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LABORATORY OF MICROBIOLOGY

TETRACYCLINE RESISTANCE IN LACTIC ACID BACTERIA ISOLATED FROM FERMENTED DRY SAUSAGES

Dissertation

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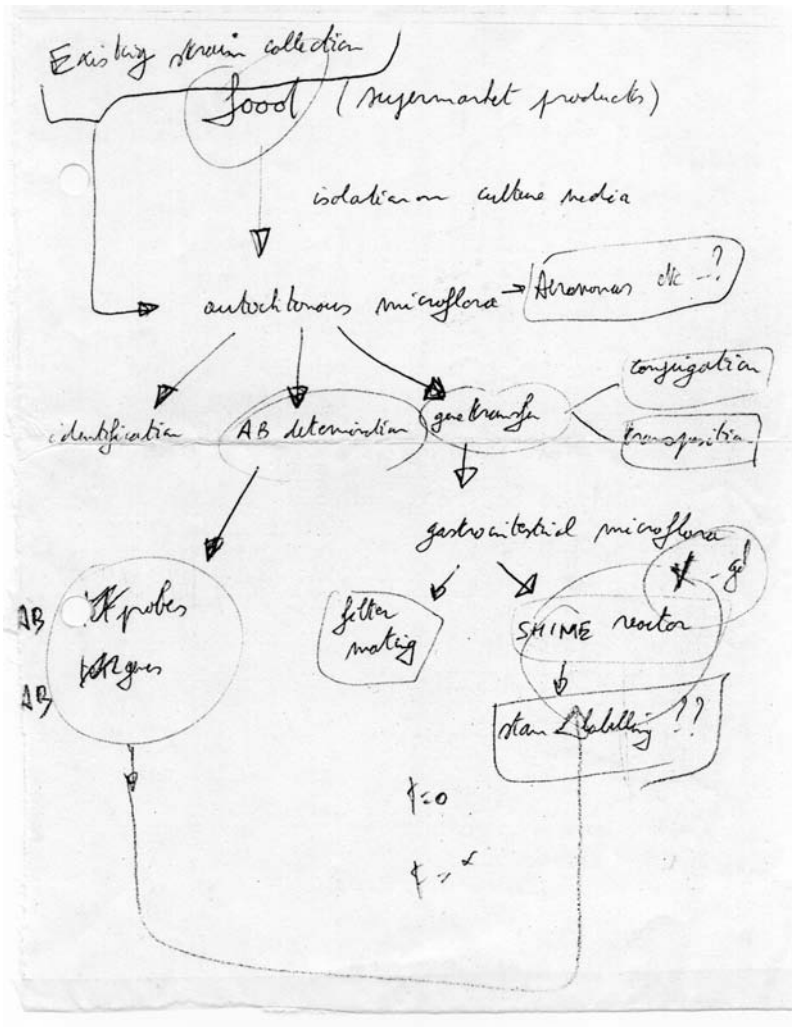
Existing strain collection
food (supermarket products)
isolation on culture media
autochtonous microflora
identification
AB determination
gene transfer
conjugation
transposition
gastrointestinal microflora
filter mating
SHIME reactor
AB
AB
pH = 0
pH = 7

Over hoe dit werk tot stand kwam…
(Een poging tot bedanken)

Deze 3 bladzijden zijn eigenlijk de belangrijkste van dit proefschrift. Niet alleen omdat bijna iedereen eerst dit stukje wil lezen, maar vooral omdat de mensen rondom mij minstens even belangrijk zijn geweest in de ontwikkeling als onderzoeker en als mens, dan de resultaten van dit onderzoek.

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Contents

List of abbreviations

AFLP	Amplified fragment length polymorphism
a_w	Water activity
BCCM	Belgian Co-ordinated Collections of Microorganisms
CFU	Colony forming units
erm	Erythromycin resistance gene
FDS	Fermented dry sausage
FEDESA	European Federation of Animal Health
GRAS	Generally regarded as safe
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
Leuc.	*Leuconostoc*
LMG	Laboratory of Microbiology Ghent
MAP	Modified atmosphere packed
MIC	Minimal inhibitory concentration
MLS	Macrolide-lincosamide-streptogramine
MRL	Maximum residue limit
MRS	de Man, Rogosa and Sharpe
MRS-S	de Man, Rogosa and Sharpe-sorbic acid
P.	*Pediococcus*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RAPD	Randomly amplified polymorphic DNA
REA	Restriction enzyme analysis
RPP	Ribosomal protection proteins
sp.	Species
spp.	Species
subsp.	Subspecies
Tc^r	Tetracycline resistant
Tc^s	Tetracycline susceptible
tet	Tetracycline resistance gene

General introduction

About 50 years ago, antibiotics were introduced for the treatment of microbial diseases. Since then, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial resistance in pathogenic bacteria. Antibiotic resistance has been shown to have occurred rarely in bacteria collected before the antibiotic era (Hughes and Datta, 1983). Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed (Levy, 1997). The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria (Levy, 1992). The solution has long been the continuous appearance of new antibiotics on the market. However, pharmaceutical companies cannot continue to deliver new antibiotics at a fast enough rate and, currently, no antibiotics belonging to a new class are expected to appear soon. Worldwide sensitising campaigns demand a less frequent and a well-considered use in order to preserve antibiotics for the future.

It is clear that hospitals offer a prime opportunity for development and transfer of antibiotic resistance (Monroe and Polk, 2000). Another focus for the development of antibiotic resistance is found in animal husbandry in which antimicrobial agents are used for prophylaxis, therapy and growth promotion. Already in the 1960s, a British committee expressed its concern about the use of antibiotics as growth promoters in the SWANN report (Anon., 1969). This has resulted in the European ban of some, and later in 1999 of most antibiotics for the use as growth promoters. The few compounds that are still allowed in the EU, represent antibiotics that are not used in human or veterinary medicine and are unlikely to exhibit cross-resistance. But in other parts of the world, no such ban exists. Moreover, the groups of antimicrobial agents currently used for animal therapy are essentially the same classes of compounds that are used in human medicine, and may also generate a reservoir of antibiotic resistant bacteria.

The human and animal microbial ecosystems are inextricably interwinded, and therefore, microbial antibiotic resistance readily crosses both ecosystems. Foods of animal origin, mainly meat products, have been suggested to be the most probable vectors of transmission of resistant bacteria to the human intestinal flora (Witte, 2000). In spite of the fairly high hygienic standards in most developed countries, faecal contamination of meat products during slaughtering cannot be avoided completely. Clonal spread of resistant bacterial strains from animals to humans is well documented for zoonotic pathogens like *Salmonella typhimurium*, but it is less documented to what extent commensals contribute in the spread of resistance determinants. However, non-pathogenic bacteria can be found on various foods in high densities as a result of the natural production process. During the past decade, it has become clear that commensal bacteria can act as reservoirs for resistance genes, and thus can play an important role in the maintenance and transfer of resistance determinants within and between bacterial populations in animal and human environments (Levy and Miller, 1989). The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria.

Most meat products are heat treated before consumption and hence no viable resistant bacteria would be expected to be present in the final product. However, the production of fermented meat products, regarded as stable and safe foods, does not include a heat treatment step, and members of the raw meat microflora that are not inhibited by the conditions created in the fermented product, such as lactic acid bacteria (LAB), might end up in the final ready-to-eat product. Although most food-associated LAB have acquired the 'Generally Regarded As Safe' (GRAS) status, and are under certain circumstances desirable as a 'protective culture', the potential health risk, due to the transfer of antibiotic resistance genes from LAB strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to pathogenic bacteria, has not been fully addressed. Moreover, LAB are also the dominating flora of other, non-fermented ready-to-eat meat products that are packed under modified atmosphere. Modified atmosphere packaging (MAP) of ready-to-eat meat products has become common practice nowadays in order to obtain fresh, refrigerated foods with an extended shelf life (Farber, 1991).

Objectives of this work

There is a need to obtain information on the extent to which commensals of foods contribute to the spread of antibiotic resistance between the animal and human environment. Therefore, the first aim of this work is to study the prevalence of antibiotic resistant LAB in MAP ready-to-eat meat products. Tetracycline resistance (Tc^r) is chosen as a focus because this agent has been widely used during the past 40 years in both humans and animals and are still important agents today, and the molecular basis of the resistance is well documented (Chopra and Roberts, 2001). Further, isolation of Tc^r LAB allow an in-depth characterization of the host, its resistance determinants and an analysis of the capacity of these food-born bacteria to transfer their resistance. Finally, it is intended to learn more on the origin and spread of Tc^r LAB and, therefore, complete process lines of fermented dry sausages (FDS) are studied.

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SHORT OVERVIEW OF THIS THESIS

Chapter 1 gives a general overview of the literature relevant to this work. In a first part, the use of antibiotics in animal husbandry, and the possible consequences and risks linked to it are discussed. Further, literature on the three focus points, tetracycline resistance, lactic acid bacteria, and fermented sausage, is one-by-one summarized. Thereby, special attention is drawn to the molecular biology of tetracycline resistance, antibiotic resistance in food-associated LAB, and the microbiology of fermented sausages.

Chapter 2 describes the prevalence of Tc^r LAB from MAP ready-to-eat meat products, including cooked ham, cooked chicken breast meat and fermented dry sausage, as well as the isolation and identification of Tc^r LAB from different fermented dry sausage end products.

Chapter 3 reports on the implementation of the rep-PCR fingerprinting technique using the $(GTG)_5$ primer as a new tool for differentiation at the species, subspecies and potentially up to the strain level of a wide range of food-associated lactobacilli.

Chapter 4 deals with the molecular analysis of the Tc^r determinants in isolates from fermented dry sausage end products and the capacity of these isolates to transfer their resistance to other LAB.

Chapter 5 describes the prevalence and diversity of the Tc^r LAB and their Tc^r determinants along the process line of fermented dry sausages.

Finally, in chapter 6, a summary, general conclusions and future perspectives are given.

1

OVERVIEW OF THE LITERATURE

1.1. Antibiotic resistance and the food chain: from the stable to the table

1.1.1. Use of antibiotics in animal husbandry

Antibiotics have been used with great success by veterinary surgeons and farmers for at least five decades. According to data compiled by the European Federation of Animal Health (FEDESA), animal use is nowadays responsible for well over one third of Europe's total antibiotic consumption (Fig. 1.1). There are three different applications for antimicrobial use in animals: therapy, prophylaxis and growth promotion.

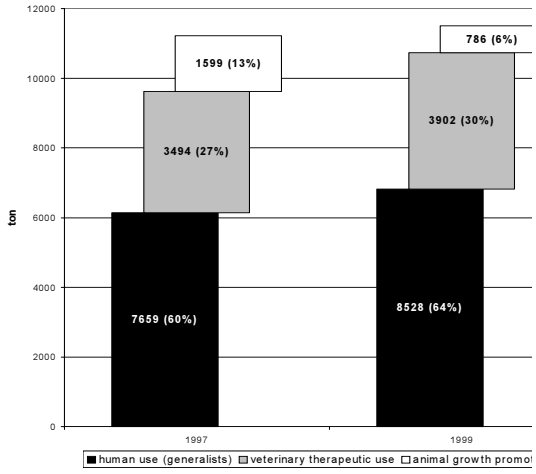

Fig. 1.1. Usage of antibiotics in humans and animals in the EU, according to data compiled by the European Federation of Animal Health (FEDESA [http://www.fedesa.be]).

Therapeutic use of antimicrobial agents is intended to control an existing bacterial infection. The main infectious diseases treated are enteric and pulmonary infections, skin and organ abscesses and mastitis. The modes of application of antimicrobial agents for therapeutic purposes differ with respect to the size of the group of animals. Individual animal treatment is commonly performed in dairy cows and calves. Whereas, for food-producing animals which are kept in larger groups, e.g. 30,000 broilers in a flock or 100 pigs in

one group, preference is given to group treatment. Moreover, it is more economical to prevent a disease, rather than to rely on treatment. Therefore, veterinary intervention in such large animal groups occurs when the first animal shows symptoms of the disease. Early medication to the entire animal group may reduce the number of sick or dead animals and may also decrease the amount of antimicrobial agents needed to treat large numbers of the symptomatically ill population, consequently reducing treatment costs. With such treatment regimes, the antimicrobials are commonly applied via feed or water. The antimicrobial agents currently used to treat or prevent bacterial infections in animals are essentially the same classes of compounds that are used in human medicine (Table 1.1).

