Antibiotic Resistance in *Lactobacillus reuteri* and *Lactobacillus plantarum*

Maria Egervärn
Faculty of Natural Resources and Agricultural Sciences
Department of Microbiology
Uppsala

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Cover: Antibiotic susceptibility testing by Etest (left; photo: M. Egervärn) and broth microdilution (right; reproduced with the permission of the National Veterinary Institute, Uppsala, Sweden).

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Abstract

Lactic acid bacteria (LAB) may act as reservoirs of antibiotic resistance genes that can be transferred via the food chain or within the gastrointestinal tract to pathogenic bacteria. This thesis provides data required for assessing the potential risk of using antibiotic resistant strains of the LAB species *Lactobacillus reuteri* and *Lactobacillus plantarum* as food processing aids or probiotics. Knowledge of the distributions of antibiotic minimum inhibitory concentrations (MICs) for a species is needed when using a phenotypic method to differentiate strains with acquired resistance from susceptible strains or strains with intrinsic resistance. Controlled and standardised conditions are required for antibiotic susceptibility testing of LAB, as demonstrated here during evaluation of the Etest and broth microdilution MIC determination methods used. Inoculum size and incubation time were varied during broth microdilution testing of the susceptibilities of 35 LAB strains to six antibiotics. An increase in either parameter resulted in elevated MICs for all species.

Antibiotic susceptibility profiles were determined for 56 *L. reuteri* and 121 *L. plantarum* strains that differed by source and spatial and temporal origin. MIC data obtained with the Etest and the broth microdilution methods corresponded well with each other. All *L. plantarum* strains were susceptible to ampicillin, gentamicin, erythromycin and clindamycin, and intrinsically resistant to streptomycin. Acquired resistance to tetracycline was associated with plasmid-bound *tet*(M).

Lactobacillus reuteri strains had acquired resistance to tetracycline (n=28), ampicillin (n=14), erythromycin/clindamycin (n=6) and chloramphenicol (n=1). This resistance was attributed to mutational *pbp* genes for ampicillin and to added *tet*(W), *erm* and *cat*(TC) genes for the antibiotics inhibiting protein synthesis. Genetic relatedness was observed among *L. reuteri* strains with high MICs for both ampicillin and tetracycline and among strains with high MICs for both erythromycin and clindamycin. The majority of the antibiotic resistant *L. reuteri* strains carried the resistance genes on plasmids. Traits of putative transfer machineries adjacent to both plasmid- and chromosome-located resistance genes were demonstrated.

Lactobacillus reuteri as a donor of resistance genes in the human gut was investigated by studying the transferability of the tetracycline resistance gene *tet*(W) to faecal enterococci, bifidobacteria and lactobacilli. No gene transfer was demonstrated under the conditions tested.

Keywords: *Lactobacillus reuteri*, *Lactobacillus plantarum*, antibiotic susceptibility testing, MIC, tetracycline resistance, *tet* genes, erythromycin resistance, *erm* genes, gene transferability.

Author's address: Maria Egervärn, National Food Administration
P.O. Box 622, 751 26 Uppsala, Sweden. *E-mail:* mia.egervarn@slv.se

Contents

List of Publications

This thesis is based on the work contained in the following papers, which are referred to in the text by their Roman numerals:

I Egervärn, M., Lindmark, H., Roos, S., Huys, G. and Lindgren, S. (2007). Effects of inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. *Antimicrobial Agents and Chemotherapy* 51(1), 394-396.

II Egervärn, M., Danielsen, M., Roos, S., Lindmark, H. and Lindgren, S. (2007). Antibiotic susceptibility profiles of *Lactobacillus reuteri* and *Lactobacillus fermentum*. *Journal of Food Protection* 70(2), 412-418.

III Flórez, A.B., Egervärn, M., Danielsen, M., Tosi, L., Morelli, L., Lindgren, S. and Mayo, B. (2006). Susceptibility of *Lactobacillus plantarum* strains to six antibiotics and definition of new susceptibility-resistance cutoff values. *Microbial Drug Resistance* 12(4), 252-256.

IV Egervärn, M., Roos, S. and Lindmark, H. (2009). Identification and characterisation of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*. Accepted for publication in *Journal of Applied Microbiology*.

V Egervärn, M., Lindmark, H. and Roos, S. Transferability of a tetracycline resistance gene from probiotic *Lactobacillus reuteri* to bacteria in the gastrointestinal tract of humans (manuscript).

Papers I-IV are reproduced with the permission of the publishers.

My contribution to the papers included in this thesis was as follows:

I Took part in planning the study and analysing the results. Performed all the laboratory work. Main writer of the manuscript.

II Took part in planning the study and analysing the results. Performed the laboratory work concerning *L. reuteri*. Main writer of the manuscript.

III Took part in planning the study and analysing the results. Performed the laboratory work on 45 of the *L. plantarum* strains. Minor part in writing the manuscript.

IV Major part in planning the study, analysing the results, performing the laboratory work and writing the manuscript.

V Major part in planning the study and analysing the results. Performed all the laboratory work. Wrote the manuscript.

Abbreviations

ACE-ART	Assessment and critical evaluation of antibiotic resistance transferability in food chain
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute (previously NCCLS)
EFSA	European Food Safety Authority
EUCAST	European Committee for Antimicrobial Susceptibility Testing
LAB	Lactic acid bacteria
LSM	LAB susceptibility test medium
MIC	Minimum inhibitory concentration (μg/mL)
MLS_B	Macrolide, lincosamide, streptogramin B compound
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
RPP	Ribosomal protection protein

1 Introduction

Lactic acid bacteria (LAB), like all other bacteria, can exchange genes to enhance their survival in antibiotic-containing habitats (Teuber *et al.*, 1999). The close contact between LAB and other bacteria, *e.g.* in the intestine, on mucosal surfaces or in food, is a precondition for horizontal gene transfer by mobile genetic elements. Ingested and indigenous LAB may therefore contribute to the pool of antibiotic resistance genes that can be transferred via the food chain or within the gastrointestinal tract to other commensal bacteria or to pathogens (Teuber *et al.*, 1999; Salyers *et al.*, 2004).

Antibiotic resistance of LAB used for food, feed and probiotic applications has recently been proposed as a hazard due to the potential risk for transfer to pathogenic bacteria. The European Commission has, as advised by the European Food Safety Authority (EFSA), requested that bacterial strains harbouring transferable antibiotic resistance genes should not be used in animal feeds (European Parliament and Council Regulation EC 429/2008; EC, 2001). No legislation exists so far regarding microorganisms intentionally added to fermented food and probiotics for human use. However, based on the precautionary principle, it is recommended that these products follow similar requirements to feed additives (EFSA, 2007). Measures based on this principle should be considered provisional until more comprehensive data concerning the risk are obtained and analysed.

The present investigation was initially performed as part of the EU project ACE-ART (CT-2003-506214; Morelli, 2008), which aimed to assess and critically evaluate antibiotic resistance transferability in the food chain. The intention was to obtain data related to phenotypic susceptibility testing, identification and characterisation of resistance genes and the potential for gene transferability of different LAB species, and through this prove or discount the current strategy based on the precautionary principle.

1.1 Aims

The overall aim of this thesis was to provide data required for assessing the potential risk of using antibiotic resistant strains of the LAB species *Lactobacillus reuteri* and *Lactobacillus plantarum* as starter cultures or probiotics. Specific objectives were to:

- Evaluate the effects of inoculum size and incubation time on the antibiotic susceptibilities of LAB using a broth microdilution method (**I**), and compare minimum inhibitory concentration (MIC) data obtained by broth microdilution with an Etest method (**II** and **III**).
- Determine antibiotic susceptibility profiles of *L. reuteri* of animal and human origin (**II**) and *L. plantarum* of dairy and vegetable origin (**III**).
- Investigate the genetic relatedness of the *L. reuteri* strains with atypical antibiotic MICs (**II**).
- Identify and characterise the resistance genes mediating atypical antibiotic MICs in *L. reuteri* and *L. plantarum* (**IV**).
- Investigate the transferability of an antibiotic resistance gene from a probiotic *L. reuteri* strain to bacteria in the intestinal tract of humans (**V**).

2 Antibiotic resistance

The rapid emergence of antibiotic resistant bacteria is a major threat to public health (ECDC, 2007; WHO, 2007b), the obvious concern being that infections might no longer be treatable with antibiotics. Effective antibiotics are also essential tools for organ transplantation, cancer chemotherapy and orthopaedic surgery (Cars *et al.*, 2008). Surveillance data have shown an increasing incidence of infections caused by antibiotic resistant pathogens in many countries (EARSS, 2007). For example, the prevalence in blood cultures of *Klebsiella pneumoniae* producing extended-spectrum betalactamases has now approached 50% in some regions of Eastern Europe (EARSS, 2007). In Sweden too, an increase in antibiotic resistant bacteria has occurred during recent years (Fig. 1). However, the situation here is better than in most other countries.

Failure of antibiotic treatment for an infection usually leads to serious consequences for the patient and is associated with increased health care costs for society. In a recent study of more than 1800 Tanzanian children with signs of systemic infection, the mortality rate from Gram-negative bloodstream infections was more than double that of malaria (44% compared with 20%) (Blomberg *et al.*, 2007). Two American studies showed that the mortality rate from bacteraemia and the hospital costs for surgery-related infections were both on average twice as high for patients infected with meticillin resistant *Staphylococcus aureus* than for patients infected with meticillin susceptible bacteria (Cosgrove *et al.*, 2003; Engemann *et al.*, 2003).

In addition, only two new antibiotic classes (oxazolidinones and lipopeptides) have been developed since the 1960s and few new drugs are currently in the pipeline (Cars *et al.*, 2008). All this has raised concerns about a 'post-antibiotic era', a period with no effective antibiotics available. Quinolones, third/fourth-generation cephalosporins and macrolides were

therefore recently classified by the WHO (2007a) as critically important antibiotics for which urgent risk management strategies are needed to preserve their efficacy within human medicine.

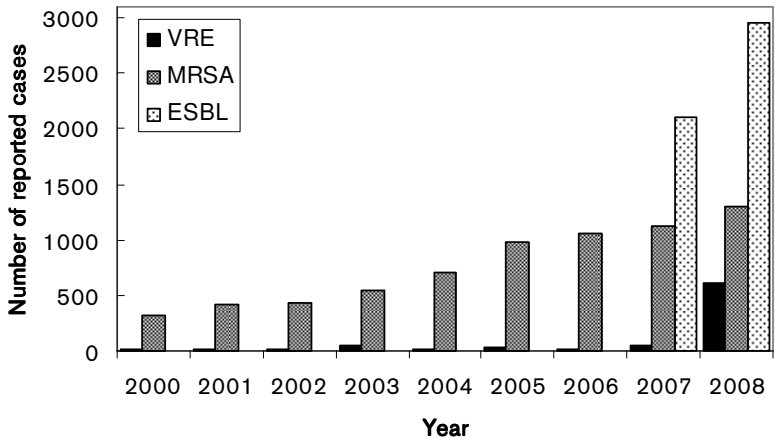

Figure 1. Number of vancomycin resistant enterococci (VRE), meticillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum betalactamase-producing *Enterobacteriaceae* (ESBL) notified annually in Sweden 2000-2008. ESBL became notifiable in 2007 (SMI, 2009).

2.1 Development of antibiotic resistant bacteria

The emergence of antibiotic resistant bacteria is primarily caused by the excessive and inappropriate use of antibiotics in human and veterinary medicine, animal husbandry, agriculture and aquaculture (Tenover & Hughes, 1996). Antibiotic prescriptions for viral infections and the use of broad-spectrum agents such as cephalosporins and fluoroquinolones when the bacteria causing the infection are unknown are examples of such misuse. Antibiotic exposure selects for resistant bacteria and kills (bactericidal drugs) or inhibits the growth (bacteriostatic drugs) of the susceptible population. Because the same antibiotic classes are used in humans and in animals, bacteria and their resistance genes can be disseminated from one host to another, *e.g.* with food as an intermediate between food-producing animals and humans (Levy, 1997). Poor hygiene in the animal and human environment, including primary production/slaughter, hospitals and the community, increases the spread of resistant bacteria, as does the crowding

of animals on farms and of old and immunocompromised people in hospitals and nursing homes. Increased travel and the worldwide distribution of food are other factors facilitating the rapid global spread (Cars *et al.*, 2008).

