There are many sources of exposure to these bacteria including probiotic preparations, fermented food products as well as the host's own microflora (Borriello *et al*., 2003). Since these organisms can adhere to epithelial lining and can survive gastric conditions, they may pose risks of translocation. They can translocate from the gastrointestinal lining to extraintestinal sites. They can enter regional lymph nodes, spleen, liver, blood vessels, and other tissues (Shou *et al*., 1994) causing systemic infections, bacteremia, septicemia and multiple organ faliure (Berg, 1992; Liong, 2008).

Indigenous microorganisms are not normally found in mesenteric lymph nodes, spleen, liver, or blood of healthy subjects. They are eliminated by the host's immune system as they attempt to translocate across the mucosal epithelium. Thus translocation of probiotic organism is not detected in most of the studies, in which probiotic organisms are administered even at high doses to healthy subjects (Liong, 2008). Lara-Villoslada *et al*. (2009) found that the strain *L. fermentum* CECT5716 orally administrated to Balb/c mice was non-pathogenic for mice even in doses 10,000 times higher (expressed per kg of body weight) than those normally consumed by humans.

Bacterial translocation does not occur commonly in healthy specific pathogen-free animals but it can be found for a long duration in germ-free mice (Ishibashi *et al*., 2001). Tanslocation was observed in sterile born mice; however, lactobacilli did not cause any harm and the organisms cleared in 2 to 3 weeks (Mogensen, 2003). *L. delbrueckii* ssp. *bulgaricus*, *L. rhamnosus*, and *B. lactis* did not translocate. Lara-Villoslada *et al*. (2007) carried out safety assessment of two probiotic strains including *L. coryniformis* CECT5711 and *L. gasseri* CECT5714 using 20 Balb/c mice which were orally treated with *L. coryniformis* CECT5711 or *L. gasseri* CECT5714 for 30 days and reported no treatment-associated bacterial translocation as these organisms were not present in liver or spleen. In another study, *L. fermentum* CECT5716, a probiotic strain isolated from human milk, was orally administered for 28 days to half of 40 Balb/c mice with a dose of 10^{10} colony forming units (cfu) per mouse per day and observed no bacteremia and no treatment-associated bacterial translocation to liver or spleen (Lara-Villoslada *et al*., 2009). Liong and Shah (2006; 2007) administered *L. casei* and *B. infantis* to 24 rats and no probiotics were detected in the spleen, liver, and kidney suggesting that the organisms were not translocated to these organs. (Tompkins *et al*., 2008) reported absence of both strains in the liver, kidneys, spleen or heart after 28-days repeated high-dose oral treatment of *E. faecium* R0026, and *Bacillus subtilis* R0179 used in Asian probiotic products, to 30 Sprague-Dawly albino rats.

Intestinal microflora of a subject also plays an important role in the prevention of probiotic translocation to internal organs. In a recent study by Gronbach *et al*. (2010), it was reported that if both intestinal microbiotia and adaptive immunity are defective, translocation across the intestinal epithelium and dissemination of probiotic bacteria such as *E. coli* Nissle could occur with potentially severe adverse effects. Although translocation of probiotic bacteria to internal organs of immunodeficient mice was observed in the study of Wagner *et al*. (1997), there was no evidence of increased inflammation or other pathologic findings in tissue sections from mice. Zhou *et al*. (2000) administered *L. acidophilus*. *B. lactis*, and *L. rhamnosus* to 78 mice at 3 levels including 5×10^7, 5×10^9, 5×10^{10} cfu per day and found that the organisms were safe, and no adverse effects were observed.

Animal model could be useful in evaluating the safety of new probiotics in immunocompromised hosts (Borriello *et al*., 2003). In most of experiments performed in mice, translocation of bacteria is usually observed in immuno-compromised subjects only but the response may vary with age of the animal. Wagner *et al*. (1997) suggested that the use of probiotic is likely to be safe for immunocompetent and immunodeficient adults, but they should be tested for safety in immunodeficient neonates.

In vitro and *in vivo* assessments of the safety of two species of *Bacillus*, including *B. subtilis* , and *B. indicus* as a food probiotic were carried out by Hong *et al*. (2008). The Natto strain of *B. subtilis* invaded and lysed cells but neither species was able to adhere significantly to any cell line. The Natto strain formed biofilms and none of strains produced any of the known *Bacillus* enterotoxins. Only *B. indicus* carried resistance to clindamycin at higher MIC than EFSA breakpoints. *In vivo* assessments of acute and chronic dosing in guinea pigs and rabbits, no toxicity was observed in animals under these conditions. The authors reported that *B. indicus* and *B. subtilis* were safe for oral use but further study is required regarding the transmissibility of clindamycin resistance of *B. indicus*.