Prophylaxis is a solely preventive measure. Its application can be to both individual animals and to groups of animals, and is widely accepted for surgical prophylaxis in animals. In dairy cows, the prophylactic intramammary administration of antimicrobials at therapeutic levels at the end of the lactation period prevents mastitis by releasing the antimicrobials in the mammary gland tissue at high concentrations for long periods. In the pork- and beef-producing industry, prophylactic use of antimicrobials occurs at key time intervals, such as weaning, or mixing of animals from different herds. Antimicrobial prophylaxis at these times is essential in many piggeries and cowhouses, as without it, frequently occurring respiratory and enteric diseases in the pigs and cattle cannot be effectively controlled. As a consequence, animal welfare would be severely compromised, the amounts of antimicrobials required for therapy would be increased and profitability would be drastically reduced. Therefore, antimicrobial prophylaxis at these key periods for disease incidence is an unavoidable measure in the current pork and beef producing systems. However, prophylactic herd treatment is criticized for providing the basis of selection of resistance.

Growth promotion may be regarded as the stimulation of an animal's growth during early life by the addition of small quantities of substances, e.g. antibiotics, to its diet. Animals receiving antibiotics in their feed gain 4 to 5% more body weight than animals that do not receive antibiotics (Anon., 1997). The mechanisms of growth promotion are still not fully elucidated, but it is supposed to be mediated by the antibacterial effect. The growth promoting effect of antibiotics in chickens was discovered by feeding mycelial mass of *Streptomyces aureofaciens*. It was later shown that residual chlortetracycline was the active ingredient which so dramatically increased growth (Jukes and Williams, 1953). Since then, the use of antibiotics as growth promoters has become widespread, in the beginning even without any restrictions. The widespread use of antibiotics as growth promoters was first criticized by the end of the 1960s, which resulted in the "Swann Report" (Anon., 1969). Although the economical benefits of using antibiotics, whether for therapeutic, prophylactic or growth promoting purposes were clearly highlighted, much concern was expressed about the possible induction of antibiotic resistance among bacteria of human and animal origin and the subsequent loss of effectiveness in the treatment of human bacterial disease. The main recommendation of the Swann Report was that the use of antibiotics for growth promotion should be restricted to antibiotics that are of economic value for livestock, that have little or no application as therapeutic agents in man or animals and that will not impair the efficacy of prescribed therapeutic drugs through the development of resistant organisms. This report was the base for the European legislation in the Directive 70/524, in which a list was published of admitted additives with their maximum and minimum dose, withdrawal period, and animal species for which they are allowed. Worldwide differences in the use and regulations of antibiotics are large. In some countries other than the members of the EU, therapeutically used antibiotics like tetracyclines and penicillins are still allowed for growth promoting. In 1986, Sweden decided to ban all antibiotics for growth promotion. The EC legislation was amended on several occasions, and since 1997 several antibiotics have been forbidden. The decisions were based on an acknowledged point of 'precautionary principle', mainly out of concern for cross-resistance with therapeutic compounds used in human medicine. Currently, only four substances are allowed for animal growth promotion in the EU, including flavophospholipol, monensin, salinomycin and avilamycin. These antibiotics represent compounds that are not used in human or veterinary therapy and are unlikely to exhibit cross-resistance.

1.1.2. RISKS OF ANTIBIOTIC USE IN ANIMAL HUSBANDRY

It is generally accepted that antibiotics should be available for use in animal husbandry, thereby considering both the economical aspect and the animal welfare. However, possible adverse effects to humans can be associated with the use of antibiotics for animals. These effects can be divided into those related to the antibacterial effects of the substances (microbiological aspects) and those related to the chemical nature of the substance (residual aspects).

1.1.2.1. Emergence and spread of antibiotic resistance

The greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens. Development of antibiotic resistance in bacteria is mainly based on two factors, the presence of resistance genes and the selective pressure by the use of antibiotics (Levy, 1992).

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

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

The **selective pressure** imposed by the use of antimicrobial agents plays a key role in the emergence of resistant bacteria. Whenever a mixed bacterial population is exposed to antimicrobial agents, it is likely that there will be bacteria that are resistant to the respective drugs at the concentration applied. Under selective pressure, the numbers of these will increase and some may pass their resistance genes to other members of the population (Aarestrup, 1999). The following factors influence the emergence of antibiotic resistance in bacteria in food producing animals: (a) the spectrum of activity of the antibiotic; (b) the number of animals exposed to antibiotics; and (c) the total amount of antibiotic used (Anon., 1999).

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

Fifty years of increasing use of antimicrobial agents has created a situation leading to an ecological imbalance resulting in the enrichment of (multiple) antibiotic resistant bacteria, both pathogenic and commensal, in human and animal habitats (Levy, 1997). It is clear that hospitals present a prime opportunity for development and transfer of antibiotic resistance (Monroe and Polk, 2000). But, there is general agreement that the exposure of animals to antibiotics selects for antibiotic resistance in animal pathogens and enteric commensal bacteria (Witte, 1998; Anon., 1999). This raises a potential risk that is present on different levels. Problems may be caused in the therapy of infections in animals through the selection for resistance among pathogenic bacteria. In addition, animals frequently harbour bacteria pathogenic for man in their intestinal tract (e.g. the zoonotic agents, *Campylobacter*, *Yersinia*, *Listeria*). Development of resistance in these zoonotic bacteria constitutes a public health risk, primarily through the increased risk of treatment failures. Furthermore, use of antibiotics will select for resistance genes in non-pathogenic bacteria, which may transfer the acquired resistance to different pathogenic bacterial species.

Many analyses state that we are facing an epidemic of bacterial resistance that is at least partially due to overuse and misuse of antibiotics. The problems caused by inappropriate use of antibiotics reach beyond the place of use (Witte, 1998). Meat products are traded worldwide, and evolving bacterial populations transgress geographical boundaries. In the countries of the developing world, which are responsible for about 25% of the world's meat production, policies regulating veterinary use of antibiotics are poorly developed or absent. In Russia, chloramphenicol is still in veterinary use, although toxic for man and animal. In Southeast Asia, use of antimicrobials in shrimp farming is unregulated (Witte, 1998).

1.1.2.2. Antibiotic residues in food

The use of antibiotics may result in residues in edible tissues, milk and eggs. De Wasch and co-workers (1998) reported that more than 5% of pork meat samples purchased from Belgian retail outlets contained residues of tetracyclines in concentrations ranging between 50 and 1000 µg/kg. The consumption of antibiotic residues by man could produce harmful effects from direct **toxicity** or from **allergic** reactions. Although a great deal of concern has been expressed about the potential risk of hypersensitivity reactions in humans consuming antibiotic residues in food, confirmed cases are extremely rare or nonexistent for most antibiotics, including beta-lactams (Dayan, 1993).

Undesirable consequence of residues in food could also result from antimicrobial activity directed against the **gastro-intestinal microflora of humans**. It would manifest itself by altering or reducing the protective barrier against infection provided by commensal gut flora. From *in vivo* studies with human volunteers it was concluded that the amount of residues left by drugs in veterinary practice or animal food supplements is too low to be a major cause of the selection of bacterial resistance in the human gut (Tancrede and Barakat, 1989; Corpet, 1987).

Technological problems may arise as a consequence of antimicrobial residues in meat used for the production of fermented dry sausage. The starter cultures used for fermentation of meat might be inhibited, resulting in a fermentation failure. The presence of penicillin (> 2 IU/g) or erythromycin (> 0.125 μg/g) has been reported to delay or stop fermentation (Holley and Blaszyk, 1997).

As for all drugs administered to food-producing animals, a **maximum residue limit** (MRL) has been established for antibiotics, and included in the European Council Regulation N° 2377/90. The purpose of the MRL was to determine the withdrawal time, which would allow levels of the antimicrobial to drop to approved levels, before milk or eggs could be sold, or before animals could be slaughtered for food. It is assumed that quantities below the MRL mean no harm to the consumer. The MRL differs depending on the tissue (muscle, liver, kidney, milk, skin, fat) and animal species. In cases in which the MRL is exceeded, either the withdrawal time was not observed or an overdose was administered. If a random check reveals residues above the MRL, the producer is responsible and juridical and economical sanctions are applied.

In general, antibiotic residues in meat and other foodstuffs can be considered as a low risk to public health.

1.1.3. ROUTES OF DISSEMINATION OF ANTIBIOTIC RESISTANT BACTERIA

Antimicrobial resistance can emerge in bacteria residing in individual animals and humans exposed to antimicrobial agents. Hospitals and animal husbandry sites are powerful foci of antibiotic selective pressure, but antibiotic treatment in the human community should be taken into consideration as well. Subsequent spread of the resistant bacteria between different environments can occur directly by skin-to-skin contact; contact with bacteria-containing

material (saliva, faeces, etc), or by the uptake of contaminated food, feed, air or water (Fig. 1.2). When reaching the new host, resistant bacteria can either colonize and infect, or remain in that particular environment for only a very short period of time. During this period, the resistant bacteria cannot only spread their resistance genes to other bacteria residing in the new host (commensals or pathogens), but can also accept resistance genes from other bacteria (Salyers, 1995).

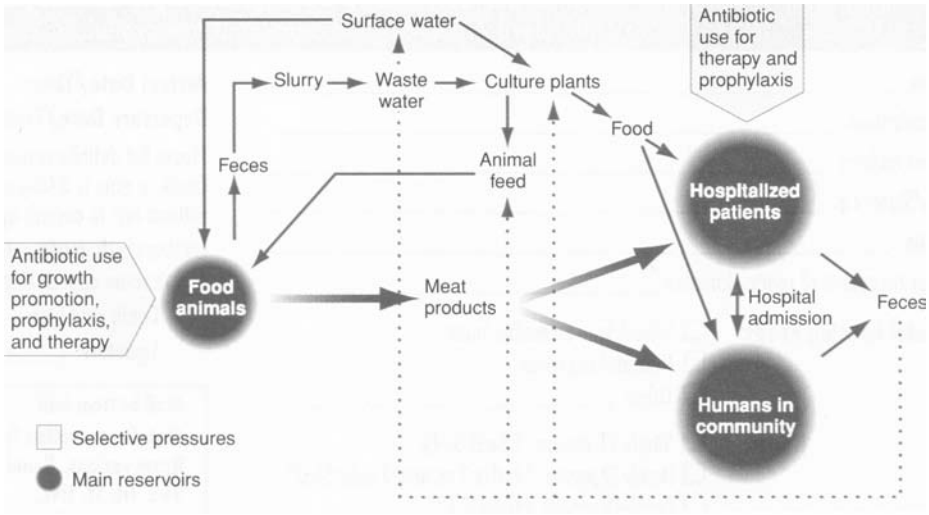

Fig. 1.2. Routes of transmission of antibiotic resistant bacteria and resistance genes. Adopted from (Witte, 2000)

Of all antibiotic resistant zoonotic bacteria causing infections in humans, *Salmonella*, *Campylobacter*, *E. coli* and *Enterococcus* are considered to be the major species that can be traced to animal sources with a high degree of certainty (Witte, 1998). Their predominant way of reaching humans is via the food chain. However, once established in a human population (not always associated with disease), such pathogens can also be spread in various ways between humans. Therefore, it is important to consider that the aforementioned zoonotic bacteria isolated from a human source may not necessarily have originated directly from animals shedding the bacteria or from contaminated animal products (Molbak *et al.*, 1999).

Compared to antibiotic resistance in pathogenic bacteria, relatively few studies have investigated acquired antibiotic resistance in non-pathogenic bacteria, including commensal bacteria of humans and animals, and plant and soil bacteria. It has been proposed that commensals may act as a reservoir for antibiotic resistance genes found in human pathogens and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations (Levy and Miller, 1989). The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. In 1998, the Reservoirs of Antibiotic Resistance network project (ROAR network [http://www.apua.org]) was set up in order to promote the studies on the selection and dissemination of non-pathogenic antibiotic resistant bacteria in humans, during food production and agricultural processes, and in the environment.

The transfer of resistant bacteria between animals and humans is often difficult to prove, and evidence of the direction of transfer is even more difficult to obtain. Since antibiotics of the same class such as tetracyclines, aminoglycosides, macrolides and beta-lactams, have been used for decades in both humans and animals, resistance to these antibiotics has also been selected for and transferred, and probably vice versa, in both groups of hosts. Once a resistance gene has become widely disseminated, it is difficult to trace it back to its origin. Characterization studies on resistance genes and plasmids in human and animal staphylococci have revealed the presence of identical resistance genes located on indistinguishable plasmids (Schwarz and Noble, 1994). Such studies produced strong evidence for the transfer of plasmids between human and animal bacteria, but in most cases it is impossible to trace where and when the original plasmid was developed, as well as the sequence of transfer events that have taken place since. There is no question that the risk of acquisition of resistant bacteria from animals is higher in humans who stay in close contact to animals or animal products, such as farmers or abattoir workers. The highest risk is for veterinarians who have daily contact with clinically ill animals that may shed resistant pathogens. Moreover, they work in an environment where a high selective pressure resulting from the use of antimicrobials, is common.

The spread of resistant bacteria from animals to humans is, in principle, possible, and there is evidence in the literature that such transfer events occurred even bilaterally (Seguin *et al.*, 1999). The frequency with which resistance properties are transferred between animals and humans is difficult to quantify. Therefore, little reliable data are available to develop a quantitative risk assessment.