To reduce the development of resistant bacteria in food-producing animals, Sweden was the first country to ban the use of antibiotics for growth promotion (Swedish act SFS 1985:295 on feed). In 1998, veterinary

Table 1. *Antibiotics, their classes, modes of action and mechanisms of resistance (modified from Guardabassi & Courvalin, 2006)*

Antibiotic	Class	Mode of action	Major resistance mechanism
Ampicillin	Betalactams	Inhibit transpeptidation step in peptidoglycan synthesis by binding to the PBPs	Betalactamases; mutations in the PBPs
Vancomycin	Glycopeptides	Bind to D-Ala-D-Ala peptidoglycan precursors, making them inaccessible to the PBPs	Peptidoglycan precursors terminating by D-Ala-D-lactate
Streptomycin, amikacin, gentamicin, kanamycin, neomycin	Aminoglycosides	Bind 30S ribosomal subunit	Aminoglycoside modifying enzymes
Linezolid	Oxazolidinones	Bind 30S ribosomal subunit	Mutation of 23S rRNA
/Oxy/tetracycline	Tetracyclines	Bind 30S ribosomal subunit	Ribosome protection; efflux
Erythromycin	Macrolides	Bind 50S ribosomal subunit	Methylation or mutation of 23S rRNA; efflux
Clindamycin	Lincosamides	Bind 50S ribosomal subunit	Methylation or mutation of 23S rRNA; efflux
Dalfopristin, quinupristin	Streptogramins	Bind 50S ribosomal subunit	Methylation or mutation of 23S rRNA; efflux
Chloramphenicol	Phenicols	Bind 50S ribosomal subunit	Chloramphenicol acetyltransferases
Trimethoprim /sulfonamide	Trimethoprim /sulfonamides	Inhibit tetrahydrofolate pathway	Mutations in the target enzymes

medicine accounted for around 50% of global consumption of antibiotics, of which the majority were used for prophylaxis and growth promotion (Wise *et al.*, 1998). Since then, the European Union has banned the use of antibiotics for the latter purpose (European Parliament and Council Regulation EC 1831/2003), but in the US and many developing countries antibiotics are still used as growth promoters.

2.2 Antibiotic targets

Antibiotics are antibacterial compounds that interfere with some structure or process that is essential to bacterial growth or survival, although in most cases harmless to the eukaryotic host harbouring the infecting bacteria. Antibiotics have three main bacterial targets: (i) cell-wall synthesis (*e.g.* betalactams, glycopeptides); (ii) protein synthesis (*e.g.* macrolides, tetracyclines and aminoglycosides); and (iii) DNA replication and repair (*e.g.* fluoroquinolones) (Walsh, 2000). Because lactobacilli are mainly associated with resistance to macrolides and tetracyclines, the mode of action and resistance mechanisms of these drugs are described in detail below. Antibiotics relevant for susceptibility testing of LAB are summarised in Table 1.

2.3 The ribosome as antibiotic target

Many chemically diverse antibiotics act by binding to the bacterial ribosome and thereby interfering with the synthesis of new proteins. High resolution atomic structures of the ribosome obtained by X-ray crystallography have recently revealed how some of these antibiotics target the ribosome (Fig. 2A), as reviewed by Poehlsgaard & Douthwaite (2005). Most of the antibiotics bind reversibly to the ribosome and are bacteriostatic at therapeutic concentrations.

Ribosomes translate the genetic code via messenger RNA (mRNA) to assemble amino acids into proteins. The bacterial ribosome (Fig. 2B) is made up of a small subunit (30S) and a large subunit (50S), both of which consist of a number of ribosomal proteins (r-proteins) and ribosomal RNAs (rRNAs), including 16S rRNA in 30S and 23S rRNA and 5S rRNA in 50S. The 30S subunit contains the A (aminoacyl) site, where the aminoacylated transfer RNA (tRNA) attaches and in which is located the decoding site, where each codon of the mRNA chain interacts with an aminoacylated tRNA anticodon. The 50S subunit contains the catalytic site, *i.e.* the peptidyl-transferase centre responsible for peptide bond formation,

as well as the tunnel through which the newly synthesised peptide chain is channelled before leaving the ribosome (Fig. 2B).

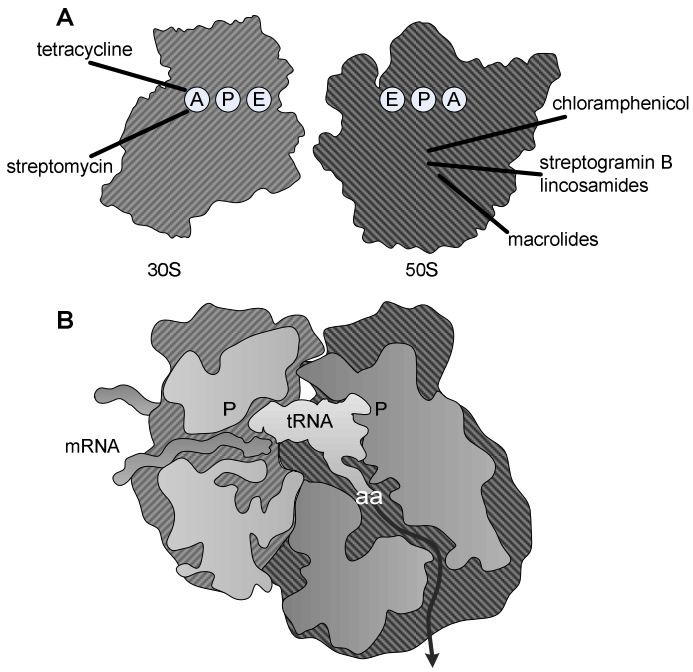

Figure 2. Structure of the small (30S) and large (50S) ribosomal subunits of bacteria with the ribosomal binding sites of some antibiotics inhibiting protein synthesis. A=aminoacyl, P=peptidyl-transferase centre, and E=exit (A). The associated subunits (70S) and the interaction with mRNA and tRNA (B). Reproduced with the permission of the publisher (modified from Poehlsgaard & Douthwaite, 2005).

2.3.1 Macrolides

Macrolides are active against Gram-positive bacteria and many Gram-negative potential pathogens such as *Campylobacter*, *Legionella*, *Chlamydia*, *Helicobacter* and *Mycoplasma*. Within Swedish veterinary medicine, macrolides are especially used for therapeutic group treatment of pigs (SVARM, 2007). This family of antibiotics has also been used non-clinically, as growth promoters in food-producing animals.

Macrolides consist of a macrocyclic lactone ring of between 14 atoms (*e.g.* erythromycin, see Fig. 3A) and 16 atoms (*e.g.* tylosin) with different substituents. Erythromycin and tylosin are naturally occurring antibiotics

synthesised by actinomycete bacteria, whereas newer macrolides such as azithromycin and clarithromycin are semi-synthetic compounds with substitutions on the lactone ring resulting in fewer side effects and better pharmacological properties. Ketolides, *e.g.* telithromycin, are the latest generation of derivatives with activity against some erythromycin resistant streptococci and staphylococci (Schulin *et al.*, 1998; Malathum *et al.*, 1999).

All macrolides target the 23S rRNA nucleotide A2058 within the 50S subunit tunnel, thereby blocking peptide chain elongation. Lincosamides (*e.g.* lincomycin and clindamycin) and streptogramin B compounds (*e.g.* quinupristin) differ chemically from macrolides, but also interact with nucleotides of the 23S rRNA, including A2058. Crystallographic data on the superposition of these so-called MLS_B antibiotics in the ribosomal tunnel show that the structures clearly overlap (reviewed by Poehlsgaard & Douthwaite, 2005).

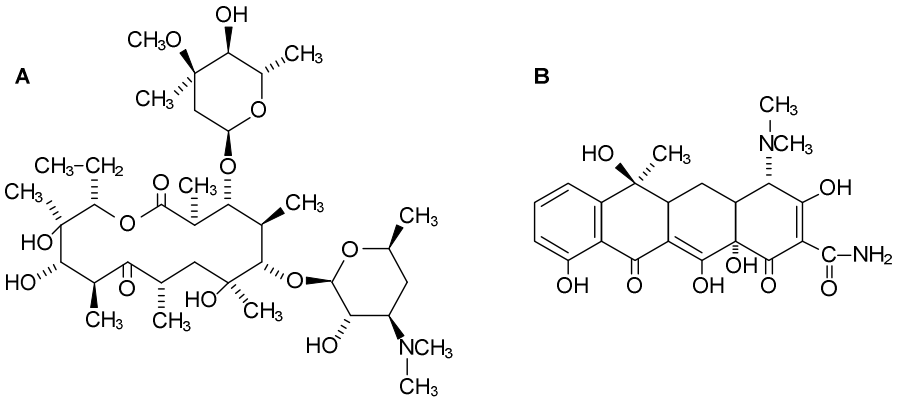

Figure 3. Chemical structure of erythromycin (A) and tetracycline (B).

2.3.2 Tetracyclines

Tetracyclines are broad-spectrum antibiotics, with activity against Gram-positive and Gram-negative bacteria, *Chlamydia* spp., *Mycoplasma* spp., *Rickettsiae* spp. and some protozoa (summarised by Roberts, 2003b). Tetracyclines are the most commonly prescribed antibiotics within Swedish human medicine after betalactams (Swedres, 2007) and are also widely used in veterinary medicine, especially for therapeutic group treatment of pigs (SVARM, 2007). Furthermore, tetracyclines have been used in horticulture (McManus *et al.*, 2002), and are still used as growth promoters in some countries, including the United States.

Tetracyclines consist of four linear fused cyclic six-membered rings with different substituents attached. Tetracycline (Fig. 3B), chlortetracycline and oxytetracyline are produced by *Streptomyces* spp., whereas newer derivatives such as doxycycline and minocycline are semi-synthetic compounds. The third and latest generation of tetracyclines is glycylcyclines, *e.g.* tigecyclin (former GAR-936), which has a bulky side chain designed to overcome resistance in some bacteria (Petersen *et al.*, 1999).

Uptake of tetracyclines into the bacterial cytoplasm favours the chelation of tetracycline with magnesium ions (Chopra & Roberts, 2001). These metal-drug complexes target the A site of the 30S bacterial ribosomal subunit, causing conformational changes and thereby physically preventing the binding of aminoacylated tRNA to the ribosome. Tetracycline primarily interacts with the H34 region of the 16S rRNA at the A-site, which is involved in the binding of aminoacylated tRNA. This was verified by crystallographic structures of the 30S ribosomal subunit, complexed with tetracycline (Brodersen *et al.*, 2000; Pioletti *et al.*, 2001). The functional relevance of the five additional binding sites found is currently not clear. The crystallographic studies in combination with a recent report on simulation of molecular dynamics (Aleksandrov & Simonson, 2008) suggest that the 'upper' part of the tetracycline molecule confers receptor binding specificity, whereas the 'lower' part controls magnesium binding.

2.4 Resistance mechanisms

Bacteria utilise four major strategies to become resistant to antibiotics (Fig. 4): Decreased intracellular antibiotic concentration by altering cell wall permeability (A) or by efflux (B); enzymatic inactivation of the antibiotic (C); and modification of the antibiotic target (D).

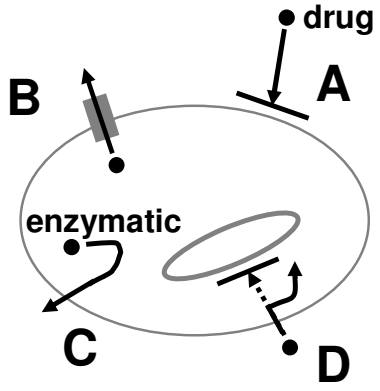

Figure 4. Major mechanisms of resistance to antibiotics in a bacterial cell.