The safety assessment of two *Bacillus* strains including *B. subtilis*, and *B. licheniformis* incorporated into a popular East European probiotic product was carried out. Both were non-hemolytic and did not produce Hbl or Nhe enterotoxins. Similarly, no *bceT* and *cytK* toxin genes were found. Study of acute toxicity in BALB/c mice demonstrated no treatment-related deaths. The oral LD_{50} for both strains was more than 2×10^{11} cfu per g. Chronic toxicity studies showed no signs of toxicity or histological changes in either organs or tissues of experimental animals. *B. subtilis* strain was sensitive to all antibiotics listed by the EFSA but *B. licheniformis* strain was resistant to chloramphenicol and clindamycin that enclosed safety risks of using *B. licheniformis* strain. However, *B. subtilis* strain was found to be non-pathogenic and safe for human consumption (Sorokulova *et al*., 2008).

Tompkins *et al*. (2008) carried out safety evaluation of 2 probiotic strains namely, *E. faecium* R0026 and *B. subtilis* R0179 used in Asian probiotic products and found absence of both diarrheal and emetic toxins in the latter strain. The authors established, on the basis of the results of this study in combination with the observations of clinical studies in both infants and adults, that these microbes were safe for use as pharmaceutical probiotics and pose low risk to the consumer.

Some of the studies have proposed beneficial effects of probiotic organisms in translocation and they have been tested to prevent bacterial translocation in animal model. The findings by Zareie *et al*. (2006) indicated that probiotic bacteria can prevent chronic stress induced intestinal abnormalities and, thereby, exert beneficial effects in the intestinal tract. Bacterial species such as enteric gram-negatives and gram-positive cocci are more prone to translocation, whereas lactobacilli appear to have a protective effect (Jeppsson *et al*., 2004). Administration of live lactobacilli including strains of *L. reuteri*, *L. plantarum* and *L. fermentum* to male Sprague-Dawley rats reduced the bacterial translocation (Adawi *et al*., 1997). This is supported by another study that showed probiotic supplementation containing *B. bifidum*, *L. acidophilus*, and *L. bulgaricus* (2×10^9 cfu per day) reduced bacterial translocation and decreased intestinal mucosal atrophy in male Sprague-Dawley rats with thermal injury (Gun *et al*., 2005). Moreover, in a rat model of small bowel syndrome, probiotic organisms decreased the bacterial translocation through mechanisms dependant on intestinal mucosal integrity (Mogilner *et al*., 2007).

Clinical cases

Documented correlations between systemic infections and probiotic consumptions are few and all occurred in patients with underlying medical conditions (Food and Agricultural Organization of the United Nations/ World Health Organization-FAO/WHO, 2002; Bernardeau *et al*., 2008). Many of the probiotic organisms have a safe history in patients receiving nutritional support, although some probiotic products have shown to increase the risk of complications in specific patient groups (Whelan *et al*., 2010).

Aguirre and Collins (1993) and Gasser (1994) have reviewed clinical cases involving LAB and bifidobacteria between 1938 and 1993, and the results are summarized in Table 3. Analysis of cases of infections revealed that out of 155 cases of infections involving LAB or bifidobacteria, 95 cases involved *Lactobacillus* spp., 33 of *Leuconostoc* spp., 18 of *Pediococcus* spp. and 9 cases involved *Bifidobacterium* spp. (Table 3) (Gasser, 1994). Endocarditis was the most frequent infection in which *Lactobacillus* species have been involved, in particular strains of species of *L. rhamnosus/casei* have been most often isolated.

Table 3. Clinical cases in which lactic acid bacteria or bifidobacteria have been isolated (Adapted from Mogensen *et al*., 2002)

Clinical outcome	Endocarditis	Bacteremia	Other infection	Total
Lactobacillus	7	8	19	34
L. acidophilus	3	3	2	8
L. casei	12	-	-	12
L. plantarum	11	2	1	14
L. rhamnosus	19	5	3	27
Bifidobacterium	-	9	-	9
Leuconostoc	2	23	8	33
Pediococcus	-	11	7	18
Total	54	61	40	155

Only about 180 cases of septicemia in humans involving LAB have been reported. In only 1 of

these cases, the identified LAB was identical with a commercially available dairy strain. *E. faecium* and *E. faecalis* are more frequently involved in clinical infection. In most cases of infection, people were reported to be infected by their own flora, however, in a few cases consumption of probiotic organisms was a potential source. About 30 cases of fungaemia have been reported in patients treated with *Saccharomyces boulardii* (Gasser, 1994), and 2 cases of infection were with food-borne *L. rhamnosus* (Mackay *et al*., 1999). In another report, 62 patients became colonized with *B. cereus* including 2 with non-fatal *Bacillus* sepsis and a death due to pneumoniae associated with the organism (Bryce *et al*., 1993).

Saxelin *et al*. (1996) studied the prevalence of bacteremia caused by *Lactobacillus* species in Southern Finland and compared the characteristics of the blood culture isolates with probiotic dairy strains. *Lactobacillus* was identified in eight of 3317 blood culture isolates; however, there was no isolate from dairy strain. In a 74-year-old woman with several years history of hypertension and non-insulin dependant diabetes mellitus, liver abscess was reported due to *L. rhamnosus* strain indistinguishable from *L. rhamnosus* strain GG (Rautio *et al*., 1999).