1.1.4. ANTIBIOTIC RESISTANT BACTERIA IN FOOD

It was suggested that the majority of antibiotic resistant bacteria in the gastro-intestinal tract of healthy humans originate from contaminated food. In an experiment by Corpet (1988), six healthy volunteers were given a control diet for three weeks, followed by a sterile diet for 2.5 weeks. During both periods, total and antibiotic resistant Enterobacteriaceae in stools were counted. A drastic drop in faecal concentrations of antibiotic resistant enterobacteria was observed during the sterile-diet period (Corpet, 1988). As animals are in many ways part of the human food chain, transfer of antibiotic resistant bacteria from animals to humans via food would be expected to occur (Teuber and Perreten, 2000). In spite of the fairly high hygienic standards in most developed countries, contamination of raw meat and milk with skin and faecal microflora cannot be avoided completely during slaughtering and milking. Enteric pathogens are readily transmitted through foods, as are antibiotic resistant pathogens and commensals. Most foods are heat treated before consumption and hence, no viable resistant bacteria would be expected to be present in the final product. However, food-borne infections with infectious doses as high as $10^6 – 10^9$ (as for salmonellosis) are relatively common. This proves that recontamination is common and viable bacteria can be present in relatively large numbers in food when consumed. The spread of resistant bacterial strains is well documented for zoonotic pathogens, but not as much for commensals (Witte, 2000). Since the early 1990s there has been a dramatic increase in antibiotic resistance in *Salmonella* and *Campylobacter* spp., and to a lesser extent in Vero cytotoxin-producing *Escherichia coli* (VTEC) O157 from cases of human infection in developed countries (Threlfall *et al.*, 2000).

An important aspect in the observed increase of antibiotic resistance in ***Salmonella*** species is due to the emergence and clonal spread of multidrug-resistant *S. typhimurium* DT104 (with chromosomally-encoded resistances towards ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline), which now appears to have an almost worldwide distribution (Threlfall, 2000). Human infection with multi-resistant DT104 has been associated with the consumption of chicken, beef, pork sausages, and meat paste, and to a lesser extent with contact with food animals. In Denmark an outbreak of multi-resistant DT104 that could be traced back to a Danish swine herd, resulted in hospitalisation of eleven patients. Two of them died because of the strain's reduced susceptibility (Molbak *et al.*, 1999). For other common serotypes of *Salmonella*, food animals were also the primary

reservoir from where they are spread through the food chain to humans (Threlfall *et al.*, 2000).

Infections with ***Campylobacter*** *jejuni* or *C. coli* are sporadic single cases resulting from the consumption of contaminated food, milk or water. Undercooked of mishandled poultry appears to be the most important source of infection (Nachamkin and Blaser, 2000). *C. jejuni* and *C. coli* are generally susceptible to a variety of antibiotics. However, increasing resistance to some antibiotics has been documented. The most common resistance phenotypes observed were tetracycline, nalidixic acid and ciprofloxacin (White *et al.*, 2002).

Although multiple resistance remains rare in **verocytoxigenic *Escherichia coli* O157** (VTEC 0157), resistance to certain antibiotics and particularly to sulphonamides and tetracyclines, is increasing in incidence (Schmidt *et al.*, 1998).

Listeriosis is an emerging food-borne disease, and numerous outbreaks that occurred during the last decade could be linked to contaminated food (Farber and Peterkin, 1991). With the exception of tetracycline resistance (Facinelli *et al.*, 1993), the proportion of ***Listeria*** spp. resistant to antibiotics remains low (Charpentier and Courvalin, 1999; Roberts *et al.*, 1996). It has been suggested that, in humans and animals, the digestive tract was the privileged site for acquisition by *Listeria* spp. of conjugative plasmids and transposons coding for resistance from *Enterococcus* and *Streptococcus* (Doucet-Populaire *et al.*, 1991).

Antibiotic resistant **enterococci** have been found in meat products, dairy products, ready-to-eat foods and even within probiotics (Teuber and Perreten, 2000; Quednau *et al.*, 1998; Pavia *et al.*, 2000; Giraffa, 2002; Giraffa and Sisto, 1997; Davies and Roberts, 1999; Wegener *et al.*, 1997; Franz *et al.*, 2001). Once ingested, antibiotic resistant enterococci can survive gastric passage and multiply, thus leading to sustained intestinal carriage. A dozen healthy volunteers were fed a single dose in milk of either glycopeptide resistant or streptogramin resistant strains of *E. faecium* obtained from raw chicken and pork. After ingestion, the resistant enterococci not only survived but also multiplied in the intestinal tract, and were present in the stool for up to 2 weeks (Sorensen *et al.*, 2001; Bertrand *et al.*, 2000).

Little information on antibiotic resistance in relation to **non-pathogenic bacteria**, the 'normal' flora in foods is available, although they may act as reservoirs of resistance genes. Antibiotic resistant coagulase-negative staphylococci (CNS) commonly found on the body of animals, may contaminate milk or meat and are subsequently to be found in fermented food made with raw material (Teuber *et al.*, 1996). Furthermore, the CNS were suggested to be a reservoir of antibiotic resistance genes which can be transferred to *Staphylococcus aureus* (Perreten *et al.*, 1998). A streptomycin-, tetracycline-, and chloramphenicol-resistant

Lactococcus lactis subsp. *lactis* was isolated from a raw milk soft cheese (2 x 10^8 CFU/ g) containing a conjugative plasmid coding for the three resistances (Perreten *et al.*, 1997b). Also other lactic acid bacteria were reported to be resistant to antibiotics (Charteris *et al.*, 1998; Orberg and Sandine, 1985; Vidal and Collins-Thompson, 1987; Raccach *et al.*, 1985; Reinbold and Reddy, 1974; Olukoya *et al.*, 1993; Katla *et al.*, 2001).

Although it can be expected that meat eaters might have higher levels of resistant coliforms, raw vegetables and salads are likely to carry large numbers of resistant bacteria caused by contamination with sewage and manure (Corpet, 1988; Fernandez-Astorga *et al.*, 1995; Levy, 1984; Linton, 1986).

1.1.5. REDUCING THE USE OF ANTIMICROBIAL AGENTS IN ANIMAL HUSBANDRY

The possession of a resistance gene can be considered beneficial to the bacterial host when residing in an environment under antibiotic pressure. However, in the absence of active antibiotic pressure, resistant genotypes may suffer a **cost of resistance**, and have growth rates that are lower than their sensitive counterparts. Mutations that confer resistance do so by disrupting some normal physiological process in the cell, thereby causing possible disadvantageous side effects. In the case of plasmid-encoded resistance functions, bacteria must synthesize additional nucleic acids and proteins; this synthesis imposes an energetic weight and the products that are synthesized may also interfere with the cell's physiology (Lenski, 1997). Resistant bacteria may therefore be metabolically weaker compared to sensitive genotypes in the absence of antibiotics. If so, then a possible strategy for containing the spread of antibiotic resistance would be to suspend the use of a particular antibiotic until the corresponding resistant genotypes had declined to low frequency. Langlois and coworkers (1986) found that swine herds deprived of all exposure to antibiotics for 14 years showed a decline of tetracycline resistance in coliforms from 94% to about 53%. However, when the swine that did not get antibiotics for so long, were placed on a truck and moved 322 km to a new location, tetracycline resistance increased to 82% in the absence of antibiotic exposure. Further, a single course of chlortetracycline raised the level of coliform resistance to levels equivalent to a control herd receiving chlortetracycline in the diet for 13 years. The authors concluded that only a complete deprivation of antibiotics would reduce the level of

antibiotic resistance in the swine herd and that was not practicable, considering the occasional need for therapy.

An important question is whether bacteria can **overcome the cost of resistance** by evolving adaptations that counteract the harmful side effects of resistance genes. In fact, several experiments have shown that the cost of antibiotic resistance may be substantially diminished or even eliminated, by evolutionary changes in bacteria over rather short periods of time (Andersson and Levin, 1999). Lenski and coworkers (1994) have shown that repeated subculture of a plasmid-containing strain under selective conditions eventually gave rise to a variant of the plasmid obtained by mutation(s) that was much more stable in the absence of selection than the original form of the plasmid. Similar reductions in the cost of chromosomal mutations that confer antibiotic resistance have been reported, including streptomycin (Schrag and Perrot, 1996) and rifampicin (Cohan *et al.*, 1994) resistance. As a consequence of this adaptation of bacteria to their resistance genes, it becomes increasingly difficult to eliminate resistant genotypes simply by suspending the use of antibiotics. Moreover, multiple resistance genes can be associated with a single mobile element, consequently the non-use of a certain antibiotic will not necessarily result in a decrease in resistance (Salyers and Amabile-Cuevas, 1997). This has been shown in the DANMAP study, where glycopeptide resistance in *Enterococcus* spp. from broilers significantly decreased after the ban of avoparcin (Bager *et al.*, 1999). In pigs, however, it stayed at similar levels due to the co-selection of multiresistance plasmids carrying the *van*A gene cluster by the use of the macrolides antibiotic tylosin (Bager *et al.*, 1999). Following a decrease of use in tylosin during 1998 and 1999, the occurrence of glycopeptide resistance in pigs decreased in 2000 (Aarestrup *et al.*, 2001).

Even with optimal antibiotic use, antibacterial resistances will probably not decline quickly and existing resistances are unlikely to vanish. Therefore, the diffusion of existing antibacterial resistance in the population should be limited, and the emergence of new strains of resistant bacteria should be avoided, by considering the extent and type of antibiotic use for both humans and animals. Worldwide, antibiotics that select for resistance against antibiotics used for human therapy should no longer be used as animal growth promoters, as it is currently the case in the EU (Witte, 1997). In addition, new classes of antimicrobials, such as ketolides, glycylcyclines, or oxazolidinones, which are currently under development or in clinical trials, have to be exclusively reserved for human therapy (Chopra, 2001). Consequently, in the near future, no new classes of antimicrobial agents are expected to become available in veterinary medicine, and veterinarians have to rely on those antimicrobial

agents currently available. In the long run, an industrial investment in **alternatives to antimicrobials** for animal growth promotion may pay off in more efficient production of food animals as well as protection of the fragile resources that are critical to successful management of infectious diseases (Witte, 1998). Alternatives such as (i) the implementation of very high standards of hygiene to improve animal health status, e.g. an all-in-all-out system of production, vaccination and (ii) the use of enzymes, probiotics or competitive exclusion products for promoting growth and feed utilisation efficiency, may actually represent additional preventive measures rather than real alternatives (Schwarz *et al.*, 2001). To retain the efficacy of the antimicrobial agents currently available for the control of bacterial infections, an accurate diagnosis, a careful choice of the respective agents and prudent use should be undertaken.

1.2. TETRACYCLINES:

MODE OF ACTION, APPLICATIONS & USE, AND MOLECULAR BIOLOGY OF RESISTANCE

1.2.1. INTRODUCTION

Discovered in the late 1940s, the tetracycline family of antibiotics has now been used for more than 40 years (Table 1.2). The tetracyclines were one of the first groups of antimicrobial agents for which the term broad spectrum was used, because they inhibit protein synthesis of a wide range of Gram-positive and Gram-negative bacteria, atypical organisms such as Chlamydiae, mycoplasmas, Rickettsiae, and protozoan parasites. Because of the spectrum of activity, the absence of major adverse side effects, and the low production cost, tetracyclines have been widely used throughout the world in fighting infections in humans, animals, fish, and plants. Given their long history of extensive use, resistance to tetracyclines has become widespread (Levy, 1992), resulting in a reduced effectiveness. Nevertheless, they retain to play important roles in both human and veterinary medicine. A new generation of tetracyclines, the glycylcyclines, is specifically being developed to overcome problems of resistance to first and second generation tetracyclines (Chopra, 2001).

Table 1.2. Principal members of the tetracycline family of antibiotics

Generation	Generic name	Origin	Year of discovery	Status
I	chlortetracycline	*S. aureofaciens*	1948	marketed
	oxytetracycline	*S. rimosus*	1948	marketed
	tetracycline	*S. aureofaciens, S. rimosus, S. viridofaciens*	1953	marketed
	demethylchlortetracycline	*S. aureofaciens*	1957	marketed
	rolitetracycline	semisynthetic	1958	marketed
	limecycline	semisynthetic	1961	marketed
II	methacycline	semisynthetic	1965	marketed
	doxycycline	semisynthetic	1967	marketed
	minocycline	semisynthetic	1972	marketed
III	glycylcyclines	semisynthetic	1993	Phase III clinical trials

Adapted from (Chopra and Roberts, 2001) *S*.: *Streptomyces*

1.2.2. MODE OF ACTION

Two different groups of tetracyclines are distinguishable by their mode of action: typical tetracyclines such as tetracycline, chlortetracyclines, doxycycline, or minocycline exhibit bacteriostatic activity, whereas some tetracycline derivatives are bactericidal (Chopra, 1994). The bacteriostatic activity of typical tetracyclines is associated with the **reversible inhibition of protein synthesis** (Schnappinger and Hillen, 1996). Atypical tetracycline derivatives have been suggested to target the cytoplasmic membrane since they cause morphological alterations of the bacterial cell and trigger release of beta-galactosidase from the cytoplasm (Oliva *et al.*, 1992). These derivatives have no therapeutic value because their action on the membrane is not specific for the prokaryotic cell, and will be excluded from further discussion.