2.4.1 Macrolide resistance

The most common macrolide resistance mechanism in both Gram-positive and Gram-negative bacteria is target site modification caused by methylation or mutations. There are currently 33 described *erm* genes, encoding methyl transferases, which specifically methylate nucleotide A2058 in the 23S rRNA of the 50S ribosomal subunit (Roberts, 2008). Addition of one methyl group by *e.g. erm*(N) confers high resistance to lincosamides, whereas addition of two methyl groups by *e.g. erm*(E) confers high resistance to all MLS_B antibiotics, including telithromycin (Liu & Douthwaite, 2002). *Erm* genes are often linked via mobile genetic elements to other resistance genes, especially genes conferring tetracycline resistance (Roberts *et al.*, 1999).

Macrolide and/or MLS_B resistance can also be caused by point mutations at A2058 or at adjacent nucleotides in the 23S rRNA and in the ribosomal proteins. Details of various mutations found in *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Haemophilus influenzae* have been reported by Franceschi *et al.* (2004). Resistance caused by chromosomal mutations generally presents a low risk of horizontal gene transfer, see below.

Another major resistance mechanism is through efflux pumps, which transport macrolides out of the cell, thereby reducing the intracellular drug concentration. So far, there are 14 described genes encoding these proteins. Efflux pumps confer diverse levels of resistance to the different MLS_B antibiotics (reviewed by Roberts *et al.*, 1999; Roberts, 2008). The *mef*(A) gene, encoding specific macrolide efflux, has been found in a variety of Gram-positive bacteria, including *S. pyogenes*, where it was first described (Clancy *et al.*, 1996). Roberts and co-workers (1999) standardised the nomenclature for genes conferring macrolide and MLS_B resistance.

2.4.2 Tetracycline resistance

Tetracycline resistance is now so widespread among bacteria that the utility of the tetracycline family has been considerably diminished. There are two ribosome-related mechanisms of tetracycline resistance in bacteria, both of which are linked to the primary binding site of the drug on the ribosome. This resistance is caused by ribosomal protection proteins (RPPs; Chopra & Roberts, 2001) or, more rarely, by mutations at nucleotide 1058 of the 16S rRNA (Ross *et al.*, 1998).

The nomenclature and characterisation of 38 *tet/otr* genes conferring /oxy/tetracycline resistance have previously been reviewed by Roberts *et al.* (2005). A 42nd *tet* gene was recently found (Brown *et al.*, 2008), reflecting the ongoing increase in knowledge in the field. Eleven of these genes (*e.g.*

tet(M), *tet*(O) and *tet*(W)) encode RPPs, which are cytoplasmic proteins with some sequence homology to elongation factors EF-Tu and EF-G. The RPPs are thought to interact with the H34 protein of the 16S rRNA at the A site, causing allosteric disruption of the primary binding site and thereby releasing tetracycline from the ribosome (Connell *et al.*, 2003). Most of these genes have been found in both Gram-negative and Gram-positive bacteria.

Resistance to tetracycline is also commonly associated with efflux proteins. There are 26 *tet* efflux genes described so far, all of which confer resistance to tetracycline and doxycycline. Two of these genes, *tet*(K) and *tet*(L), are primarily found in Gram-positive bacteria. Efflux genes and genes encoding RPPs are both commonly associated with mobile genetic elements (Roberts, 2005).

2.5 Transfer of resistance genes

Antibiotic resistance traits are passed to daughter cells during replication of a bacterial strain (clone), so-called vertical or clonal dissemination. Horizontal transmission can provide a bacterial host of a different strain, species or even genus with genetic information that can be transiently needed, such as antibiotic resistance genes (Courvalin, 2006). Antibiotic exposure favours bacteria that have acquired resistance determinants at the expense of the susceptible population. Subsequently, wild-type bacteria stop proliferating or die, enabling the resistant bacteria to increase in abundance.

2.5.1 Horizontal gene transfer

There are three known mechanisms for horizontal transfer of resistance genes: conjugation, transformation and transduction. Conjugation is believed to be the most important mode of transfer within the gastrointestinal microbiota (reviewed by Licht & Wilcks, 2006). By this mechanism mobile genetic elements such as plasmids and transposons are transferred from one live bacterium to another through a protein tunnel that temporarily physically connects the bacteria. In Gram-negative bacteria, cell to cell contact is achieved by the formation of sex pili, whereas in Gram-positive bacteria it is achieved through uncharacterised cell surface structures (Grohmann *et al.*, 2003).

Both plasmid-encoded and chromosome-located resistance genes can be transferred via conjugation. Conjugative plasmids are larger than approximately 15 kb and are self-transmissible due to the presence of the origin of transfer (*oriT*) gene and transfer (*tra*) genes. Unlike plasmids,

conjugative transposons are chromosomal elements that cannot replicate by themselves, but can move within the genome, *e.g.* from plasmid to plasmid or from chromosome to plasmid and *vice versa* (Salyers *et al.*, 1995). Smaller, non-conjugative plasmids that carry mobilisation (*mob*) genes and the *ori*T sequence can be mobilised by taking advantage of the transfer machinery provided by conjugative elements (Francia *et al.*, 2004). Studies on the *in vivo* conjugative transfer of resistance genes in the gastrointestinal microbiota of various animals have recently been reviewed by Licht and Wilcks (2006).

Horizontal gene transfer can also occur by transformation, where DNA released from one bacterium is taken up by a competent bacterium, and by transduction, where DNA is transferred from one bacterium to another via bacteriophages (Licht & Wilcks, 2006). In both cases, the DNA generally must incorporate into the recipient genome by homologous recombination (Frost *et al.*, 2005; Thomas & Nielsen, 2005). This implies narrow-host-range gene transfer, which is in contrast to the much broader host range of *e.g.* conjugative transposons (Salyers *et al.*, 1995). However, non-homologous recombination is possible during both transformation and transduction, *e.g.* if plasmid DNA is acquired (Frost *et al.*, 2005; Thomas & Nielsen, 2005). The frequency of gene transfer depends on the genetic material to be transferred, the transfer mechanism, the concentrations of donor and recipient strains and the contacts between these, and the selection pressure (Donohue *et al.*, 1998).

2.6 Intrinsic and acquired resistance

Bacterial resistance to antibiotics can either be intrinsic or acquired (Courvalin, 2006). Intrinsic resistance is an inherent trait of bacteria and can be due to reduced permeability of certain antibiotics across the cell wall, absence of the antibiotic target or the presence of low affinity targets. Glycopeptide resistance of heterofermentative lactobacilli is an example of intrinsic resistance and is caused by peptidoglycan dipeptides terminating with D-lactate instead of D-alanine, the cell wall target precursor for glycopeptide activity (Billot-Klein *et al.*, 1994; Klein *et al.*, 2000). In contrast, a bacterial strain can acquire resistance either by mutation in indigenous genes or by the uptake of exogenous resistance genes by horizontal transfer from other bacteria. By their nature, intrinsic resistance and resistance due to chromosomal mutation pose a low or even negligible risk for horizontal spread. Added resistance genes, especially those carried by mobile genetic elements, may be more easily transferred between bacteria.

2.6.1 Microbiological breakpoints

Bacteria not responding even to maximum doses of a given antibiotic during an infection are defined as clinically resistant. Clinical breakpoints, based on pharmacokinetic, clinical and microbiological data, are used by clinicians to advise on antibiotic therapy in patients (EUCAST, 2000). Bacteria that have acquired resistance mechanisms can grow at a higher antibiotic concentration than the more susceptible wild-type population and are defined as microbiologically resistant. Consequently, the resistant bacteria exhibit a higher MIC, *i.e.* the lowest concentration of an antibiotic that inhibits bacterial growth.

Microbiological breakpoints, referred to as epidemiological cut-off values by the European Committee for Antimicrobial Susceptibility Testing (EUCAST), are used for distinguishing between strains with and without acquired resistance genes (White *et al.*, 2001). This categorisation is specific for each species and antibiotic and is based on the distribution of antibiotic MICs for a representative number of bacterial strains; 300-600 according to White *et al.* (2001). Theoretically, a uniform MIC distribution in the lower antibiotic concentration range indicates that all strains are susceptible; a uniform distribution with a high MIC for all strains may be due to an intrinsic trait; and a bimodal MIC distribution indicates that the strains with a high, atypical MIC may have acquired resistance. However, this differentiation is not always clear-cut in practice.

2.6.2 Phenotypic methods

Several methods have been used for phenotypic assessment of the antibiotic susceptibility of bacteria. Agar dilution and broth microdilution (right figure on front page) are dilution methods in which bacterial strains are tested for their ability to produce visible growth on a series of agar plates or in broth in microtitre wells containing serial two-fold dilutions of different antibiotics. The MIC is read as the first plate or well without growth. Dilution methods are the reference methods for antibiotic susceptibility testing in aerobic (CLSI, 2008) and anaerobic bacteria (CLSI, 2007).

The Etest (left figure on front page) is a commercial product and comprises a preformed, predefined gradient of antibiotic concentrations on a plastic strip. When the strip is applied to an inoculated agar plate, the gradient is transferred to the agar and established along the strip. The MIC is read where the edge of the elliptic inhibition zone formed intersects the Etest strip. Another diffusion method is disc diffusion, which comprises a disc containing a specific antibiotic concentration. Radial diffusion of the antibiotic into the inoculated agar creates an inhibition zone, the diameter

of which is subsequently measured. In contrast to the latter method, the Etest and both dilution methods give MICs, *i.e.* quantitative data on the bacterial strains tested.

3 Lactobacilli

3.1 Occurrence and taxonomy

Lactobacilli are fastidious bacteria, found in a variety of nutrient-rich environments such as meat and dairy products, plant material, animal and human mucosal surfaces, and sewage and manure (Hammes & Hertel, 2006).

The genus *Lactobacillus* is a heterogeneous group of LAB, exemplified by the genome G+C content ranging from 32 to 54% of the species included (Schleifer & Ludwig, 1995). Genetic characterisation has also revealed that the former classification of lactobacilli into obligately homofermentative, facultatively heterofermentative and obligately heterofermentative (summarised by Hammes & Vogel, 1995) is only partly connected to phylogeny. Sequencing of the 16S rRNA genes has been used to divide the more than one hundred currently identified *Lactobacillus* spp. into seven (Hammes & Hertel, 2006) or 12 (Felis & Dellaglio, 2007) phylogenetic groups. However, the grouping of the genus may be subject to future changes as more whole-genome sequences become available and new species are continuously identified (Claesson *et al.*, 2008). The phylogenetic diversity of lactobacilli is displayed in Fig. 5.

3.1.1 Occurrence and taxonomy of *L. reuteri* and *L. plantarum*

Lactobacillus reuteri is an obligate heterofermenter belonging to the *L. reuteri* phylogenetic group, whereas *L. plantarum* is a facultative heterofermenter belonging to the *L. plantarum* phylogenetic group (Hammes & Hertel, 2006). These bacteria occur naturally in the gastrointestinal, vaginal and oral tracts of humans and other warm-blooded animals, and *L. plantarum* is also found naturally in plant material (Hammes & Hertel, 2006).