In a study by Kalliomäki *et al*. (2001), *L. rhamnosus* GG was given to 132 women who were at high risk of their babies developing atopic dermatitis. There was no report of adverse effects in mothers indicating that the probiotic organism was safe. Reports by Salminen *et al*. (2002) suggest that *L. rhamnosus* GG has been used widely in Finland since late 1980s and despite the long term use of this probiotic organism, there has been only few cases of bacteremia (0.05 cases per 100 000 cases).

Whelan and Myers (2010) reviewed of total of 1966 articles, of which they found 72 to fulfil the inclusion criteria. There were 20 case reports of adverse events in 32 patients, all of which were infections due to *L. rhamnosus* GG or *Saccharomyces boulardii*. The risk factors included central venous catheters and disorders associated with increased bacterial translocation. There were 52 articles reporting 53 trials in which 4131 patients received probiotic organisms. Most trials showed either no effect or a positive effect on outcomes related to safety (e.g., mortality and infections). Only 3 trials showed increased complications, which were largely non-infectious in nature and in specific patient groups (e.g., transplant and pancreatitis).

Cannon *et al*. (2005) reviewed 241 clinical cases of *Lactobacillus* infections and found 129 cases of bacteremia and 73 cases of endocarditis. *L. casei* and *L. rhamnosus* were most common species and the overall mortality was reported nearly 30%. Patients of all ages and both gender were afftected. The main underlying conditions were recognized as cancer, diabetes, transplantation particularly of liver, abscesses, and hypertension. Husni *et al*. (1997) reviewed 45 cases of *Lactobacillus* infections occuring over 15 years and the organisms causing infections were characterized. The common underlying conditions were cancer (40%), recent surgery (38%), and diabetes mellitus (27%). One in 39 deaths was attributed to *Lactobacillus* bacteremia. Cannon *et al*. (2005) recognized a very small percentage (1.7%) of cases associated with heavy dairy consumption, where 3 cases were associated with endocarditis and 1 with a liver abscess. A case of aortic valve endocarditis caused by *L. casei* in a 53-year-old immunocompetent patient with past history of rheumatic fever was reported by Zé-Zé *et al*. (2004). Noticeably clinical symptoms appeared after a dental extraction and the patient's diet included several tubs of yogurts per day. Presterl *et al*. (2001) reported a young man having diet comprising large quantities of probiotic yogurt developed endocarditis and septic arthritis caused by *L. rhamnosus*. However the contradictory findings were reported by Wallet *et al*. (2002), where a case of endocarditis due to *L. casei* subsp. *rhamnosus* was found in 73-year-old man without previous history of dental manipulation or daily yogurt intake. In relation to a consumption of about 20 million tons of fermented milk annually, the above numbers are negligible (Mogensen, 2003). There is no foundation for safety concern in relation to probiotic dairy products on the market today. Probiotic organisms are generally considered safe. As evidenced by epidemiologic studies, bacteremia or sepsis from lactobacilli is extremely rare. Numerous probiotic organisms have a long history of safe use and no health concerns have been observed. A long history of safe use is still the most credible safety test.

Conclusions

Selective pressure of using antibiotic in both human and animal treatment, and dissemination of antibiotic resistance bacteria has the possibility to aggravate acquisition and spread of resistant genes. In this context, probiotic organisms are considered to pool the resistant genes and transfer these to pathogenic bacteria. In order to eliminate this possibility, MIC of the most relevant antimicrobials for each strain used as a probiotic organism, food or feed additives could be determined using protocols given by EFSA and on firm genetic grounds. Several studies regarding

the antibiotic susceptibilities of LAB, bifidobacteria have been reviewed but only few have determined the genetic basis of these resistances. Majority of resistance found in the species of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Streptococcus* and *Bacillus* were of intrinsic type. Resistances to tetracycline, vancomycin and erythromycin were frequent in these species and some showed to harbour genes *tet*(W), *tet*(M), *van*(A) and *erm*(B) mostly on chromosome with only few on plasmid or transposon. Intrinsic resistance, and resistance due to mutation of chromosomal genes present a low risk of horizontal dissemination, and such strains should be acceptable for food consumption. However, acquired resistance mediated by added genes may present a risk for public health. Starter culture bacteria in dairy products do not appear to represent an important source for the spread of genes encoding resistance to antimicrobial agents. However antibiotic resistance profiles of novel strains used as starters or probiotics in dairy products must be checked for fermented dairy products. In case of *Enterococcus* strains, resistance genes *van*(A), *tet*(L), and *tet*(M) were often detected and 2 reports have found enterococci to transfer *tet*(M) to *E. faecalis* or *Listeria* strains and *van*(A) to a commercial strain *L. acidophilus*.

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