In order for tetracyclines to interact with their targets these molecules need to **traverse one or more membrane systems** depending on whether the susceptible organism is Gram-positive or Gram-negative (Schnappinger and Hillen, 1996). Tetracyclines traverse the outer membrane of Gram-negative bacteria through porin channels, probably chelating a Mg^{2+} ion. The cationic metal ion-antibiotic complex is attracted by the Donnan potential across the outer membrane, leading to accumulation in the periplasm, where the metal ion-tetracycline complex probably dissociates. A weakly lipophilic molecule diffuses through the lipid bilayer regions of the inner (cytoplasmic) membrane. Similarly, the electroneutral, lipophilic form is transferred across the cytoplasmic membrane of Gram-positive bacteria. Uptake of tetracyclines across the cytoplasmic membrane is energy dependent and driven by the ΔpH component of the proton motive force ($PMF = \Delta pH + \Delta\psi$), consequently the antibacterial activity is influenced by pH and Mg^{2+} concentration in the extracellular medium.

The **molecular biochemistry** of the mode of action of tetracyclines is not completely understood. They probably act by reversible binding to the bacterial 30S ribosomal subunit and thereby preventing the attachment of aminoacyl-tRNA to the ribosomal receptor, resulting in an inhibition of protein synthesis. Further research on the ribosome-tetracycline interaction and its correlation with the inhibition of protein synthesis is necessary to reveal the molecular mechanism (Chopra and Roberts, 2001).

The absence of a major anti-eukaryotic activity explains the selective antimicrobial properties of the tetracyclines. At the molecular level, this selectivity results from relatively weak inhibition of protein synthesis by 80S ribosomes and poor accumulation in mammalian cells (Chopra and Roberts, 2001).

1.2.3. APPLICATIONS & USE OF TETRACYCLINES

Tetracyclines have been used extensively since their introduction in the early 1950s. They are the second most used group of antibiotics after the penicillins and they still have different applications in various fields.

Humans. Nowadays, tetracyclines are still applied for treatment of infections by Chlamydiae (lymhogranuloma), Rickettsiae (rickettsiosis), *Leptospira* spp. (leptospirosis), *Borrelia* spp. (lyme disease, relapsing fever), *Bartonella quintana* (trench fever) and to treat acne (Sanford*et al.*, 2002). Mainly doxycyclines are used and are of value primarily in the prophylaxis and treatment of community-acquired infections, rather than for nosocomial infections. A recent report on the use of antibiotics in Dutch hospitals supports this view (Janknegt *et al.*, 2000). New applications of tetracyclines include treatment of stomach ulcers caused by *Helicobacter pylori* (one of the three components in a triple formulation), treatment of rheumatoid arthritis, and treatment of infections with methicillin-resistant *Staphylococcus aureus* (Hunter and Hill, 1997).

Animals. The agricultural market for tetracyclines far exceeds the use for humans. This is particularly true in fish farming. In Germany for instance, oxytetracycline and chlortetracycline are the only antibiotics licensed for use in aquaculture (Hunter and Hill, 1997). In some countries, regulations on the use of antimicrobials may exist, but are not always effectively enforced, in others no regulatory regime exists (WHO, 1999). In addition, the tetracyclines have applications for the treatment of infections in poultry, cattle, sheep, and swine. In some cases, e.g. for therapeutic treatment of large numbers of poultry, the antibiotics are added directly to feed or water or can be administered in aerosols. According to data compiled by the European federation of animal health (FEDESA [http://www.fedesa.be]) tetracyclines are the most frequently used antibiotics in animal husbandry (66% of the total amount, corresponding to 2294 tons/year). Tetracyclines are also used for treatment of infections in domestic pets (Kordick *et al.*, 1997). Although tetracyclines were banned as growth promoters in Europe in the early 1970s, no such ban has been imposed in other parts of the world such as the United States and Australia (Chopra and Roberts, 2001). According to data collected from a survey by the Animal Health Institute (representing companies in the US that make medicines for pet and farm animals) the volume of antibiotics

used in animals in the US in 1999 amounts to 9280 tons of active ingredient. Approximately 15% is used as growth promoters, of which the majority are antibiotics banned in the EU. Tetracyclines, the second most used antibiotics, count for 16% of the total amount (corresponding to 1470 tons), whereof 5,4% (80 tons) is used as animal growth promoters.

Other uses. Tetracyclines are (i) sprayed onto fruit trees and other plants to treat infection by *Erwinia amylovora* (lethal yellowing), (ii) injected in palm trees to treat mycoplasma infections, and (iii) used to control infection of seeds by *Xanthomonas campestris* (black rot) (Levy, 1992). They also have applications in the treatment of insects of commercial value, e.g. oxytetracycline is used to treat foulbrood disease of the honeybee, which is caused by either *Bacillus larvae* or *Streptococcus pluton* (Levy, 1992).

1.2.4. RESISTANCE TO TETRACYCLINES

Bacterial resistance to tetracyclines was first reported in *Shigella dysenteriae* in 1953, shortly after their discovery (Roberts, 1996). Prior to this, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines, as illustrated by the finding that among 433 different members of the *Enterobacteriaceae* collected between 1917 and 1954, only 2% were tetracycline resistant (Hughes and Datta, 1983). The emergence of resistance has followed the introduction of these agents for human, animal, and agricultural use. Tetracycline resistance (Tc^r) has now become widespread in both Gram-negative and Gram-positive species due to acquisition of tetracycline resistance genes (*tet* genes) located on transposons or plasmids. So far, three different bacterial strategies of Tc^r have been identified, and more than 30 different genes have been reported.

1.2.4.1. Nomenclature of tetracycline resistance determinants

Currently, two *tet* genes are considered to belong to the same class and are given the same gene designation if they have ≥ 80% of their amino acid sequence in common (Levy *et al.*, 1999). The correct nomenclature is as shown in Table 1.3. A total of 29 classes of *tet* genes and four classes of oxytetracycline resistance (*otr*) genes have been described and characterized (Table 1.4). There is no inherent difference between a *tet* and an *otr* gene. The

otr genes were first identified in oxytetracycline-producing *Streptomyces*, and thus the nomenclature reflects the organisms first shown to carry the particular gene.

Table 1.3. Nomenclature of tetracycline resistance determinants

		Structural [c]		Regulatory (repressc	
Class [a]	Determinant [b]	Gene	Protein	Gene	Prote
n	Tet n	*tet* (n)	Tet(n)	*tetR* (n)	TetR(

Adopted from (Levy *et al.*, 1999); [a] Class n is used as an example, where n is a lette not R) or a number (30, 31, 32, etc.); [b] Note the space between Tet and n; [c] In the multiple structural genes, the following format is used *tetA* (n), *tetB* (n), etc.

1.2.4.2. Mechanisms of tetracycline resistance

Resistance to tetracyclines is primarily due to acquisition of *tet* genes rather than to mutation of existing chromosomal genes. There are three mechanisms by which organisms become resistant to tetracyclines (Table 1.4): (i) reduction of the intracellular concentration of tetracycline (efflux proteins), (ii) protection of the ribosome as the antibiotic target (ribosomal protection proteins), and (iii) inactivation of the antibiotic by modifying enzymes.

Tetracycline specific efflux proteins. The efflux proteins are the best studied Tet determinants. They belong to the major facilitator superfamily (MFS), of which products include over 300 individual proteins (Paulsen *et al.*, 1996). All the tetracycline efflux genes (n = 20) code for membrane-associated proteins which export tetracycline from the cell. Export of tetracycline reduces the intracellular drug concentrations and thus protects the ribosomes within the cell. Most of these efflux proteins confer resistance to tetracycline but not to minocycline or glycylcyclines. An exception is Tet(B), which confers resistance to both tetracycline and minocycline but not to glycylcyclines. However, laboratory-derived mutations in *tet*(A) and *tet*(B) have led to glycylcyclines resistance, suggesting that bacterial resistance to this group of drugs may develop over time and with clinical use (Chopra and Roberts, 2001). Each of the efflux genes code for an approximately 46-kDa membrane-bound efflux protein with either 12 (Gram-negative) or 14 (Gram-positive) predicted transmembrane α-helices. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient. Tetracycline efflux proteins share amino acid and protein structure similarities with other efflux proteins involved in multiple-drug resistance, quaternary ammonium resistance, chloramphenicol, and quinolones resistance (Sheridan and Chopra, 1991).

Table 1.4. Mechanisms of resistance for characterized *tet* and *otr* g

Genes coding for	Fou
Efflux proteins	
tet (A), *tet* (B), *tet* (C), *tet* (D), *tet* (E)	C
tet (F), *tet* (G), *tet* (H), *tet* (I), *tet* (J)	
tet (Y), *tet* (30), *tet* (31), *tet* (34)[d], *tet* (35)	
tet P(A)[c], *tet* (V)[c], *tet* (Z), *tet* (33)	C
otr (B)[d]	G + & *Str*
tet (K), *tet* (L)	G - & G + &
tcr 3[b]	*Strept*
Ribosomal protection proteins	
tet P(B)[c], *tet* (S), *tet* (T), *tet* (32)[c]	G
tet (M), *tet* (O), *tet* (W), *tet* (Q)	G - &
otr (A)[d]	G + & *Str*
tet[b]	*Strept*
Emzymatic inactivation of tetracycline	
tet (X)	C
Unknown[e]	
tet (U)	G
otr (C)[d]	*Strept*

a/ adapted from (Chopra and Roberts, 2001) and supplemented (Melville et al., 2001; Nonaka and Suzuki, 2002; Teo et al., 2(positive, G-: Gram-negative; b/ these genes have not been given (Levy et al., 1999); c/ so far only reported in anaerobic species; d resistance gene; e/ *tet* (U) has been sequenced but does not appea

Ribosomal protection proteins. Ribosomal protection is the most widespread of the Tc^r mechanisms. Ribosomal protection proteins (RPP) are cytoplasmic proteins (72-kDa) which protect the ribosomes from the action of tetracycline, doxycycline and minocycline. They confer a wider spectrum of resistance to tetracyclines than is seen for bacteria carrying tetracycline efflux proteins. The RPP have homology to elongation factors EF-Tu and EF-G (Taylor and Chau, 1996). The greatest homology is seen at the *N*-terminal area, which contains the GTP-binding domain. Current data suggest that the ribosomal protection proteins bind to the ribosome. This causes an alteration in ribosomal conformation which prevents tetracycline from binding to the ribosome, without altering or stopping protein synthesis. The hydrolysis of GTP may provide the energy for the ribosomal conformational change.

The Tet(M) and Tet(O) proteins are the most extensively characterized members of the ribosomal protection group. It has been assumed that the other proteins in the RPP group have GTPase activity and interact with tetracycline and the ribosomes in similar ways, because of the similarities at the amino acid sequence level. Based on the amino acid sequence comparison, the ribosomal protection proteins can be divided into three groups. The first group includes Tet(M), Tet(O), Tet(S), Tet(32), and Tet(W). The second group includes the Tet(Q) and Tet(T) proteins, while the third group consists of TetB(P) and Otr(A). For most *tet* genes, only one representative from each class has been sequenced. One exception is the *tet*(M) gene, which has been sequenced from a number of Gram-positive and Gram-negative species. By comparing these sequences, a mosaic structure was detected which could be traced to two distinct alleles (Oggioni *et al.*, 1996). The two alleles displayed a divergence of 8% and a different %G+C content. The block structure of these genes provides evidence for the contribution of homologous recombination to the evolution and the heterogeneity of the *tet*(M) locus.

Enzymatic inactivation of tetracycline. The only example of tetracycline resistance due to the enzymatic alteration of tetracycline is coded by the Tet X determinant, found in the Gram-negative anaerobe *Bacteroides* sp. (Speer *et al.*, 1991). The gene product was shown to be a 44-kDa cytoplasmic protein that chemically modifies tetracycline in the presence of both oxygen and NADPH. Sequence analysis indicated that this protein has amino acid homology with other NADPH-requiring oxidoreductases. It does not function in the natural anaerobic *Bacteroides* host, but has been shown to function after cloning in *E. coli* (Speer *et al.*, 1991).