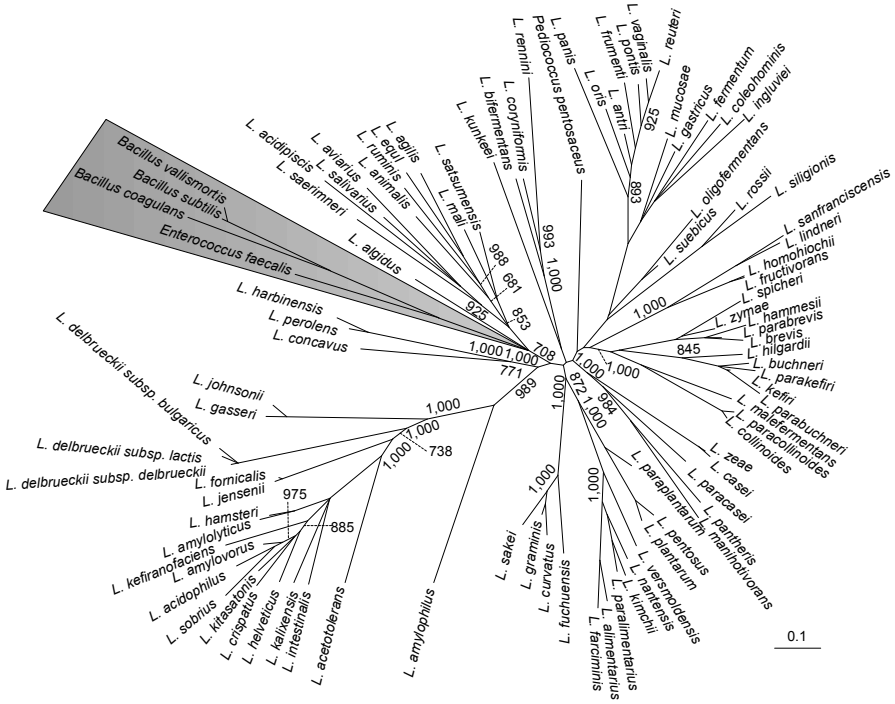

Figure 5. Evolutionary relationship between lactobacilli based on a neighbour-joining tree of 16S rRNA gene sequences. Bootstrap values above 600 are indicated. Outgroups are shaded in grey. Scale bars indicate 0.1 nucleotide substitutions per site. Reproduced with the permission of the publisher (modified from Ventura *et al.*, 2009).

3.2 Industrial applications

The two major applications of lactobacilli are as starter cultures of food and feed and as probiotics. In fermentations, lactobacilli are either present as natural contaminants, added as a component of a previous batch or added as a pure or mixed culture. The purpose is to affect flavour and texture and to improve the safety and shelf-life of the final product. Examples of fermented products inoculated with lactobacilli include meat products, dairy products, sourdough, vegetables and silage (Hammes & Hertel, 2006).

Probiotics are defined as 'live organisms, which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001). Species of *Lactobacillus* and *Bifidobacterium* are the bacteria most commonly used as probiotics (Saxelin *et al.*, 2005). Specific human strains of

e.g. L. casei, *L. johnsonii*, *L. rhamnosus*, *L. plantarum* and *L. reuteri* have been shown to be protective against a variety of gastrointestinal infections and allergic disorders (reviewed by Saxelin *et al.*, 2005; Britton & Versalovic, 2008). However, the mechanisms of action of these bacteria are just beginning to be understood. Putative probiotic mechanisms are related to production of antimicrobial compounds, interference with pathogens in terms of competition for nutrients or mucosal attachment, enhancement of intestinal barrier function and immunomodulation (Saxelin *et al.*, 2005; Britton & Versalovic, 2008).

3.2.1 Industrial applications of *L. reuteri* and *L. plantarum*

Lactobacillus plantarum is commonly used as an inoculant for fermented meat, milk and dairy products as well as for vegetables and silage (Hammes & Hertel, 2006). Both *L. reuteri* and *L. plantarum* are associated with lactic acid fermentations of sour dough (Vogel *et al.*, 1994; Hufner *et al.*, 2008) and selected strains are used as probiotics (Casas & Dobrogosz, 2000; Molin, 2001).

Lactobacillus reuteri (strain ATCC 55730) has been shown in several clinical studies to improve outcome in various disorders such as acute rotavirus diarrhoea (Shornikova *et al.*, 1997), colic in babies (Savino *et al.*, 2007), IgE-associated allergy (Abrahamsson *et al.*, 2007), infectious illness in nursery infants (Weizman *et al.*, 2005) and *Helicobacter pylori* infection (Francavilla *et al.*, 2008). The underlying primary mechanisms conferring the antipathogenic properties of *L. reuteri* are currently not known (Britton & Versalovic, 2008). Bacteria of the *L. reuteri* species produce a broad-spectrum antimicrobial compound, reuterin (3-hydroxy-propionaldehyde), when using glycerol as an electron acceptor (Talarico *et al.*, 1988). Reuterin could potentially be applied for decontamination and preservation of meat (El-Ziney *et al.*, 1999), but whether this compound is partly responsible for the probiotic effects of *L. reuteri* remains to be proven.

3.3 Safety aspects

Lactobacilli have a long history of safe use as food and feed processing aids, and, as previously mentioned, certain *Lactobacillus* strains confer a health benefit on humans and animals. Despite the ingestion of large numbers of lactobacilli with fermented food and their wide distribution in high numbers in the human microbiota, very few adverse clinical effects have been reported, justifying this safety status (Hammes & Hertel, 2006). However, lactobacilli may function as a reservoir of antibiotic resistance

genes (Teuber *et al.*, 1999) (see below), and some strains exhibit certain metabolic activities considered disadvantageous with regard to consumer safety (reviewed by Bernardeau *et al.*, 2006). Metabolites such as D-lactate and biogenic amines can be produced and accumulate in fermented dairy products. Platelet aggregation and bile salt deconjugase activities are other examples of lactobacilli properties of concern (Bernardeau *et al.*, 2006). However, there is currently insufficient evidence available to link some of these metabolic activities to significant safety risks (Connolly *et al.*, 2005; Vankerckhoven *et al.*, 2008).

Lactobacilli have in rare cases caused infections such as bacteraemia and endocarditis (e.g. Salminen *et al.*, 2004; Cannon *et al.*, 2005). The incidence of lactobacilli-induced bacteraemia was less than 1% of the total number of bacteraemia cases reported each year in Sweden between 1998 and 2004 (Sullivan & Nord, 2006). Cannon *et al.* (2005) reviewed 241 cases of *Lactobacillus*-associated infection reported worldwide between 1950 and 2003. The majority of these infections occurred in immunocompromised or severely ill patients. *Lactobacillus rhamnsous* and *L. casei* were the most frequently isolated species, followed by *L. plantarum* and *L. acidophilus*. In four of the cases, the infection was related to heavy dairy consumption and for one patient, with a liver abscess, the probiotic strain *L. rhamnosus* GG was reported as the causative agent (Rautio *et al.*, 1999). The same strain, originally isolated from the human intestine, caused 11 (12%) cases of *Lactobacillus* bacteraemia reported between 1990 and 2000 in Finnish patients, all but one having a severe underlying illness (Salminen *et al.*, 2004). However, there was no correlation between the increased probiotic use of *L. rhamnosus* GG and the incidence of lactobacilli bacteraemia in Finland during 1990-2000 (Salminen *et al.*, 2002). In most cases of *Lactobacillus* infection, the host's own microbiota is likely to be the source of infection (Vesterlund *et al.*, 2007). One case of bacteraemia caused by an *L. reuteri* isolate has been reported (Vesterlund *et al.*, 2007).

3.3.1 Safety assessment of lactobacilli

As previously mentioned, legislation exists regarding antibiotic resistant LAB used for feed applications (European Parliament and Council Regulation EC 429/2008), but not for food and probiotic applications. Due to the wide use of lactobacilli as starter cultures and probiotics for humans and to the increasing application of novel strains, especially for probiotic purposes (Temmerman et al., 2003), there is a strong need for valid safety assessments prior to commercial use. At present, there are several unofficial guidelines, which vary in their recommendations, but which taken together

suggest assessing properties related to systemic infections, harmful metabolic activity, excessive immune stimulation and transferability of resistance genes (Adams, 1999; Marteau, 2001; FAO/WHO, 2002). However, more recent guidelines consider transferable antibiotic resistance to be the major hazard concerning commercially used lactobacilli (EFSA, 2007; Bernardeau *et al.*, 2008; Vankerckhoven *et al.*, 2008). Currently, these guidelines are not mandatory because they have not been adopted by any authority and hence it is up to the probiotic or starter producer to decide on the safety assessment procedure for a novel strain.

Antibiotic resistance in lactobacilli has been heavily debated during the last decade. Lactobacilli intentionally added to the food chain should not carry transferable antibiotic resistance genes according to EFSA (2007). Thus, such traits are currently a 'no go area' in the development of new strains. Alternatively, curative strategies could be applied to health-promoting or starter lactobacilli strains to remove plasmids carrying unwanted antibiotic resistance genes (Huys *et al.*, 2006; Rosander *et al.*, 2008). One such example is the commercial probiotic *L. reuteri* strain DSM 17938, which was derived from *L. reuteri* ATCC 55730 by the removal of two resistance plasmids (Fig. 8) without losing any probiotic characteristics (Rosander *et al.*, 2008).

3.4 Antibiotic resistance in lactobacilli

Until 1999, there were only a few systematic studies assessing acquired antibiotic resistance in LAB, including lactobacilli. Teuber *et al.* (1999) therefore suggested that the potential of commensal bacteria to transfer their antibiotic resistance genes from food to the indigenous human microflora should be investigated. Since the initial publication of the regulation on the use of antibiotic resistant microorganisms as feed additives (EC, 2001) and the report by Danielsen & Wind (2003) opposing the microbiological breakpoints defined by this regulation, antibiotic susceptibility profiles in terms of wild-type MIC distributions have been reported for several individual *Lactobacillus* spp. (e.g. Klare *et al.*, 2007; Danielsen *et al.*, 2008; Huys *et al.*, 2008; Papers II and III).

Heterofermentative lactobacilli are, as previously mentioned, intrinsically resistant to glycopeptides such as vancomycin, whereas most obligate homofermentative species are susceptible (Danielsen & Wind, 2003). Lactobacilli are generally susceptible to penicillins, and more resistant to cephalosporins such as ceftriaxone, a third-generation cephalosporin (Danielsen & Wind, 2003). The resistance mechanism is not fully

elaborated, but cell wall impermeability and non-specific multidrug transporters may be involved (Ammor *et al.*, 2007). Lactobacilli seem to be intrinsically resistant to quinolones, *e.g.* ciprofloxacin and nalidixic acid, by a currently unknown resistance mechanism (Hummel *et al.*, 2007). Resistance to other inhibitors of nucleic acid synthesis such as trimethoprim and sulphonamides has also been reported as an intrinsic feature (Katla *et al.*, 2001). However, this was probably due to thymidine in the growth medium, which is antagonistic to antibiotic activity (Danielsen *et al.*, 2004).

Lactobacilli are generally susceptible to all protein synthesis inhibitors except aminoglycosides. Intrinsic resistance to the latter group of antibiotics is attributed to the absence of cytochrome-mediated electron transport, enabling antibiotic uptake (Charteris *et al.*, 2001). However, aacA-aphD as well as aadE and aphA3, encoding aminoglycoside modifying enzymes, have previously been found in *L. acidophilus* and *L. salivarius* strains isolated from animal faeces (Tenorio *et al.*, 2001) and in *L. curvatus* from an unpasteurised milk cheese (Danielsen *et al.*, 2005), respectively. Acquired resistance determinants for tetracyclines, chloramphenicol and MLS_B antibiotics have been found in lactobacilli isolates from a variety of habitats. Identical genes mediating resistance to these antibiotics have also been found in *e.g.* streptococci and enterococci, showing that there is no barrier between lactobacilli and pathogenic species (Teuber et al., 1999). Tetracycline resistance genes *tet*(K, L, M, O, Q, S, W, 36) have been reported in various *Lactobacillus* species (Chopra & Roberts, 2001; Roberts, 2005; Ammor *et al.*, 2007). The overall most frequently found *tet* gene, *tet*(M), has previously been identified in strains of *L. plantarum*, *L. alimentarius*, *L. curvatus*, *L. casei*, *L. acidophilus*, *L. gasseri*, *L. crispatus* and *L. sakei* (Gevers *et al.*, 2003a; Klare *et al.*, 2007), and more recently also in *L. reuteri* (van Hoek *et al.*, 2008). Ammor *et al.* (2008) recently identified a plasmid-encoded *tet*(L) gene and a chromosome-located transposon-associated *tet*(M) gene in a single *L. sakei* strain.

MLS_B resistance in lactobacilli is frequently associated with the presence of *erm*(B) and in a few cases *erm*(C), *erm*(G) and *erm*(T) (Roberts, 2003a; Ammor *et al.*, 2007). Besides *erm* genes, acquired resistance to lincosamides has been caused by *lnu*(A) in the probiotic strain *L. reuteri* ATCC 55730 (Kastner *et al.*, 2006) and to streptogramins A by *vat*(E-1) in an *L. fermentum* strain from unpasteurised milk (Gfeller *et al.*, 2003), respectively. Macrolide resistance in lactobacilli caused by a point mutation at A2058 in the 23S rRNA has so far only been found in an *L. rhamnosus* strain of human origin (Florez *et al.*, 2007). Plasmid-encoded genes mediating chloramphenicol resistance (*cat*) have been identified in *L. plantarum* from pork (Ahn *et al.*,

1992), *L. reuteri* from chicken (Lin *et al.*, 1996) and in three *L. johnsonii* from calf, pig and turkey (van Hoek *et al.*, 2008). The gene of the *L. plantarum* strain was similar to a streptococcal *cat* gene and could be transferred by conjugation with a helper plasmid to a *Carnobacterium* strain (Ahn *et al.*, 1992). Transferable multiresistance in lactobacilli is rare so far, with the exception of *erm-vat-tet* resistance in an *L. fermentum* strain (Gfeller *et al.*, 2003) and *erm-vat-tet-aad-aph-sat* resistance in an *L. curvatus* strain (Danielsen *et al.*, 2005), both isolated from cheeses made from unpasteurised milk.

Lactobacilli, like other bacteria, have probably acquired most antibiotic resistance genes by conjugation (Ammor *et al.*, 2007), but transfer of resistance genes by transduction has also been reported (Morelli *et al.*, 2004). Natural transformation has not been described in lactobacilli (Ammor et al., 2007), although genes involved in uptake of free DNA have been found in *e.g. L. reuteri* (Båth *et al.*, 2005; H. Jonsson, pers. comm. 2009). Several mobilisable plasmids have been described, *e.g.* the tetracycline resistance plasmid pMD5057 of *L. plantarum* 5057 (Danielsen, 2002) and the erythromycin and streptogramin A resistance plasmid pLME300 of *L. fermentum* ROT1 (Gfeller *et al.*, 2003). There are to date no reports linking conjugative transposons and antibiotic resistance in lactobacilli (Ammor *et al.*, 2007).

In vitro and *in vivo* conjugal transfer of the introduced, broad-host-range plasmid pAMβ1 has previously been demonstrated between different lactobacilli and between lactobacilli and enterococci, lactococci or streptococci, respectively (Tannock, 1987; Morelli *et al.*, 1988; McConnell *et al.*, 1991; Vogel *et al.*, 1992). In food, fermented sausages have been the site of observed transfer of pAMβ1 between *L. curvatus* strains (Vogel et al., 1992). Interspecies gene transfer to *E. faecalis* of mobilisable *erm* or *tet*-containing plasmids from food-related *L. plantarum* has been demonstrated *in vitro* (Gevers *et al.*, 2003b; Feld *et al.*, 2008) and in the intestinal tract of gnotobiotic rats (Jacobsen *et al.*, 2007; Feld *et al.*, 2008). Transfer to *E. faecalis* of an *erm* gene in an *L. reuteri* strain from pig has also recently been demonstrated *in vitro* (Ouoba *et al.*, 2008). However, no *erm* gene transfer from *L. plantarum* has been observed *in vivo* in the presence of a surrounding microbiota, either with or without selection pressure (Feld et al., 2008). Mater *et al.* (2008) recently showed that probiotic lactobacilli may also acquire antibiotic resistance *in vivo*. However, the extent to which lactobacilli contribute to dissemination of antibiotic resistance genes in the human gut is not clear and this issue thus needs further attention with regard to consumer safety.

4 Results and discussion

In the following chapter, the main findings of the papers included in this thesis are summarised and discussed.

4.1 Antibiotic susceptibility testing (II and III)

Distributions of antibiotic MICs for a representative set of strains within a species are needed when using a phenotypic method to assess the presence of acquired resistance genes (White *et al.*, 2001). Antibiotic susceptibility profiles based on wild-type MIC distributions were determined for 56 *L. reuteri* strains of animal and human origin (Paper II), 56 *L. fermentum* strains of dairy origin (not discussed further here; Paper II), and for 121 *L. plantarum* strains of dairy and vegetable origin (Paper III). During the compilation of *L. reuteri* and *L. plantarum* strains for this thesis, efforts were made to obtain a wide distribution in terms of source, year of isolation, geographical origin and clonal diversity. The source and spatial and temporal origin of the *L. reuteri* strains are shown in Fig. 6.

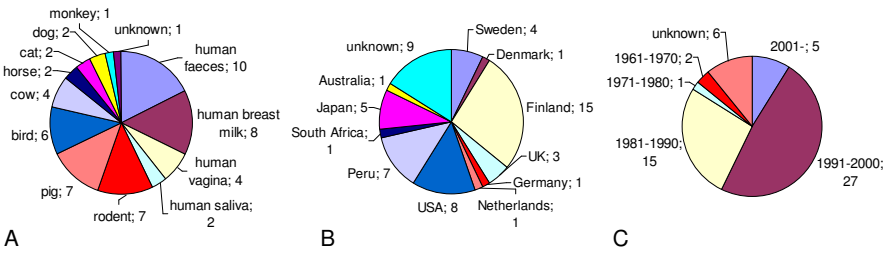

Figure 6. Source (A), geographical origin (B) and year of isolation (C) of the 56 *L. reuteri* strains.

Species confirmation was conducted by sequence analysis of the 16S rRNA gene and subtyping by rep-PCR genomic fingerprinting using the primer $(GTG)_5$, a method that has previously been successfully applied in lactobacilli for this purpose (Gevers *et al.*, 2001). Because *L. plantarum*, *L. pentosus* and *L. paraplantarum* are genotypically closely related and thus have nearly identical 16S rRNA gene sequences (Quere *et al.*, 1997), identification of *L. plantarum* strains was also confirmed by a species-specific multiplex PCR (Torriani *et al.*, 2001).

The antibiotics tested throughout the studies were ampicillin, tetracycline, erythromycin, clindamycin, streptomycin and gentamicin. The susceptibility of eight additional antibiotics for which EFSA (2005) also defined microbiological breakpoints was tested for *L. reuteri* (Table 2).

4.1.1 Evaluation of a broth microdilution method (I)

Besides testing a variety of strains belonging to the same species, standardised and reliable testing procedures are needed for accurate recognition of strains harbouring acquired resistance genes (White *et al.*, 2001). There is currently no standard method for antibiotic susceptibility testing of *Lactobacillus* spp. At present, the Clinical and Laboratory Standards Institute (CLSI) recommends broth microdilution for susceptibility testing of clinical *Lactobacillus* isolates that cause endocarditis and bacteraemia (Jorgensen & Hindler, 2007). However, the suggested testing medium is blood-supplemented Müeller-Hinton, which does not support the growth of all *Lactobacillus* species (Huys *et al.*, 2002; Klare *et al.*, 2005). In a recent report, no growth was obtained for 3 out of 20 food-related lactobacilli isolates tested as recommended by the CLSI guideline (Ge *et al.*, 2007). Consequently, a variety of methods have been applied for lactobacilli, such as broth microdilution (Flórez *et al.*, 2005; D'Aimmo *et al.*, 2007), agar dilution (Chou *et al.*, 2004; Korhonen *et al.*, 2007), Etest (Danielsen & Wind, 2003; Hummel *et al.*, 2007) and disc diffusion (Temmerman *et al.*, 2003; Kastner *et al.*, 2006). In many of these studies, the testing medium used was MRS, which can exert an antagonistic effect on certain antibiotics (Huys *et al.*, 2002; Klare *et al.*, 2005). LAB susceptibility test medium (LSM; isosensitest 90% (v/v) and MRS 10% (v/v), pH 6.7) was developed by Klare and co-workers (2005) to overcome the disadvantages of previously used media. Other factors that may limit the reproducibility and comparability of MIC data between different laboratories are inoculum size, incubation time, incubation temperature and composition of the atmosphere (White *et al.*, 2001). This led to the study described in Paper I,

in which the effects of inoculum size and incubation time on broth microdilution susceptibility testing of some LAB were evaluated.

MICs for 29 LAB reference strains (27 *Lactobacillus*, 1 *Streptococcus thermophilus* and 1 *Lactococcus lactis*) and six clinical *Lactobacillus* isolates against six antibiotics were determined using a commercial microdilution panel at inoculum densities ranging from 3×10^4 to 3×10^7 CFU/mL and at 24 and 48 h of incubation. The *Lactobacillus* reference strains encompassed different phylogenetic groups and sugar fermentation pathways (Table 1 in Paper I). Increased inoculum size and extended incubation time both resulted in elevated antibiotic MICs for all LAB species tested, underlining the importance of controlled and standardised conditions for susceptibility testing of LAB. An inoculum size of 3×10^5 CFU/mL and an incubation time of 48 h were recommended to assess the antibiotic susceptibility of LAB using broth microdilution and LSM.

Standard operating procedures for antibiotic susceptibility testing of lactobacilli using broth microdilution and an Etest method were elaborated within the ACE-ART project as a first step toward standardised methods. These were based on intra- and interlaboratory tests performed within the ACE-ART project (G. Huys, pers. comm. 2009), the use of LSM and the results obtained in Paper I for broth microdilution. The MIC distributions obtained in Papers II and III were subsequently determined according to these protocols. Currently, the broth microdilution protocol is under evaluation at the International Dairy Federation (IDF) for use as an international ISO/IDF standard method (Danielsen & Seifert, 2008).

4.1.2 Comparison of Etest and broth microdilution MICs (II and III)

Information concerning the comparability of different methods for antibiotic susceptibility testing of lactobacilli is limited. All 56 *L. reuteri* strains and 72 of the *L. plantarum* strains were therefore tested for their responses to six antibiotics with both the Etest and the broth microdilution assay. The need for MIC methods in combination with previous experience of these methods in the ACE-ART project were the reasons for using them in this thesis.

For *L. reuteri*, 86% of the 258 strain-antibiotic combinations resulting in MICs within the test range with both methods were within the accuracy limit of MIC determination tests, *i.e.* ± one $\log_2$ dilution step (CLSI, 2005; Table 2 in Paper II). The MIC agreement was less pronounced for ampicillin and clindamycin. Similar results were obtained for *L. plantarum* for 329 MICs within the test range (data not shown). The correlation of MICs determined by Etest and broth microdilution on/in LSM has

previously been reported for LAB such as *L. paraplantarum* (Huys *et al.*, 2008). Only 56% of the MIC data were within the accuracy limit, with generally two $\log_2$ dilution steps higher MICs obtained by Etest than by broth microdilution for the aminoglycosides tested. However, for *Streptococcus thermophilus* (Tosi *et al.*, 2007) and members of the *L. acidophilus* group (Mayrhofer *et al.*, 2008), the percentage of MICs falling within one $\log_2$ dilution step was approximately 80%, with the highest discrepancies obtained for clindamycin and tetracycline and, as in Papers II and III, with generally higher MICs obtained by broth microdilution than by Etest. Taken together, MICs obtained by the two methods are comparable and either method could thus be used to assess the presence of acquired antibiotic resistance genes.

In my opinion, broth microdilution provides a simple method to determine MICs for a large number of strains and antibiotics, whereas the Etest could be more suitable for testing single strains. However, resistant and susceptible strains were generally more clearly separated by Etest in the present investigation due to the wider and more precise (MICs between the $\log_2$ dilution steps) antibiotic concentration range of the Etest. However, the Etest in particular needs trained eyes to determine correct MICs.

4.2 Antibiotic resistance in *L. reuteri* (II and IV)

The susceptibility of 56 *L. reuteri* strains to 14 antibiotics was assessed by Etest and/or broth microdilution (Paper II). Strains exhibiting atypical MICs were subsequently screened by real-time PCR and/or a DNA microarray assay for the presence of known resistance genes (Paper IV). Antibiotic susceptibility ranges and identified resistance genes are summarised in Table 2. The distribution of MICs was uniform for most antibiotics, with MICs in the lower range for linezolid, gentamicin and netilmycin and in the upper range for amikacin and streptomycin. All MICs for kanamycin, vancomycin and trimethoprim were high, with strains exhibiting MICs above the maximum concentration tested. Bimodal distributions of MICs were obtained for ampicillin, chloramphenicol, tetracycline, erythromycin, clindamycin and dalfopristin-quinupristin.