Other/unknown mechanisms of resistance. The plasmid-borne Tet(U) determinant that provides low-level resistance to both tetracycline and minocycline in *Enterococcus faecium*, was tentatively categorized as related to the ribosomal protection protein family (Ridenhour *et al.*, 1996). However, the predicted protein of only 105 amino acids had little sequence identity to any other tetracycline-resistant protein, and the mechanisms are thus listed as unknown (Table 1.4). The *otr*C gene from *Streptomyces* sp. has not been sequenced, and its mechanism is unknown. A new Mg^{2+}-dependent oxytetracycline resistance gene *tet*(34) was recently reported in *Vibrio* (Nonaka and Suzuki, 2002). The amino acid sequence of the ORF was homologous to sequences of several bacterial xanthine-guanine phosphoribosyltransferases (XPRT), which act in purine nucleotide synthesis. Mg^{2+} binding

site residues and the active site were highly conserved in XPRT and the ORF of Tet 34.

Other efflux systems. Bacteria have a number of innate chromosomally-encoded proteins, which transport molecules in and out of the cell. Some of these efflux pumps exhibit an extremely wide specificity covering many antibiotics, chemotherapeutic agents, detergents, dyes, and other inhibitors. These proteins have been divided into groups which include the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) family, the small multidrug resistance (SMR) family, and the ATP-binding cassette (ABC) transport family (Nikaido, 1998). The MFS, RND and SMR families use the proton motive force as the driving force for efflux. In contrast, the ABC transporters use ATP hydrolysis. Some, but not all of these efflux pumps confer resistance to tetracycline. Examples of these are the Acr system found in *E. coli*, the multiple Mex systems in *Pseudomonas aeruginosa* and related operons in *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Campylobacter jejuni* and *Neisseria gonorrhoeae*, and the *mar* locus and the *emr*E gene in *E. coli* (Chopra and Roberts, 2001).

Point mutations. The first ribosomal mutation giving rise to clinical tetracycline resistance was described in 1998 in isolates of *Propionibacterium* (Ross *et al.*, 1998). A change of a guanine to a cytosine at position 1058 in the 16S rRNA was found to be associated with an increase in the MIC to tetracycline and doxycycline, and was not seen in any susceptible strain. This region of the 16S rRNA, known as helix 34, is important for peptide chain termination and translational accuracy. The mutation was re-created in *rrn*B, the *E. coli* gene for 16S rRNA, and cloned on a multicopy plasmid (Ross *et al.*, 1998). An *E.* coli strain bearing this plasmid was more resistant to tetracycline and had a longer lag-phase if grown without the drug, the latter reflecting a slight loss of ribosome function. Mutations which alter the permeability of the outer membrane porins and/or lipopolysaccharides can also affect bacterial susceptibility to tetracycline and other agents (Schnappinger and Hillen, 1996). How often these mutations occur and whether they are of clinical importance has not been established.

1.2.4.3. Incidence and distribution of tetracycline resistance

Tet determinants are found in a variety of bacteria isolated from man, animals, food and environment (Chopra and Roberts, 2001). The majority of these determinants have been associated with plasmids and/or transposons. Furthermore, tetracycline itself is able to promote the mobility of some elements by stimulating the frequency of conjugation (Hunter and Hill, 1997). These genetic properties of resistance determinants and the continued use and misuse of tetracycline in medicine, veterinary medicine, and agriculture have probably caused (or at least stimulated) their **distribution to virtually all groups of bacteria** formerly susceptible to tetracyclines. The widespread distribution of specific *tet* genes such as *tet*(B) or *tet*(M) support the hypothesis that the *tet* genes are exchanged by bacteria from many different ecosystems. The *tet*(B) gene has the widest host range of the Gram-negative *tet* genes and has been identified in more than 20 Gram-negative genera, while *tet*(M) is found in more than 25 genera including Gram-negative and Gram-positive bacteria. It was suggested that some genes, such as *tet*(E), may have a more limited host range because they are located on non-mobile plasmids, which reduces opportunities for transfer to other species and genera (DePaola and Roberts, 1995). Obligatory intracellular pathogens such as Chlamydiae and Rickettsiae have not yet acquired tetracycline resistance. Since these bacteria grow only inside cells, it would require that cells be infected with two genera to allow gene exchange into the obligate intercellular pathogen. Therefore, tetracyclines remain antimicrobial agents of primary choice to treat infections with Chlamydiae and Rickettsiae (Sanford*et al.*, 2002).

Based on current data, most *tet* genes may be divided in "Gram-negative *tet* genes" and "Gram-positive *tet* genes" (Chopra and Roberts, 2001) (see also Table 1.4). The **"Gram-negative *tet* genes"** are those which have (so far) been found exclusively in Gram-negative bacteria, *i.e.* *tet*(A) – *tet*(E), *tet*(F), *tet*(G), *tet*(H) – *tet*(J), *tet*(Y), *tet*(30), and *tet*(31). These genes have higher G+C contents (> 40%) than those of Gram-positive origin. All of the Gram-negative *tet* genes encode efflux proteins and do not express well if moved into Gram-positive hosts. The **"Gram-positive *tet* genes"** are those which are usually found in Gram-positive species, but more importantly, have relatively low G+C contents (< 35%). The genes exclusively found in Gram-positive bacteria are *tet*P(A), *tet*P(B), *tet*(S), *tet*(T), *tet*(U), *tet*(V), *tet*(Z), *tet*(32), *otr*(B), and *otr*(A). Other *tet* genes, such as *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(W), and *tet*(Q), are found in both Gram-positive and in an increasing number of Gram-negative species. The ribosomal protection genes are generally thought to be of Gram-

positive origin but are now often found in a variety of aerobic and anaerobic Gram-negative species (Roberts, 1997). The Gram-positive genera and *Streptomyces* often have individual isolates that carry multiple *tet* genes, which can have either the same mode of action (efflux or ribosomal protection), or different modes of action (efflux and ribosomal protection) (Roberts, 1997). This is uncommon in Gram-negative bacteria. The reason for this is unknown, but a similar situation exists for the carriage of other antibiotic resistance genes (Roberts, 1996).

Commensal bacteria have the same *tet* genes, plasmids, and transposons as their disease-producing counterparts among the opportunistic and pathogenic bacteria, e.g. *Haemophilus* (Marshall *et al.*, 1984), *Neisseria* (Knapp *et al.*, 1988), Bacteroides (de Barbeyrac *et al.*, 1991), *Bacillus* (Sakaguchi and Shishido, 1988) and *Streptococcus* (Fitzgerald and Clewell, 1985). However, these commensal bacteria have not yet been as extensively examined as bacteria causing human diseases. Nevertheless, it has been proposed that commensal bacteria may act as a reservoir for *tet* and other antibiotic resistance genes found in human pathogens and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations (Roberts, 1994).

The Gram-negative efflux determinants are normally found on transposons inserted into a diverse group of plasmids from a variety of incompatibility groups, with restricted or broad host ranges (Roberts, 1997). The Tet E determinant differs from the Tet A, Tet B, Tet C, and Tet D because it is associated with large plasmids that are neither mobile nor conjugative (DePaola and Roberts, 1995). The Gram-positive Tet K and Tet L determinants are found on small transmissible plasmids that can become integrated into the chromosome (McMurry and Levy, 2000). The ribosomal protection determinants Tet S and Tet O can be found on conjugative plasmids, or in the chromosome, where they are not self mobile (Charpentier *et al.*, 1994; Luna and Roberts, 1998). The Tet M determinant is often associated with conjugative chromosomal elements of the Tn*916*-Tn*1545* family, which code for their own transfer (Franke and Clewell, 1981; Courvalin and Carlier, 1987). This group of elements form circular intermediates, which are essential for both intracellular transposition and intercellular conjugative transfer (Flannagan *et al.*, 1994). The two transposons Tn*916* and Tn*1545* differ in size (18 *versus* 25.2 kb, respectively) and in the antimicrobial resistance which they encode (resistance to tetracycline and to tetracycline/erythromycin/kanamycin, respectively). Despite these differences, the two transposons are similar and even identical in many respects, e.g. in the sequence of termini, and by the integrase and excisase genes that encode transposition functions. There appears to be few if any limits to the types of

bacterial hosts into which conjugative transposons will transfer *in vitro*. The Tn*916* family was found naturally or could be transferred in the laboratory into over 50 different species representing 24 bacterial genera (Clewell *et al.*, 1995). Evidence of the transfer *in vivo* exists as well, as it was demonstrated that Tn*1545* could transfer from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tracts of gnotobiotic mice (Doucet-Populaire *et al.*, 1991). The Tn*916* family can mobilize plasmids in trans, *i.e.* the transposon provides all the proteins needed for mating and the plasmid provides the proteins that nick the plasmid and initiate plasmid transfer (Clewell *et al.*, 1995).

1.2.4.4. Determination of resistance

The main objective of susceptibility testing is to predict the outcome of treatment with the antimicrobial agents tested. The implication of the result "susceptible" is that there is a high probability that the patient will respond to treatment with a specific concentration of that antimicrobial agent. The result "resistant" implies that this treatment is likely to fail. In this regard, a lot of efforts have been put into the establishment of susceptibility testing methods for clinical microorganisms, including the publication of breakpoints to interpret the susceptibility testing results. But different **guidelines** exist for performing antimicrobial susceptibility testing created by national breakpoint committees, e.g. British Society of Antimicrobial Chemotherapy (BSAC), Commissie Richtlijnen Gevoeligheidsbepalingen (CRG), Deutscher Institut für Normung (DIN), Mesa Española de Normalización de la Sensibilidad y Resistencia a Los Antimicrobianos (MENSURA), Norwegian Working Group on Antibiotics (NWGA), Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM), and Swedish Reference Group of Antibiotics (SRGA). In 1997 a EUropean Committee on Antimicrobial Susceptibility Testing (EUCAST) was constituted to achieve consensus on the practice of antimicrobial susceptibility testing by bringing together the national committees and work out standardised methods and breakpoints. One of their objectives is to work together with the National Committee for Clinical Laboratory Standards (NCCLS, US) to achieve international consensus on susceptibility testing. One shortcoming is that all published performance standards up to now are optimised only for a limited spectrum of organisms, mainly clinical organisms, and it is not likely that the same methods, reference tables, etc. will be applicable to others.

Antimicrobial susceptibility testing may be performed reliably either by dilution or diffusion methods (Jorgensen and Turnidge, 1999). The **dilution methods** determine the minimal inhibitory concentration (MIC) of an antimicrobial agent required to inhibit or kill a microorganism. Procedures for determining MICs can be carried out by either agar- or broth-based methods. Antimicrobial agents are usually tested at twofold serial dilutions, and the lowest concentration that inhibits the visible growth of an organism is regarded as the MIC. The broth-based method can also be performed in microplates, which can contain several antimicrobial agents to be tested simultaneously and which are suitable for automated spectrophotometric reading of the susceptibility results. The **disk diffusion method** allows categorization of bacterial isolates as susceptible, resistant or intermediate to a variety of antimicrobial agents. To perform the test, commercially prepared filter paper disks impregnated with a specified amount of an antimicrobial agent are applied to the surface of an agar-based culture medium that has been inoculated with the test organism. The drug in the disk diffuses through the agar upon contact with its surface. As the distance from the edge of the disk increases, the concentration of the antimicrobial agent decreases logarithmically, creating a drug concentration gradient in the agar medium surrounding the disk. The disk diffusion method has the advantage that it is relatively inexpensive, flexible regarding the selection of antimicrobial agents used for testing, and technically simple to perform. However, only qualitative results are obtained, whereas the dilution methods produce quantitative results that also can be used to categorise in susceptible, resistant or intermediate. The quantitative results may be useful in the delineation of degrees of resistance among isolates. The **gradient diffusion method** (Etest, AB Biodisk, Sweden) is a method for quantitative antimicrobial susceptibility testing in which a preformed antimicrobial gradient from a plastic-coated strip diffuses into an agar medium inoculated with the test organism. In this test, the MIC is read directly from a scale on the strip, at the point where the ellipse of organism growth inhibition intercepts the strip. There is a good agreement between the MICs obtained by the Etest and those obtained by reference dilution methods. The Etest combines the simplicity and flexibility of the disk diffusion test with the ability to determine the MICs. However, Etest strips are much more expensive than the disks used for diffusion testing.

There is no standard methodology for antimicrobial susceptibility testing applicable to all organisms, because different species may require different culture conditions, and may differ in the breakpoints for categorization as susceptible, resistant or intermediate. Phenotypic resistance relates to arbitrarily chosen breakpoints and depends upon the experimental

conditions, including (i) medium composition, (ii) concentration of inoculum, and (iii) incubation parameters.

As the molecular basis of antimicrobial resistance has been partly or fully elucidated for many antimicrobial agents, genetic methods for assessing antimicrobial resistance have been developed (Cockerill, 1999). Compared to conventional susceptibility methods, the **genetic methods** have some advantages. (1) They assess the genotype rather than the phenotype (*i.e.* the expression of the genotype under artificial or laboratory conditions), and are therefore independent of regulation of expression and, very important, of the culture conditions. (2) They can be performed both on isolates and on samples. (3) They are faster. (4) They are also applicable to slow-growing or non-culturable organisms. (5) Their outcome is the presence or absence of resistance rather than a categorization of resistance. (6) And they can be easily standardised. However, disadvantages of genetic testing methods are that: (a) different assays are required for each antibiotic resistance gene, (b) these methods only detect what one specifically is looking for, and will not detect new, unknown forms of antibiotic resistance, (c) and that intrinsic resistance is not detected. The latter type of resistance can be genetically undetectable because of lack of a specific target as is the case of impermeability to the drugs. The major techniques used for genetic detection of antibiotic resistance are DNA probe hybridisation and PCR.