Intrinsic resistance to vancomycin and aminoglycosides such as streptomycin and kanamycin has been reported as a general feature for lactobacilli (Danielsen & Wind, 2003), and stems from the absence of the peptidoglycan D-alanine target precursor and the lack of a cytochrome-mediated transport system required for aminoglycoside uptake, respectively. However, the reduced susceptibility to trimethoprim reported here and by

others (Charteris *et al.*, 1998; Klein *et al.*, 2000; Klare *et al.*, 2007) is not caused by an intrinsic trait, but is probably due to thymidine in the growth medium, which is antagonistic to antibiotic activity (Danielsen *et al.*, 2004).

Table 2. *Antibiotic susceptibility ranges in terms of distribution of typical and atypical MICs for* L. reuteri *obtained by Etest and identified resistance genes.*

Antibiotic	Wild-type MIC range (μg/mL) (n)	Atypical MIC range (μg/mL) (n)	Identified resistance genes (n)
Ampicillin	≤0.12-2 (42)	8-32 (14)	Mutational *pbp*'s[a]
Vancomycin	>256 (49)		
Amikacin	4-64 (49)		
Gentamicin	0.5-4 (56)		
Kanamycin	16->256 (49)		
Netilmycin	0.25-4 (49)		
Streptomycin	8-64 (56)		
Erythromycin	0.25-2 (50)	>256 (6)	*erm*(B) (4), *erm*(C) (1), *erm*(T) (1)
Clindamycin	≤0.12-2 (50)	>256 (6)	*erm*(B) (4), *erm*(C) (1), *erm*(T) (1)
Dalfopristin-quinup.[b]	0.25-1 (46)	8-16 (3)	*erm*(B) (3)
Tetracycline	4-32 (28)	128->256 (28)	*tet*(W) (24)
Chloramphenicol	2-4 (48)	128 (1)	*cat*(TC) (1)
Linezolid	1-4 (49)		
Trimethoprim	>256 (49)		

[a](Rosander *et al.*, 2008)

[b]Dalfopristin-quinupristin

For ampicillin, atypical MICs of 8-32 μg/ml were obtained for almost one-third of the strains. This is in contrast to the common opinion of lactobacilli being susceptible to penicillins in general (Danielsen & Wind, 2003; Hummel *et al.*, 2007). The genetic mechanism conferring high ampicillin MICs in strains of *L. reuteri* has been subject to investigation (Rosander *et al.*, 2008) since the publication of Paper II. Five genes encoding penicillin-binding proteins (Pbp), the target of betalactam antibiotics, from three susceptible strains (DSM 20015, DSM 20016, and ATCC 55148) and three less susceptible strains (ATCC 55730, ATCC 55149, and CF48-3A1) have been sequenced. Point mutations generating amino acid substitutions in the corresponding proteins Pbp1a, Pbp2a and/or Pbp2x have been found to be correlated with the atypical ampicillin MICs and are suggested to cause the resistance. This resembles the identified cause of penicillin resistance in

streptococci (Hiramatsu *et al.*, 2004). The *pbp* genes are located on the chromosome and regarded as non-transferable (Rosander *et al.*, 2008).

For chloramphenicol, the wild-type distribution ranging up to 4 μg/mL was in agreement with a previous susceptibility study of *L. reuteri* (Klare *et al.*, 2007). *Lactobacillus reuteri* strain 5010, isolated from dog and exhibiting a 30 times higher chloramphenicol MIC harbours a plasmid-located *cat*(TC) gene encoding a chloramphenicol acetyltransferase. However, the gene is not identical to the plasmid pTC82 encoded *cat*(TC) gene of *L. reuteri* G4 from chicken (Lin *et al.*, 1996), as demonstrated by the negative result using an additional set of primers designed by Cataloluk and co-workers (2004) and covering the whole *cat*(TC) gene (data not shown). Another *L. reuteri* strain, ATCC PTA6127, isolated in 1994-95 from a Peruvian dog and displaying a similar rep-PCR fingerprint but with MIC 4 μg/mL for chloramphenicol, was negative in the first PCR screening to the chloramphenicol resistance gene tested. Chloramphenicol is used for certain life-threatening infections such as typhoid fever, but it can cause fatal aplastic anaemia at therapeutic doses in humans, limiting its use within human medicine. However, chloramphenicol is used for several disease conditions in domestic animals (Schwarz *et al.*, 2004), which could be a plausible explanation for the occurrence of the chloramphenicol resistant *L. reuteri* strain isolated from dog.

4.2.1 Tetracycline resistance in *L. reuteri*

In total, 28 strains displayed MICs above 64 μg/mL for tetracycline with both Etest and broth microdilution. The wide range of high MICs obtained was in agreement with a previous study assessing antibiotic susceptibility of 43 *L. reuteri* strains isolated from piglets (Korhonen *et al.*, 2007). Based on the appearance of the tetracycline MIC distribution, it was first believed that the *L. reuteri* strains harboured different tetracycline resistance genes conferring diverse levels of susceptibility, as reviewed by Chopra & Roberts (2001). However, real-time PCR revealed the presence of *tet*(W) in 24 of the 28 *L. reuteri* strains with atypical MIC for tetracycline. None of the other five tetracycline resistance genes tested (*tet*(K), *tet*(L), *tet*(M), *tet*(O), and *tet*(S)) were found in any strain including the four *tet*(W) negative strains Cow 10, ATCC 55148, MF2-3 and MF14-C.

The *tet*(W) gene is commonly found in human and animal intestinal Gram-positive bacteria, such as various species of *Bifidobacterium, Butyrivibrio, Mitsuokella* and *Fusobacterium* (Kazimierczak *et al.*, 2006; van Hoek *et al.*, 2008). Since the genotypic data of the ACE-ART project became available (van Hoek *et al.*, 2008), it is evident that *tet*(W) is also

found in various *Lactobacillus* species such as *L. amylovorus*, *L. brevis*, *L. crispatus*, *L. gallinarum*, *L. johnsonii, L. paracasei* and *L. reuteri*. In Papers II and IV, we demonstrated that *L. reuteri*, displaying 40-42% G+C content (Hammes & Hertel, 2006), is frequently associated with *tet*(W), whereas the closely related species *L. fermentum*, displaying 52-54% G+C content (Hammes & Hertel, 2006), is susceptible to tetracycline. Interestingly, this is in contrast to the proposed theory that *tet*(W), which has a much higher G+C content (53%) than other ribosome-protection-type *tet* genes, is generally associated with bacterial hosts with a similar G+C-content, such as bifidobacteria and *Mitsuokella* (Scott *et al.*, 2000).

Comparison of MICs and $(GTG)_5$-PCR genomic fingerprinting data showed that 14 of the 16 *L. reuteri* strains with high MICs for both ampicillin and tetracycline displayed highly similar rep-PCR fingerprints. Three strains of this so-called group B (Fig. 1 in Paper II) were further characterised. According to size, all three strains seemed to carry the same four plasmids and a *tet*(W) probe hybridised to the same plasmid of approximately 12 kb. We therefore presumed that all strains of group B, widely distributed in terms of source and geographical origin, contained the same tetracycline resistance plasmid. The other ten strains with atypical MIC for tetracycline were evenly scattered throughout the dendrogram. The apparent genetic heterogeneity of these strains was further demonstrated by the mixed localisation of the *tet*(W) gene on plasmids or on the chromosome, as determined by Southern blot and/or the $\Delta\Delta C_t$ PCR method described in detail in Paper IV.

The conservation of the *tet*(W) gene sequences from different isolates is remarkably high (Scott *et al.*, 2000), as was further confirmed here by the sequence analysis of a chromosome-located *tet*(W) gene and a plasmid-bound *tet*(W) gene identified in *L. reuteri* strains from pig (PA-16) isolated in the 1970s and from human breast milk (ATCC 55730) isolated in 1990, respectively. The whole 1.9 kb gene differs by only two nucleotides in the two *L. reuteri* strains and by 38 or 40 nucleotides compared with the rumen anaerobe *Butyrivibrio fibrisolvens* (data not shown), where *tet*(W) was first identified on a chromosomal transposon (Barbosa *et al.*, 1999). The regions surrounding *tet*(W) vary in different species of gut bacteria, but contain a conserved core region of 2.6 kb, including the resistance gene, as reported by Kazimierczak *et al.* (2006). The flanking regions of the two *L. reuteri* *tet*(W) genes described in this thesis showed 95-96% similarity to the conserved 657-bp upstream region, but did not contain the conserved 43-bp region downstream of *tet*(W) (Fig. 7).

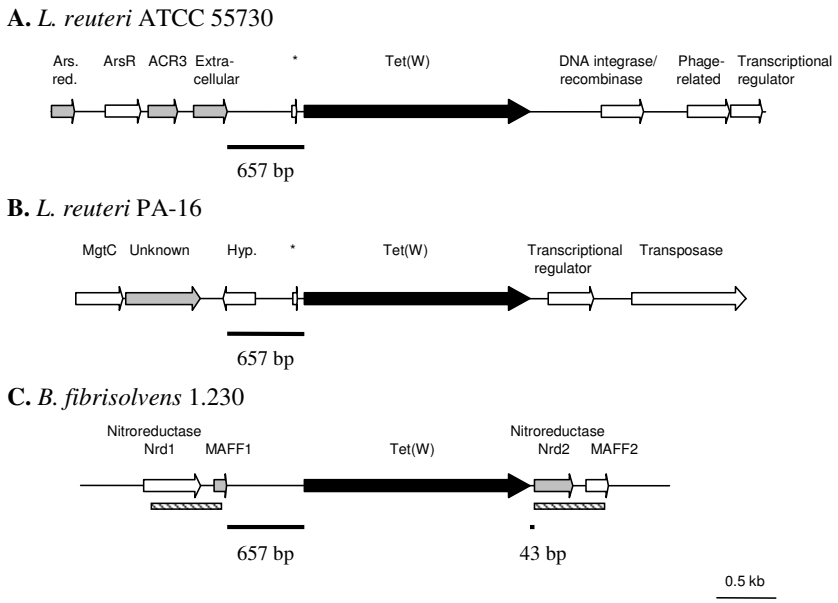

Figure 7. Organisation of the regions surrounding *tet*(W) of *L. reuteri* ATCC 55730 (A) and *L. reuteri* PA-16 (B) described in this thesis, and of *Butyrvibrio fibrisolvens* 1.230 on transposon Tn*B1230* (Melville *et al.*, 2004; Kazimierczak *et al.*, 2006) (C). Grey arrows indicate pseudogenes. Hyp. = Hypothetical protein; Ars. red. = Arsenite reductase; ArsR = Arsenite transcriptional regulator; ACR3 = Arsenite efflux pump; MgtC = MgtC/SapB transporter; ⋆ = *Tet*(W)-regulatory peptide; MAFF= a 46-aa protein designated MAFF to represent the first four amino acids. In Tn*B1230*, *tet*(W) is flanked by two identical direct-repeat DNA sequences, indicated as diagonal striped rectangular boxes.

Whether the widespread presence of *tet*(W) in genetically diverse *L. reuteri* strains is due to repeated uptake of the gene/plasmid or to a common ancestor becoming *tet*(W) positive and some strains having lost their *tet*(W) gene over time is an open question. An argument for the former hypothesis is that *tet*(W) has been found in many species present in the gastrointestinal tract of both humans and animals (Scott *et al.*, 2000) and is often associated with conjugative transposons (Roberts, 2005). Differences with respect to flanking regions of the two sequenced *tet*(W) genes would also suggest multiple independent acquisitions. However, although sequence analysis of the 12-kb-plasmid harbouring *tet*(W) in *L. reuteri* ATCC 55730 revealed a downstream integrase, no known origin of transfer or any described *tra* or *mob* genes were found. Alternatively, only the *tet*(W) gene has been transferred. As suggested by Kazimierczak *et al.* (2006), the conserved

surrounding region might function as a mini transfer cassette that has become incorporated into larger mobile elements.