In case of Tet determinants of which representatives of all known classes have been sequenced (Levy *et al.*, 1999), genetic methods for detecting tetracycline resistance have been extensively applied (Tenover *et al.*, 1995). Not only class-specific primers were developed and validated (Pang *et al.*, 1994; Marshall *et al.*, 1983; Guillaume *et al.*, 2000; Roberts *et al.*, 1993; Charpentier *et al.*, 1994; Gascoyne-Binzi *et al.*, 1994; Aminov *et al.*, 2002), but also mechanism-specific degenerated primers have been reported for detection of all RPP genes (Clermont *et al.*, 1997). In addition, these degenerated primers allow to detect new members of the *tet* gene family, e.g. *tet*(T) (Clermont *et al.*, 1997), and *tet*(32) (Melville *et al.*, 2001). More recently, PCR primers were described for a culture-independent study of the molecular ecology of tetracycline resistance in samples of the rumen of cows, and in swine feed and faeces (Aminov *et al.*, 2001). In general, the choice of method has to be based on the experimental set-up and is ideally a combination of phenotypic and genetic methods.

1.3. Lactic acid bacteria:

identification and typing, and a host for acquired antibiotic resistances

1.3.1. Introduction

Lactic acid bacteria (LAB) comprise a heterogeneous group of Gram-positive, non-spore-forming strictly fermentative bacteria. They occur as cocci, coccobacilli or rods and generally lack catalase, although pseudo-catalase activity has been reported in rare cases. Hexoses are converted mainly to lactic acid (homofermentatives) or to lactic acid, carbon dioxide, ethanol and/or acetic acid (heterofermentatives). LAB are commonly found in foods (dairy products, fermented meat, sour dough, fermented vegetables, silage, beverages), on plants, in sewage, but also in the genital, intestinal and respiratory tracts of man and animals. The LAB in foods belong to the genera of *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997).

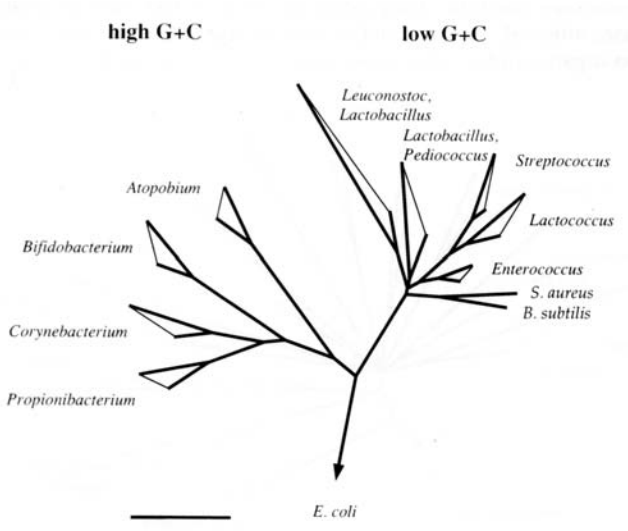

Fig. 1.3. Phylogenetic tree of Gram-positive bacteria based on 16S rRNA sequence comparison. The bar indicates 10% expected sequence divergence. Adopted from (Schleifer and Ludwig, 1995)

Based on 16S and 23S rDNA sequence data, the Gram-positive bacteria form two lines of descent (Fig. 1.3). One phylum consists of Gram-positive bacteria with a DNA base composition of less than 50 mol% G+C, the so-called *Clostridium* branch, whereas the other branch (Actinomycetes) compromises organisms with a G+C content that is higher than 50 mol%. The typical LAB have a G+C content of less than 50 mol%. While the genus *Bifidobacterium* is considered to be a member of the LAB from a physiological point of view, based on the high DNA G+C content and from 16S rRNA data it is quite clear now that bifidobacteria belong to the Actinomycetes branch, comprising also *Propionibacterium* and *Brevibacterium* (Fig. 1.3). There is little correlation between traditional classification and phylogenetic relatedness of LAB. The morphologically distinct genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* are phylogenetically intermixed (Schleifer and Ludwig, 1996).

The LAB play a prominent **role in many aspects of food** development and health. Food fermented with LAB is an important part of the human diet, including a wide variety of fermented dairy products (e.g. cheese, yoghurt), fermented sausages, vegetables and olives, sour dough breads, soda crackers, silage etc. (Wood, 1998). These organisms are particularly suitable as antagonistic microorganisms in foods because they are capable of inhibiting other food-borne bacteria by e.g. production of organic acids, hydrogen peroxide and/or bacteriocins (De Vuyst and Vandamme, 1994; Holzapfel *et al.*, 1995). Some species of LAB are claimed to have a health or nutritional benefit; e.g. improved nutritional value of food, control of intestinal infections, improved digestion of lactose, control of some types of cancer, and control of serum cholesterol levels (Gilliland, 1990). Therefore, their use as probiotics, *i.e.* dietary and therapeutic adjuncts, for man and animals is receiving increased attention in the last decade.

With the exception of some streptococci, LAB are not considered to be pathogenic to man and animals. However, there have been reports of the involvement of LAB in human clinical infection (Aguirre and Collins, 1993). In the majority of these clinical cases, patients had a history of underlying disease, should be considered as immunocompromised and/or may have been treated with antibiotics. Therefore, some LAB may fall into the category of opportunistic pathogens. Nevertheless, there is no evidence to doubt the safety of ingesting large numbers of LAB in fermented foods, and because of this long history of safe use, the '**Generally Regarded As Safe**' (GRAS) status has been ascribed to food-associated LAB.

1.3.2. IDENTIFICATION AND TYPING OF LACTIC ACID BACTERIA

The classical approach to bacterial taxonomy of LAB was based on morphological and physiological features. This was expanded to include chemotaxonomic markers (e.g. cellular fatty acids), whole-cell protein analysis and other characteristics of the cell. An improved classification and identification is very much dependent on genotypic information. Genotypic methods such as sequencing of rDNA, ribotyping, randomly amplified polymorphic DNA (RAPD), rep-PCR fingerprinting, amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE) of whole digested chromosomal DNA now constitute an important part of modern LAB taxonomy. Below, a concise overview is presented of the most important techniques used for classification and identification of LAB.

1.3.2.1. Phenotypic methods

The conventional phenotypic approach in LAB taxonomy still has its place in applied (food) microbiology laboratories. Different key tests have been widely adopted and nowadays morphological characterization as well as physiological, metabolic/biochemical and chemotaxonomic methods are used. Simple **physiological tests**, such as growth at different temperatures, acid, alkaline and salt tolerance and gas production are useful for genus differentiation. The determination of **carbohydrate fermentation patterns** is used in standard phenotypic tests to differentiate species. Although very useful, one should be aware of the limitations of this method, notably the large degree of variation within species, interlaboratory variation and poor reproducibility (Pot *et al.*, 1994a). However, databases prepared from results using standardized, commercially available systems (e.g. API 50 CH, API systems, France) are valuable due to the increased standardization and the accumulation of large numbers of strains. The use of identification systems based on biochemical and physiological characteristics results often in disappointing identification results and misidentification.

The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), or **protein profiling**, has proved to be extremely reliable for identification on the species and/or subspecies level provided that a database of digitised and normalized protein patterns of all known species

of LAB is available (Pot *et al.*, 1994b). For some species, the discriminatory power of protein profiling is limited, as witnessed within the *Lactobacillus acidophilus* complex (Gancheva *et al.*, 1999), and the closely related species *Lactobacillus plantarum*, *Lb. pentosus* and *Lb. paraplantarum* (Torriani *et al*., 2001).

1.3.2.2. Genotypic methods

DNA based techniques enable an improved insight in the identity of microorganisms on different levels, varying from genus to strain level depending on the methods used. In general they have the advantage over phenotypic identification methods of not being influenced by the culture conditions. The direct **sequencing of the 16S rRNA** genes by PCR technology is one of the most powerful methods in the classification of an unknown strain in one single step. However, there are some pitfalls (Vandamme *et al.*, 1996; Rossello-Mora and Amann, 2001), e.g. some clearly different species may have the same 16S rDNA sequence (Fox *et al.*, 1992) and the reliability of some sequences in the databases can be questioned. Also, it is still not clear to what extent there exists interoperon sequence variation (within the same clone) and/or strain variation within species (Nubel *et al.*, 1996).

Reliable strain typing methods will become increasingly important in the study of the performance of LAB starter cultures and cultures used as additives in functional food type products. Genotypic methods used for strain typing include PFGE of whole digested chromosomal DNA, ribotyping, plasmid profiling and the PCR-based fingerprinting methods such as RAPD, and AFLP.

PFGE of digested chromosomal DNA is often considered the "golden standard" of molecular typing methods because it displays by far the greatest discriminatory power and the highest reproducibility (Tenover *et al.*, 1995). However, this rather laborious method requires a species-specific approach (different restriction enzymes and electrophoresis conditions) and is consequently mainly used to study the intra-species diversity and/or clonal relatedness. Another PCR-independent typing method is **ribotyping**, which combines restriction enzyme analysis of chromosomal DNA with the use of rDNA probes, thereby discriminating between various species (Johansson *et al.*, 1995a; Rodtong and Tannock, 1993; Zhong *et al.*, 1998; Bjorkroth and Korkeala, 1996; Lyhs *et al.*, 1999). The discriminatory power of the method depends on the number and type of oligonucleotide probes and restriction enzymes used. In a comparison of PFGE and ribotyping, it was concluded that PFGE was an efficient method to differentiate genetically closely related *Lb. acidophilus*

strains whereas ribotyping was particularly useful for revealing heterogeneities between strains with lower homology (Roussel *et al.*, 1993). The variation in the number and the sizes of plasmids harboured by strains of the same species, *i.e.* **plasmid profiling**, is useful as a tool for typing in LAB, because most strains of this group seem to contain multiple plasmids (Dykes and von Holy, 1994; Ahrne *et al.*, 1989; Hill and Hill, 1986; Tannock *et al.*, 1990). However, this typing method is affected by the ability of the strains to loose or gain plasmids making surveillance over longer time spans unreliable.

When a high-throughput, high discriminatory power both on the species and intra-species level and low cost is opted for, than PCR-based genomic fingerprinting techniques are believed to have the most potential (Olive and Bean, 1999). **RAPD fingerprinting** is by far the most used PCR-based technique for identification of LAB. RAPD has been used successfully to differentiate LAB at the intra-species level (Johansson *et al.*, 1995b; Berthier and Ehrlich, 1999), at the inter-species level in enterococci (Descheemaeker *et al.*, 1997), pediococci (Nigatu *et al.*, 1998), and lactobacilli (Du Plessis and Dicks, 1995; Gancheva *et al.*, 1999; Daud Khaled *et al.*, 1997; Nigatu *et al.*, 2001), and at the inter-genus level for strains isolated from dairy products (Cocconcelli *et al.*, 1995; Moschetti *et al.*, 2001) and meat products (Yost and Nattress, 2002). However, primers with a high discriminatory power and a broad applicability within a large group of LAB species have not been described. Moreover, because RAPD primers are not directed against a particular genetic locus, the resulting band patterns often exhibit a poor reproducibility (Olive and Bean, 1999; Meunier and Grimont, 1993). Unless standardized (including DNA polymerase and thermal cycler), the RAPD method is not suitable for the construction of identification databases. Another PCR-based technique, **AFLP** has been reported to be a more reproducible tool to discriminate strains at the species and the intra-species level (Janssen *et al.*, 1996), and its use is increasing (Torriani *et al.*, 2001; Gancheva *et al.*, 1999; Bruinsma *et al.*, 2002; Borgen *et al.*, 2002; Vancanneyt *et al.*, 2002).

1.3.3. ANTIBIOTIC RESISTANCE IN LACTIC ACID BACTERIA

Only recently, LAB have gained interest regarding the spread of antibiotic resistance (Teuber *et al.*, 1999). Although most food-associated LAB bear the 'Generally Regarded As Safe' (GRAS) status because of their long history of safe use, they may serve as hosts of acquired antibiotic resistance genes, *i.e.* resistance genes located on conjugative or mobilizable plasmids and transposons, which can be transferred to other (pathogenic) bacteria.

Different types of antibiotic resistance. Intrinsic resistance, in contrast with acquired resistance, poses no hazard in non-pathogenic LAB, because it is not horizontally transferable. However, the available data on intrinsic resistances in LAB are relatively scarce. Enterococci are intrinsically resistant to cephalosporins and low levels of aminoglycoside and clindamycin (Teuber *et al.*, 1999; Knudtson and Hartman, 1993). Lactobacilli, pediococci and *Leuconostoc* spp. have been reported to have a high natural resistance to vancomycin, a property that is useful to separate them from other Gram-positive bacteria (Hamilton-Miller and Shah, 1998; Simpson *et al.*, 1988). Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin trimethoprim/sulphamethoxazole, and vancomycin (Danielsen and Wind, 2002). For a number of lactobacilli a very high frequency of spontaneous mutation to nitrofurazone (10^{-5}), kanamycin and streptomycin was found (Curragh and Collins, 1992). From these data it is clear that inter-genus and inter-species differences exist, and consequently identification at species level is required in order to interpret phenotypic susceptibility data.

Mobile genetic elements. Plasmids are common in LAB, and differences are found in size, function and distribution (Davidson *et al.*, 1996; Wang and Lee, 1997). The functions found on plasmids include hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins and exopolysaccharides, and resistance to antibiotics, heavy metals and phages. At least 25 species of lactobacilli contain native plasmids (Wang and Lee, 1997), and often appear to contain multiple (from 1 to 16) different plasmids in a single strain. R-plasmids encoding tetracycline, erythromycin, chloramphenicol, or macrolide-lincomycin-streptogramin resistance have been reported in *Lb. reuteri* (Vescovo *et al.*, 1982; Axelsson *et al.*, 1988; Lin *et al.*, 1996; Tannock *et al.*, 1994), *Lb. fermentum* (Ishiwa and Iwata, 1980; Fons *et al.*, 1997), *Lb. acidophilus* (Vescovo *et al.*, 1982), and *Lb.*

plantarum (Ahn *et al.*, 1992; Danielsen, 2002) isolated from raw meat, silage and faeces. Most of these R-plasmids had a size smaller than 10 kb (5.7 – 18 kb). The reported prevalence of antibiotic resistance genes such as erythromycin, vancomycin, tetracycline, chloramphenicol, and gentamicin resistance genes, on transferable genetic elements in enterococci is more extensive, both on plasmids (Christie *et al.*, 1987; Rice *et al.*, 1998; West and Warner, 1985; Clewell *et al.*, 1974; Murray *et al.*, 1988) and transposons (Perreten *et al.*, 1997a; Clewell *et al.*, 1995; Rice and Marshall, 1994). A multiple antibiotic resistance plasmid was reported in a *Lactococcus lactis* strain isolated from cheese (Perreten *et al.*, 1997b), encoding streptomycin, tetracycline and chloramphenicol resistance.

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

Antibiotic resistance in LAB food isolates. There have been few systematic studies to investigate acquired antibiotic resistance in LAB from food. Most data exist on opportunistic pathogenic enterococci, while the number of reports on lactococci and lactobacilli is limited. A lot of attention has been paid to glycopeptide resistance in **enterococci**. Vancomycin resistant enterococci (VRE) have emerged in the last decade as a frequent cause of nosocomial infections, mostly in the U.S, and it has been associated primarily with the use of glycopeptides in hospitals (Jett *et al.*, 1994; Moellering, 1998). Of considerable concern is the possibility that VRE, selected and enriched by the use of avoparcin (with cross-resistance to vancomycin) as a growth promoter in animal husbandry, are spread via the food chain (Wegener *et al.*, 1997; Klein *et al.*, 1998; Pavia *et al.*, 2000; Van Den Braak *et al.*, 1998; Giraffa and Sisto, 1997). A comparison of VRE from poultry and VRE from humans by PFGE typing, did not reveal genetic overlap (Van Den Braak *et al.*, 1998). Sequencing of the vancomycin resistance genes, on the other hand, showed full sequence conservation in more than 50% of the strains suggesting that dissemination of the resistance genes carried on transferable elements may be of greater importance than clonal dissemination of resistant strains. Because of this concern, the use of avoparcin as growth promoter in Europe has been banned (in Denmark in 1995, in the rest of Europe in 1997), resulting in a significant decline of VRE between the end of 1995 and the first half of 1998 in broilers (Bager, 2000). Somewhat surprisingly, this ban appears not to have such an effect in pigs (Bager, 2000). Enterococcal food isolates (mainly *E. faecalis* and *E. faecium*) were analysed for resistances to a broader range of different antibiotics using phenotypic susceptibility testing, both in raw meat (Klein *et al.*, 1998; Quednau *et al.*, 1998; Knudtson and Hartman, 1993) and fermented milk and meat products (Teuber and Perreten, 2000; Franz *et al.*, 2001). Their data suggest a high prevalence of (multiple) antibiotic resistant enterococci in foods, which nevertheless were mostly susceptible to the clinically relevant antibiotics ampicillin and vancomycin. An overview of antibiotic resistances reported in the **other food-associated LAB** is given in Table 1.5, which can be summarized by stating that only a limited number of papers reported the prevalence of antibiotic resistance in mainly *Lactobacillus* spp. isolated from raw meat and fermented food products. A few studies have reported an overall susceptibility to antimicrobial agents (with exception of intrinsic resistances) in strains used as meat starter cultures (Raccach *et al.*, 1985; Holley and Blaszyk, 1997) or dairy starter cultures (Katla *et al.*, 2001; Reinbold and Reddy, 1974).

Reviewing the scarce literature on antibiotic resistance in LAB resulted in the following observations. A great diversity in methods for susceptibility testing has been used, including disc diffusion, agar dilution, broth microdilution, and Etest. Whereas genotypic detection and identification of resistance genes provides direct evidence, phenotypic methods are more problematic. The first problem one is confronted with is the choice of medium for susceptibility testing of LAB. The recommended growth media by the National Committee for Clinical Laboratory Standards (Mueller-Hinton agar) (NCCLS, 2002) and by the British Society for Antimicrobial Chemotherapy (Iso-Sensitest agar) (Andrews, 2001) do not support growth of all LAB. MRS medium, that generally supports the growth of LAB much better, is not always compatible to the Iso-Sensitest medium for use in susceptibility testing, as was recently reported for various classes of antibiotics (Huys *et al.*, 2002). Furthermore, there are as yet no guidelines available for the interpretation of susceptibility test results of commensal or food-associated bacteria. Contributions to establish microbiological breakpoints based on MIC determinations (by Etest) have recently been made for a number of lactobacilli (Felten *et al.*, 1999; Zarazaga *et al.*, 1999; Danielsen and Wind, 2002). An important conclusion of these latter publications is that the natural levels of resistance can differ between different species of the same genus. Identification to the species level is important in order to enable a correct interpretation of the susceptibility results.

Table 1.5. Overview of reported antibiotic resistances in food-associated lactic acid bacteria other than enterococci

Foods	Species	Resistance	Remarks	Reference
Raw meat products				
Poultry	*Lb. reuteri* G4	*cat*	MIC of 256 µg/ml; located on plasmid (pTC82); 95% sequence similarity to *cat* from *S. aureus* plasmid pC194	Lin *et al*. (1996)
	Lb. reuteri 100-63	*erm* (T)	MLS resistance; located on plasmid (pGT633); transferable to different gram-positive bacteria	Tannock *et al*. (1994)
Raw ground pork	*Lb. plantarum* caTC2R	Cm^r	Located on non-conjugative plasmid (pCaT); co-mobilizable by pAM 1 to *Carnobacterium piscicola*	Ahn *et al*. (1992)
Raw ground pork and beef	*Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Lb. brevis*, *Leuc. mesenteroides* (total of 67 isolates)	Tetracycline (69%); Chloramphenicol (3%); Methicillin (85%)	Phenotypic susceptibility testing (disc diffusion)	Vidal and Collins-Thompson (1987)
Fermented products				
Raw milk soft cheese	*Lc. lactis lactis* strain K214	*str-tet* (S)-*cat*	Located on plasmid (pK214; 29.8 kb); *tet* (S) has 99.8% sequence similarity to *tet* (S) from *Listeria monocytogenes*	Perreten *et al*. (1997)
Greek cheese	*Lb. acidophilus* ACA-DC 243	Penicillin	Phenotypic susceptibility testing (disc diffusion)	Charteris *et al*. (1998)
Yoghurt starter cultures	*S. thermophilus* and *Lb. delbreuckii* subsp. *bulgaricus*	Novobiocin, cloxacillin, oxacillin, polymyxin B, neomycin, lincomycin, doxycycline	Phenotypic susceptibility testing (disc diffusion)	Sozzi and Smiley (1980)
Nigerian fermented foods and beverages	*Lb. pentosus*, *Lb. acidophilus*, *Lb. casei*, *Lb. cellobiosus*, *Lb. brevis*, *Lb. plantarum*, and *Lb. jensenii* (total of 50 isolates)	Cloxacillin (80%); Penicillin (77.5%); Ampicillin (47.5%); Tetracycline (42.5%); Chloramphenicol (20%); Erythromycin (17.5%)	Phenotypic susceptibility testing (disc diffusion)	Olukoya *et al*. (1993)
Others				
Maize silage	*Lb. plantarum* 5057	*tet* (M)	Located on plasmid (pMD5057; 10.9 kb); MIC > 256 µg/ml	Danielsen (2002)

Lb.: *Lactobacillus*; *Lc*.: *Lactococcus*; *Leuc*.: *Leuconostoc*, *S*.: *Streptococcus*; *cat*: chloramphenicol acetylase gene; *str*: streptomycin adenylase gene; *tet*: tetracycline resistance gene; *erm*: erythromycine resistance gene; MIC: minimal inhibitory concentration; MLS: macrolide-lincosamide-streptogramine

1.4. Fermented dry sausage:

manufacture and microbiology

1.4.1. Introduction

Fermented sausages are cured meat products that are shelf stable (without cooling) and are commonly consumed without application of any heating process. They probably originated in the countries around the Mediterranean Sea (Zeuthen, 1995). The Romans knew that ground meat with added salt, sugar and spices turns into an appetizing product with a long shelf life if prepared and ripened properly. Apparently the normal winter climate in the Mediterranean countries is favourable for sausage ripening. In contrast, salting and drying of unground meat was the traditional way of meat preservation in other European countries. The microbiological stability and some organoleptic properties are owed to a fermentation carried out by LAB, micrococci and moulds. Traditionally, the ground meat was pre-salted in order to promote development of LAB. Alternatively, the '**back slopping**' method was used in which a small amount of meat from successful batches (before fermentation) was mixed with fresh meat. Intensive research into the microbiology and chemistry of sausage ripening was triggered when traditional empirical methods of manufacture no longer met the requirements of large-scale, low-cost industrial production. This type of research commenced in the United States in the 1930s, whereas in Europe the first studies were published in the 1950s. Jensen and Paddock (1940) were the first to describe the addition of LAB (*Lb. plantarum*, *Lb. brevis*, and *Lb. fermentum*) as a **starter culture** in the production of dry sausages. They used both pure and mixed cultures and claimed that the bacteria reduced the ripening time, prevented the development of faulty products and improved the flavour. Furthermore, the acid produced by the fermentation of added sugars contributed to the control of pathogens or spoilage microorganisms and also improved texture. Nowadays, lactobacilli and pediococci are most commonly used as starter cultures for the production of fermented meat products in Europe and USA, respectively (Jessen, 1995).

Production and consumption figures are currently highest in Germany, Italy, Spain and France (Fisher and Palmer, 1995). In fact, these four countries account for approximately 95% of the estimated EU production of fermented sausages. In Germany most of the fermented sausages are smoked, while in Italy, France and Spain air-dried, spicy sausages predominate (Fisher and Palmer, 1995). Other types of fermented sausages emerged as a consequence of advanced meat processing techniques and the availability of refrigeration.

1.4.2. MANUFACTURE OF FERMENTED SAUSAGE

The manufacture of fermented sausage is a complicated and a labour-intensive process (Vösgen, 1994). The slightest deviation in bringing together the meat, spices or other ingredients or in the conditions applied determines a constant variation in the quality of the products. From the microbiological point of view, fermented sausages can be characterized on the basis of water activity, a_w (semi-dry or dry), and surface treatment (mould-ripened or no mould growth) (Lücke, 1998). Additional criteria for classification include the casing diameter, the degree of comminution of the ingredients, the type of the raw meat, the fat content and type of tissue used, as well as spices, seasonings, starter culture and other non-meat ingredients used.