4.2.2 Erythromycin resistance in *L. reuteri*

Six *L. reuteri* strains with clearly higher MICs for erythromycin than the majority of strains also had atypical MICs for clindamycin, indicating cross-resistance. Indeed, four of the strains were positive for *erm*(B) and one strain each was positive for *erm*(C) and *erm*(T), as determined with real-time PCR. The resulting dimethylation of the overlapping binding site of the 50S ribosomal subunit confers high resistance to all MLS_B antibiotics (Liu & Douthwaite, 2002), thus also explaining the increased MICs to dalfopristin-quinupristin (a mixture of streptogramin A and B). The presence of *erm*(B) was in agreement with previous studies of three (1048, 1068, 8557:1) of the erythromycin resistant strains (Axelsson *et al.*, 1988; S. Ahrné, pers. comm. 2009).

Comparison of MICs and $(GTG)_5$-PCR genomic fingerprinting data (Fig. 1 in Paper IV) showed that the six strains with atypical MICs for erythromycin and clindamycin were clustered together in the dendrogram, although they did not form a separate group. All *erm* genes were plasmid-encoded and except for *erm*(B) in strains 8557:1 and 1068, they were located on plasmids of different sizes, as determined by Southern blot (data not shown), the $\Delta\Delta C_t$ PCR method and/or reported previously (Axelsson *et al.*, 1988; S. Ahrné, pers. comm. 2009). The unique plasmid profiles of the *erm* positive strains imply that the erythromycin resistance was not spread clonally, but rather taken up in separate events.

Similarly to the *L. reuteri* strain LMG 18391, the presence of both *erm*(B) and *tet*(W) has recently been reported in an *L. paracasei* strain (Huys *et al.*, 2008) and two *L. crispatus* strains (Klare *et al.*, 2007). We found that the two genes were located on the same plasmid, as determined by Southern blot (data not shown for *erm*(B)). However, it remains to be determined whether the genes are linked to a conjugative transposon, which is often the case with linked *erm*(B) and *tet*(M) (Roberts *et al.*, 1999). The other strain (PA-16) with atypical MICs for both tetracycline and erythromycin/clindamycin carried *tet*(W) and *erm*(C). Sequence analysis of the *erm*(C) gene and its flanking regions by direct genome sequencing and a subsequent BLASTP search in Genbank revealed an rRNA methylase with high similarity (95-99% amino acid identity) to *erm*(C) genes present in various *Staphylococcus* species. The gene, which is usually located on small plasmids (<5 kb) in staphylococci, was found in the *L. reuteri* strain on a plasmid of approximately 20 kb. The transposases located downstream of

the *erm*(C) gene and the chromosome-located *tet*(W) gene of the same strain may be part of transfer machineries, facilitating the spread to other strains.

The macrolide tylosin was the most commonly used antimicrobial agent in pig farming in the European Union until it was banned as an animal growth promoter in 1999. Today it is still used for therapeutic purposes (A. Franklin, pers. comm. 2009). Consequently, bacteria such as enterococci and staphylococci isolated from pigs are frequently resistant to macrolides (Aarestrup & Carstensen, 1998). Here, we found that four of the six *L. reuteri* strains that tested positive for an *erm* gene were originally isolated from pigs, although only six of the 56 strains tested in this thesis were from this source. In contrast, Korhonen *et al.* (2007) previously reported that none of the 43 *L. reuteri* strains isolated from 30-day-old piglets displayed atypical MICs for erythromycin or clindamycin.

4.3 Antibiotic resistance in *L. plantarum* (III and IV)

The susceptibility of up to 121 *L. plantarum* strains to six antibiotics was assessed by Etest and/or broth microdilution (Paper III and Table E1 of the preceding errata list). Strains with atypical antibiotic MICs were subsequently screened by real-time PCR and/or a DNA microarray assay for the presence of known resistance genes (Paper IV). Antibiotic susceptibility ranges and identified resistance genes are summarised in Table 3. A uniform MIC distribution was obtained for ampicillin, erythromycin and gentamicin, with MICs up to 2 μg/mL, 1-2 μg/mL and 8-16 μg/mL, respectively, depending on the method used. For streptomycin, all strains had MICs in the upper test range or above the maximum concentration tested. Thus, further testing using higher streptomycin concentrations would be needed to define a microbiological breakpoint for *L. plantarum* to this antibiotic. However, the increased MICs, which are probably due to an intrinsic trait (Danielsen & Wind, 2003), are in accordance with previous results for *L. plantarum* and other *Lactobacillus* species, as is the higher observed susceptibility to gentamicin compared with streptomycin (Danielsen & Wind, 2003; Korhonen *et al.*, 2008; Paper II). A distribution with MICs up to 8 μg/mL, but without a clear peak, was obtained for clindamycin. The wide range, covering seven $\log_2$ dilution steps, could be due to interlaboratory discrepancies despite the same protocols being used in the four participating laboratories.

Table 3. *Antibiotic susceptibility ranges in terms of distribution of typical and atypical MICs for* L. plantarum *obtained by Etest, and identified resistance genes.*

Antibiotic	Wild-type MIC range (μg/mL) (n)	Atypical MIC range (μg/mL) (n)	Identified resistance genes (n)
Ampicillin	≤0.12-2 (66)		
Gentamicin	0.25-8 (66)		
Streptomycin	2->256 (121)		
Erythromycin	≤0.12-2 (121)		
Clindamycin	≤0.12-8 (73)		
Tetracycline	2-32 (117)	128->256 (4)	*tet*(M) (2)

4.3.1 Tetracycline resistance in *L. plantarum*

A bimodal distribution of high MICs was obtained for tetracycline with both Etest and broth microdilution. Four strains displayed a tetracycline MIC above 64 μg/mL by Etest and thus deviated from the wild-type population, which displayed MICs of up to 32 μg/mL. The genes conferring tetracycline resistance in the two strains isolated from Italian silage in 1999 were subsequently identified by real-time PCR and localised by Southern blot and/or the $\Delta\Delta C_t$ PCR method (Paper IV). Both these *L. plantarum* strains displayed the same plasmid profile and were positive for *tet(*M), the most widely distributed *tet* gene in terms of genera (Roberts, 2005), including lactobacilli. The *tet(*M) and *tet*(S) are the only *tet* genes found so far in *L. plantarum* (Danielsen, 2002; Gevers *et al.*, 2003a; Huys *et al.*, 2006). When located in this species, *tet(*M) has been found on a plasmid with a size of approx. 10 kb (Danielsen, 2002; Gevers *et al.*, 2003a), which was also the case in the present study. Thus, the two strains harboured potentially transferable resistance genes and should in this regard not be commercially used as feed additives (European Parliament and Council Regulation EC 429/2008; EC, 2001).

The remaining two *L. plantarum* strains with atypical MIC for tetracycline were isolated from Italian or Spanish dairy products in 2002. One of the strains has not been screened for tetracycline resistance genes, whereas the other strain was negative to the tetracycline resistance genes tested by the DNA microarray assay (data not shown).

4.3.2 Tentative microbiological breakpoints for *L. plantarum*

The microbiological breakpoints defined by EFSA (2005) for lactobacilli strains used as feed additives are divided into three categories:

heterofermentative, obligately homofermentive and the species *L. plantarum*. This has been found to be inadequate in many cases, especially for the large *L. delbrueckii* group (Korhonen *et al.*, 2008). Tentative microbiological breakpoints, referred to as susceptibility-resistance cut-off values in Paper III, were proposed for *L. plantarum* to emphasise the need for breakpoints for individual LAB species (Paper III and Table E2 of the preceding errata list). The *L. plantarum* breakpoints defined in Paper III and by EFSA were conflicting for all antibiotics except tetracycline. Thus, the same four strains with atypical MIC for tetracycline were considered resistant using either breakpoint, whereas *e.g.* eight strains that were susceptible to clindamycin were considered resistant using the EFSA breakpoints. However, the EFSA breakpoints have recently been updated and are now in agreement with the cut-offs defined in Paper III using broth microdilution for all antibiotics except clindamycin (EFSA, 2008).

4.4 Phenotypic versus genotypic data (II, III, and IV)

The results of the molecular screening correlated well with MIC data on *L. reuteri* and *L. plantarum*, except for one *tet*(W) positive *L. reuteri* strain with a tetracycline MIC of 16 μg/mL and four presumably *tet* negative *L. reuteri* strains with atypical tetracycline MIC (≥256 μg/mL). A weaker hybridisation signal for the *tet*(W) oligonucleotide was observed on the microarray for the former strain compared with control strains, indicating the presence of a partial or mutated *tet*(W) gene, or a gene that is similar, rather than identical to *tet*(W). Sequence analysis of the *tet*(W) gene in this phenotypically susceptible strain could further elucidate why the gene is non-functional.

Two of the four strains with atypical tetracycline MIC were also tested by microarray analysis, but were negative to the 33 *tet* genes included in the array. For these strains, there might be other underlying resistance mechanisms such as the presence of multidrug efflux pumps removing tetracycline from its target, as has previously been demonstrated in bifidobacteria for other antibiotics (Margolles *et al.*, 2006). In either case, this shows that phenotypic and molecular tools are both needed to guarantee the presence or absence of acquired resistance genes in strains intended for use in food, feed and probiotic applications, as has also been pointed out by others (Hummel *et al.*, 2007; van Hoek *et al.*, 2008).

4.5 *In vivo* transferability of an *L. reuteri tet*(W) gene (V)

As stated in the introduction, the potential contribution of lactobacilli to the spread of antibiotic resistance genes in the human gut is poorly addressed. We therefore investigated the transferability of the tetracycline resistance gene *tet*(W) from the formerly commercially available probiotic *L. reuteri* strain ATCC 55730 to bacteria in the intestinal tract of humans. In a double-blind clinical study, seven subjects consumed *L. reuteri* ATCC 55730 harbouring a plasmid-encoded *tet*(W) gene (*tet*(W)-reuteri). The control group of seven other subjects consumed *L. reuteri* DSM 17938 derived from the ATCC 55730 strain by the removal of two plasmids (Fig. 8), one of which contained the *tet*(W)-reuteri gene (Rosander *et al.*, 2008).

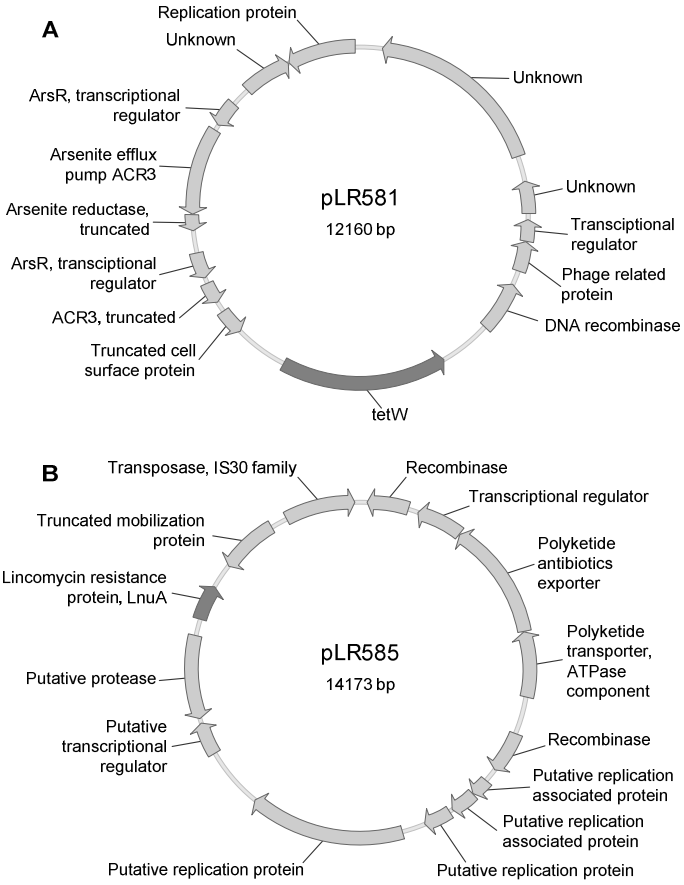

Figure 8. The commercial probiotic *L. reuteri* DSM 17938 was derived from *L. reuteri* ATCC 55730 by the removal of two resistance plasmids, pLR581 harbouring a *tet*(W) gene (A) and pLR585 harbouring an *lnu*(A) gene (B).

In total, 5×10^8 CFU of *L. reuteri* were ingested in the form of chewable tablets each day for 14 days and faecal samples were collected on four occasions on Days -7 and 0 (baseline), 14 and 28, *i.e.* after a two-week washout period (Fig. 9).

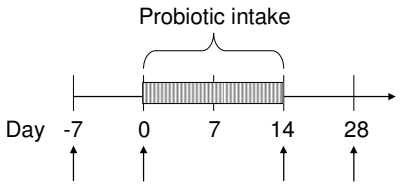

Figure 9. Probiotic intake (shaded field) and collection of faecal samples (arrows).

Both *L. reuteri* strains were detectable at similar levels in faeces by culture after 14 days of ingestion in 13 of the 14 subjects, but not after a two-week washout period, indicating that the strains survive but are only transiently present in the intestine. Colonisation of the human gastrointestinal tract would increase the probability of donor-recipient encounters facilitating gene exchange, but temporary presence of the donor bacteria is sufficient for conjugative gene transfer to occur at least, as reviewed by Licht & Wilks (2006). The *tet*(W)-reuteri plasmid appears to be non-conjugative and non-mobilisable (Paper IV). Furthermore, the risk of horizontal transfer by transformation in the gastrointestinal tract is regarded as negligible (van den Eede *et al.*, 2004). However, several phage related genes have been identified in the draft genome sequence of the ATCC 55730 strain (Båth *et al.*, 2005; H. Jonsson, pers. comm. 2009), thus not excluding transduction as a possible mechanism of transfer.

To distinguish between *tet*(W)-reuteri and *tet*(W) genes present in the faecal microbiota, a real-time PCR method for allelic discrimination was developed in Paper V (Fig. 10). This was necessary due to the wide distribution of *tet*(W) in different bacterial species of human faeces in combination with the high conservation of *tet*(W) nucleotide sequences from different isolates (Scott *et al.*, 2000; Kazimierczak *et al.*, 2006; Paper IV). A *tet*(W)-reuteri or *tet*(W) signal produced for two strains harbouring either type of gene showed that the method could be used for distinguishing between the *tet*(W) gene types. However, testing different ratios of *tet*(W)-reuteri to *tet*(W) showed that a *tet*(W)-reuteri signal was detectable in the presence of a 100-fold higher concentration of *tet*(W) but not in a 1000-fold higher concentration. Furthermore, a *tet*(W)-reuteri signal was produced from the faecal *L. reuteri* isolates tested of those subjects

having ingested the *tet*(W)-positive strain. Thus the method could be used to detect the gene in faecal material.

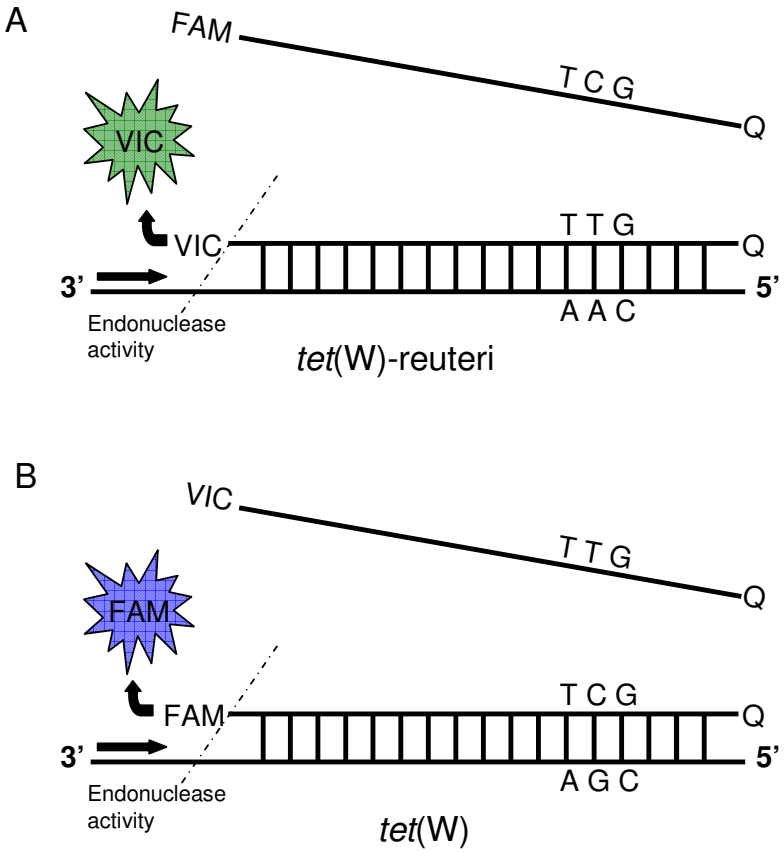

Figure 10. Principle of the real-time PCR allelic discrimination method developed in Paper V. The technique is dependent on the competition between two probes labelled with the same quenching fluorophor but different reporter fluorophors, VIC and FAM. The VIC probe was specific for *tet*(W)-reuteri, with an A in position 109 of the resistance gene (A) and the FAM probe was specific for other previously described *tet*(W) genes, with a G in that position (B). During PCR-amplification, primers (not shown) and the exact-matched probe bind and the probe is subsequently hydrolysed by the endonuclease activity of the Taq polymerase. This releases the corresponding reporter fluorophor from its quencher and results in an increase in VIC or FAM fluorescence, *i.e.* a *tet*(W)-reuteri or *tet*(W) signal. The design of primers and probes is described in detail in Paper V.

After enrichment and isolation of bacterial colonies in/on genus-specific tetracycline supplemented media, DNA was extracted and the presence of *tet*(W)-reuteri was screened by the real-time PCR method developed. A lower C_t value compared with the baseline and the control group obtained by the *tet*(W)-reuteri detector for the Day 14 and/or Day 28 faecal sample DNA from a subject having ingested *tet*(W) positive *L. reuteri* would indicate *tet*(W)-reuteri gene transfer. However, no *tet*(W)-reuteri signal was produced from any of the DNA samples. Thus no transfer events were demonstrated under the conditions tested, suggesting that transfer of the *tet*(W)-reuteri gene during intestinal passage of the probiotic *L. reuteri* did not occur or occurred at low frequencies undetectable by the method used. As indicated by the *tet*(W) signals produced, this gene was present in the faecal material from all subjects on one or more sampling occasions, thus verifying its common occurrence within the tetracycline resistant faecal populations of the three genera investigated.

An additional objective was to assess the proportion of tetracycline resistant enterococci, bifidobacteria and lactobacilli present in faeces. The baseline level was 5-12% for one of the eight subjects tested, but in most cases it was less than 0.1%. Our results are in agreement with findings from a similar study in Finland by Saarela *et al.* (2007), but in contrast to previous findings on the antibiotic susceptibility of faecal strains in Spain, France and Denmark, which suggested a high natural prevalence of tetracycline resistant bifidobacteria, lactobacilli and enterococci in human faeces (Aarestrup *et al.*, 2000; Delgado *et al.*, 2005; Moubareck *et al.*, 2005). Variation in antibiotic use could be a plausible explanation for the higher levels observed in France and Spain (Cars *et al.*, 2001).

5 Conclusions

The main findings of this thesis were:

- Controlled and standardised conditions are required for antibiotic susceptibility testing of LAB.
  - Increased inoculum size or extended incubation time resulted in elevated antibiotic MICs for all LAB strains using broth microdilution.
  - An inoculum of 3×10^5 CFU/mL and an incubation time of 48 h were recommended for assessing the antibiotic susceptibility of LAB by this method.
  - The results obtained have contributed to the development of an international ISO standard method for broth microdilution.
  - The MIC data obtained with the Etest and the broth microdilution method were in close agreement for both *L. reuteri* and *L. plantarum*.

- All *L. plantarum* strains tested (n=121) were susceptible to ampicillin, gentamicin, erythromycin and clindamycin, and intrinsically resistant to streptomycin. Acquired resistance to tetracycline was associated with plasmid-bound *tet*(M).

- The members of *L. reuteri* tested (n=56) were susceptible to linezolid, trimethoprim and the aminoglycosides gentamicin and netilmycin, and intrinsically resistant to vancomycin and the aminoglycosides amikacin, kanamycin and streptomycin.

- *Lactobacillus reuteri* strains with atypical MICs for both tetracycline and ampicillin were linked to the same genotype, and the strains with

atypical MICs for erythromycin and clindamycin were clustered together, although not forming a separate group.

- *Lactobacillus reuteri* strains had acquired resistance to tetracycline (n=28), ampicillin (n=14), erythromycin/clindamycin (n=6), and chloramphenicol (n=1). This resistance was attributed to mutational *pbp* genes for ampicillin and to added *tet*(W), *erm* and *cat*(TC) genes for the antibiotics inhibiting protein synthesis.

- The majority of the antibiotic resistant *L. reuteri* strains harboured plasmid-encoded resistance genes. Traits of putative transfer machineries adjacent to both plasmid- and chromosome-located resistance genes were demonstrated.

- A maximum of two acquired resistance genes were found per *L. reuteri* strain.

- Transfer of the *tet*(W) gene from the probiotic strain *L. reuteri* ATCC 55730 to faecal enterococci, bifidobacteria and lactobacilli was non-detectable under the conditions tested, although transfer at low frequencies cannot be excluded.

5.1 Future perspectives

LAB intentionally added to the food chain should not carry transferable antibiotic resistance genes (EFSA, 2007). In the absence of reliable scientific data on the potential transfer of antibiotic resistant LAB strains, such strains should be avoided as food processing aids and probiotics according to the precautionary principle. This thesis provides data required to assess the possible risk of using antibiotic resistant strains of the LAB species *L. reuteri* and *L. plantarum* as starter cultures or probiotics. However, more data would be of value for further assessment. In particular, more transferability studies in the human gut of both conjugative and non-conjugative antibiotic resistance plasmids/transposons are needed to determine the potential for horizontal transfer. In my opinion, such studies should primarily focus on *erm* positive lactobacilli due to the clinical importance of macrolides. Sequencing of flanking regions of the remaining plasmid-encoded or chromosome-located *tet*(W) genes found in *L. reuteri* could reveal more about the acquisition of this apparently common gene within this species.

The considerable increase in available antibiotic susceptibility data for lactobacilli through *e.g.* ACE-ART (Korhonen *et al.*, 2008) and PROSAFE (Klare *et al.*, 2007) resulted in an recent update of the EFSA breakpoints for LAB used as feed additives and a subsequent split-up of the breakpoints for individual LAB species (EFSA, 2008). A regulation for LAB added to fermented food and probiotics for human use will probably be proposed in the near future. Imposed restrictions have to be based on scientific data, such as these, so that the current strategy based on the precautionary principle can be proven or discounted. Phenotypic tests and molecular tools are both needed to scientifically guarantee the presence or absence of acquired resistance genes in potential starter/probiotic candidates, as pointed out in this thesis and by others (Hummel *et al.*, 2007; van Hoek *et al.*, 2008).

The MIC data presented in this thesis in combination with the results of others will hopefully be used for risk management strategies by organisations such as EUCAST to define rational microbiological breakpoints for the species *L. reuteri* and *L. plantarum*. In this thesis, the antibiotics included in the susceptibility testing were those recommended at the time by EFSA (2005). Resistance to quinolones and third/fourth- generation cefalosporins has, as previously mentioned, been reported as an intrinsic feature in lactobacilli. In my opinion, these antibiotics should potentially be tested on a number of *Lactobacillus* species as more pathogenic bacteria become resistant to the clinically important antibiotics.

Finally, antibiotic resistance of LAB could also be regarded as a beneficial property. A resistant probiotic strain that is co-administered with an antibiotic may reduce the gastrointestinal side effects related to antibiotic treatment (Courvalin, 2006). By identifying strains with potentially non-transferable resistance genes, this field of application might gain wider acceptance and thus have a greater impact in the future.

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