An outline of the manufacture of fermented sausages is shown in Table 1.6. In principle, there is no limit to the use of **raw meats** from different animal species, and sausages made from or added with beef, poultry, turkey, horse, goose and deer meat in addition to pork may be common. However, pork is by far the prime source of raw material for most sausage processors worldwide. The process starts with chilled or partially frozen raw meat that is **comminuted** in a meat grinder or cutter. Fatty tissue, most frequently firm pork back fat, is comminuted in the frozen state and than added to the mixture. The size of the particles in the sausage determines the product type. Curing salt, carbohydrates, starter culture and seasonings are then mixed in. Fermentable carbohydrates are added as substrates for the starter culture. Due to the post-mortem glycogenolysis, glycogen is degraded by meat enzymes to glucose and further to lactic acid, resulting in a lack of carbon source for the starter culture; therefore addition of sugars is required. The mixture is than stuffed into **casings**, which determine the product shape and size. Natural casings as well as casings made from modified collagen and/or cellulose are most frequently used. They must allow evaporation

Table 1. 6. Outline of the manufacture of fermented sausages[a]

Process steps	Additives	Semi-dry sausages	D mc
Meat/fat		Meat temperatu pH 5.5 – 5	
Comminution and mixing	<------ Salt	Target a_w 0.955	
	<------ Curing agents		
	<------ Sugars	0.5 – 0.8%	(
	<------ Starter culture	LAB, Micrococ Staphyloco	
	<------ Seasonings		
Filling	<------ Casings		
Surface inoculu	<------ Starter	Mould cult	
Fermentation		20 – 25 °C, 2 – 3 days, target pH < 5.3	 tar (
Smoking			
Ageing		< 15 °C target a_w 0.93	 tar
Storage		15 °C	

of water from the sausage and penetration of smoke, and they must follow the shrinkage of the sausage during drying. Further, they are stored in the **ripening chamber**, operated under controlled temperature, humidity and air flow conditions. Sausage ripening is subdivided into fermentation (lactic acid formation and concomitant processes) and ageing (drying, aroma formation, etc.), and the two main purposes are lowering the pH and lowering the a_w. For dry sausages with long shelf life and for mould-ripened sausages, fermentation temperatures are usually below 22°C. Semi-dry sausages are usually fermented at 22–26 °C, and for American-style semi-dry sausages, even higher fermentation temperatures (38–40 °C) along with shorter fermentation time are applied. The higher the temperature the

more rapid the fermentation will proceed, but this holds a higher risk of growth of undesired microorganisms if the fermentation is not strictly controlled. While the pH of the sausage decreases near the isoelectric point of meat, the water holding capacity of meat decreases. This favours the drying and consequently the weight losses of sausage, which result in the firm texture and sliceability of the end product. Sausages are usually aged at 12–15 °C for two weeks (Lücke, 1998).

At present, **modified atmosphere packing** (MAP) of ready-to-eat products, such as sliced cold meat products, has become common practice as food manufacturers have attempted to meet consumer demands for fresh, refrigerated foods with extended shelf life (Farber, 1991). In MAP meat products, the aerobic spoilage organisms are significantly suppressed by the presence of CO_2, which results in an autochthonous microflora that is largely dominated by LAB (Borch *et al.*, 1996). For packaging of meat products with low a_w values, such as fermented sausages that are shelf stable but might still be subject to spoilage by mould growth and chemical spoilage (oxidations), mixtures of high percentages of N_2 (80 – 90%) and a low percentage of CO_2 (10 – 20%) are most useful. The combination of an oxygen-free atmosphere and the activity of CO_2 will inhibit possible mould growth (Debevere, 1991). For comparison, meat products with high a_w, such as cooked meat products are mainly subject to spoilage by LAB and *Brochotrix thermosphacta*. These Gram-positive bacteria can only be efficiently inhibited by high concentrations of CO_2. Mixtures of high percentages of CO_2 (50 – 70%) and a lower percentage of N_2 (50 – 30%) are successful (Debevere, 1991).

1.4.3. MICROBIOLOGY OF FERMENTED SAUSAGE

The microflora of refrigerated raw meat largely consists of Gram-negative, oxidase-positive rods, particularly psychrotrophic pseudomonads (Holzapfel, 1998). Psychrotrophic Enterobacteriaceae are also present, while Gram-positive organisms including LAB usually occur only in small numbers. If the meat is processed into raw sausage mixture, the water activity is reduced to 0.96 – 0.97, and the oxygen present within the mixture is rapidly consumed. Thus, the pseudomonads, which require oxygen and are usually sensitive to salt and nitrite, are inhibited. Similarly, the competitiveness of the Enterobacteriaceae is reduced at low oxygen tension, low pH and in the presence of salt. This results in a shift in

microflora composition towards the LAB and catalase-positive cocci (micrococci and staphylococci). Although this natural shift in microflora towards LAB is the basis of the traditional manufacture of fermented sausages and has been applied for centuries, the most dependable fermentations come from the addition of a starter culture. This will make sure that undesired or hazardous acid-sensitive bacteria are suppressed even if few LAB are present in the raw materials. Figure 1.4 shows the behaviour of the most important groups of microorganisms during normal raw-sausage ripening.

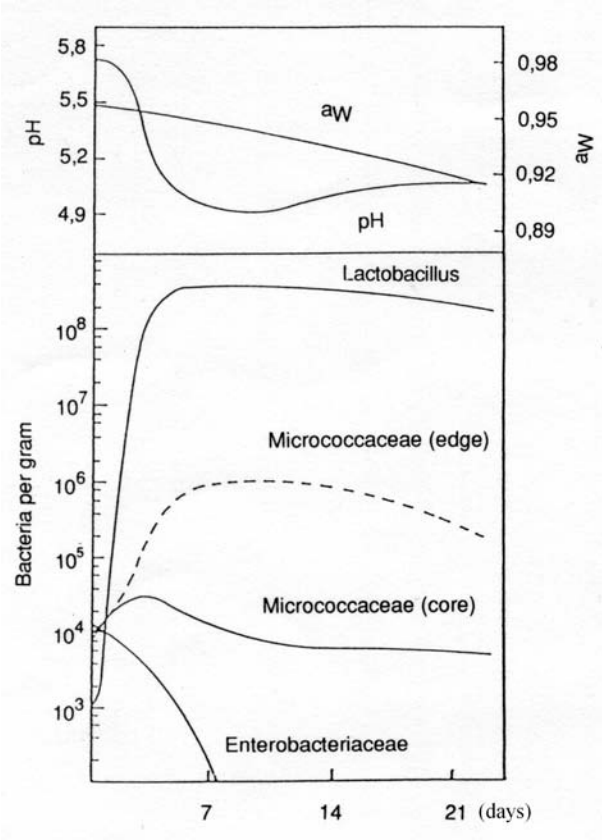

Fig. 1.4. Diagrammatic representation of 'normal' ripening of fermented sausage. Adopted from (Lücke, 1998)

Table 1.7. Starter cultures for sausage fermentation [a]

Microbial group	Species available as starters [b]	Desired metabolic activities	Benefits
Lactic acid bacteria	*Lb. plantarum* *Lb. pentosus* *Lb. sakei* *Lb. curvatus* *P. pentosaceus* *P. acidilactici*	Formation of lactic acid	Inhibition spoilage ba of colour f
Catalase-positive cocci	*St. carnosus* *St. xylosus* *M. varians*	Nitrate reduction	Colour for stabilizatio
		Nitrite reduction	Removal o
		Oxygen consumption, peroxide destruction	Delay of ra stabilizatio
		Formation of carbonyls and esters	Aroma and developme
Moulds	*Pen. nalgiovense* *Pen. chrysogenum*	Surface colonization	Suppressio moulds; fa
		Oxygen consumption	Delay of ra stabilizatio
		Lactate oxidation, degradation of proteins and amino acids	Flavour de
Yeasts	*Deb. hansenii*	Oxygen consumption	Delay of ra stabilizatio
		Not known in detail	Aroma and developme

a/ Adopted from (Lücke, 1998); b/ *Lb*.: *Lactobacillus*; *P*.: Pediococcus, *St*.: St
Micrococcus; *Deb*.: Debaryomyces; *Pen*.: Penicillium

1.4.3.1. Starter cultures for meat fermentation

The role of and benefits from different groups of microorganisms available as starter cultures for sausage fermentation are compiled in Table 1.7. The presence of 10^6–10^7 rather than 10^2 active LAB per gram of fresh sausage mixture leads to a more predictable and more rapid pH decrease and to earlier development of firmness, and may improve product safety. ***Lactobacillus* and *Pediococcus*** are the two genera of LAB used for acidification of meat to prepare fermented sausages. Although a very high number of preparations are commercially available, the number of species is limited. LAB strains currently most employed are *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *P. pentosaceus* and *P. acidilactici* (Jessen, 1995). In addition, **catalase-positive cocci** (*Staphylococcus*, *Micrococcus*/*Kocuria*) are essential for nitrate reduction and aroma formation, and they play a role in protecting the

product from detrimental effects of oxygen. **Yeast and mould** strains are available as starter cultures for air-dried sausages. *Debaryomyces hansenii* appears to be the only yeast species in meat starter cultures because it grows at low a_w and can affect colour and flavour when added to the sausage mixture. Moulds contribute to the characteristic aroma, flavour and appearance of air-dried sausages. However, colonization of the sausage surface with the 'wrong' mould leads to unsatisfactory product quality and increases the risk of mycotoxin formation. The sausage should therefore be dipped into a suspension of conidia of an appropriate starter mould, such as *Penicillium nalgiovense* and *Pen. chrysogenum*, before ripening. Starter cultures are distributed frozen or freeze-dried. Combinations of LAB with catalase-positive cocci proved most useful for fermented sausages and are now most widely used throughout Europe.

1.4.3.2. Hurdles in fermented sausage

Fermented dry sausages are microbiologically stable and safe products. This is achieved by the combination and timing of different factors referred to as the 'hurdle effect' (Leistner, 1995). Figure 1.5 shows that there are several hurdles in a raw sausage, which take effect one after the other in a specific sequence.

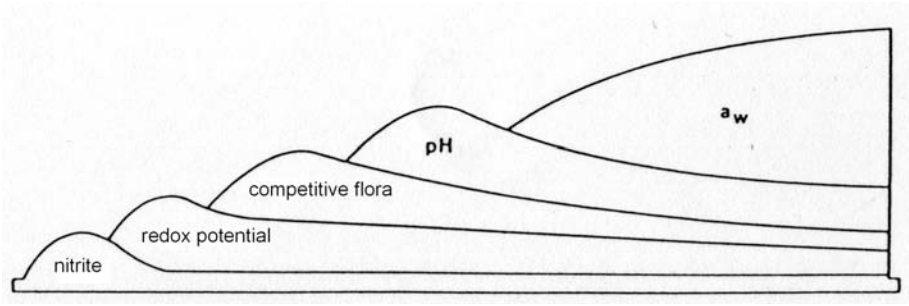

FIG. 1.5. Sequence of hurdles in fermented sausage. Adopted from (Leistner, 1995)

The addition of **nitrite** to the raw sausage mix, together with the nitrite-curing salt, or which is formed from added nitrate by microorganisms, is particularly important at the start of fermentation for the microbiological stability of the product, especially for inhibiting any salmonellae and many other pathogens e.g. *E. coli*, *Listeria monocytogenes*, etc., that may be present in the raw sausage mix. The main functions of nitrite are the development of the

curing colour and the inhibition of auto-oxidative processes leading to rancidity. This is a short-lived hurdle, because nitrite is broken down to oxides of nitrogen during the course of raw-sausage fermentation, and in the end product only a few mg nitrite per kg dry sausage may be traced.

The next important hurdle is the **redox potential** (E_h). During the first day of fermentation the growth of microbes in sausage material uses up all the oxygen mixed in the sausage matrix during the chopping. This reduces the E_h making the nitrite more effective as a bactericidal substance (nitrite is more effective under anaerobic conditions), and restricts the growth of aerobic spoilage bacteria (particularly members of the family Pseudomonadaceae). Under conditions of reduced redox potential, the desirable lactic acid bacteria have a selective advantage over other microorganisms.

After the E_h, the competitive flora becomes the most important hurdle in a raw sausage during fermentation. These are **lactic acid bacteria**, which suppress undesirable microorganisms, such as pathogens (*Listeria*, *Salmonella*, and pathogenic *Staphylococci*) and spoilage bacteria (*Pseudomonas*), by the production of lactic acid, H_2O_2 and possibly also through the formation of bacteriocins, and by competing for available nutrients (De Vuyst and Vandamme, 1994; Holzapfel *et al*., 1995).

The **pH value** is undoubtedly a very important hurdle in the stability of many raw sausages. Particularly with fast-ripened products that still contain a lot of water and, therefore have a relatively high a_w, the pH is an important hurdle. How fast and how far the pH in a raw sausage drops can be affected by the amount of added sugar, the ripening temperature and the type of starter culture.

The **a_w value** of a raw sausage continues to fall as ripening progresses, so that the a_w is the only hurdle in a raw sausage which steadily increases in importance. How quickly and how far the a_w in a raw sausage falls is influenced by the recipe and the ripening temperature, but above all by the relative air humidity in the ripening chamber relative to the ripening time.

It is important to note that 'lowering' one hurdle may be compensated for by 'fortifying' another hurdle. For example, increasing the initial a_w within certain limits may be compensated by a higher rate of acid formation, or a lower fermentation temperature.

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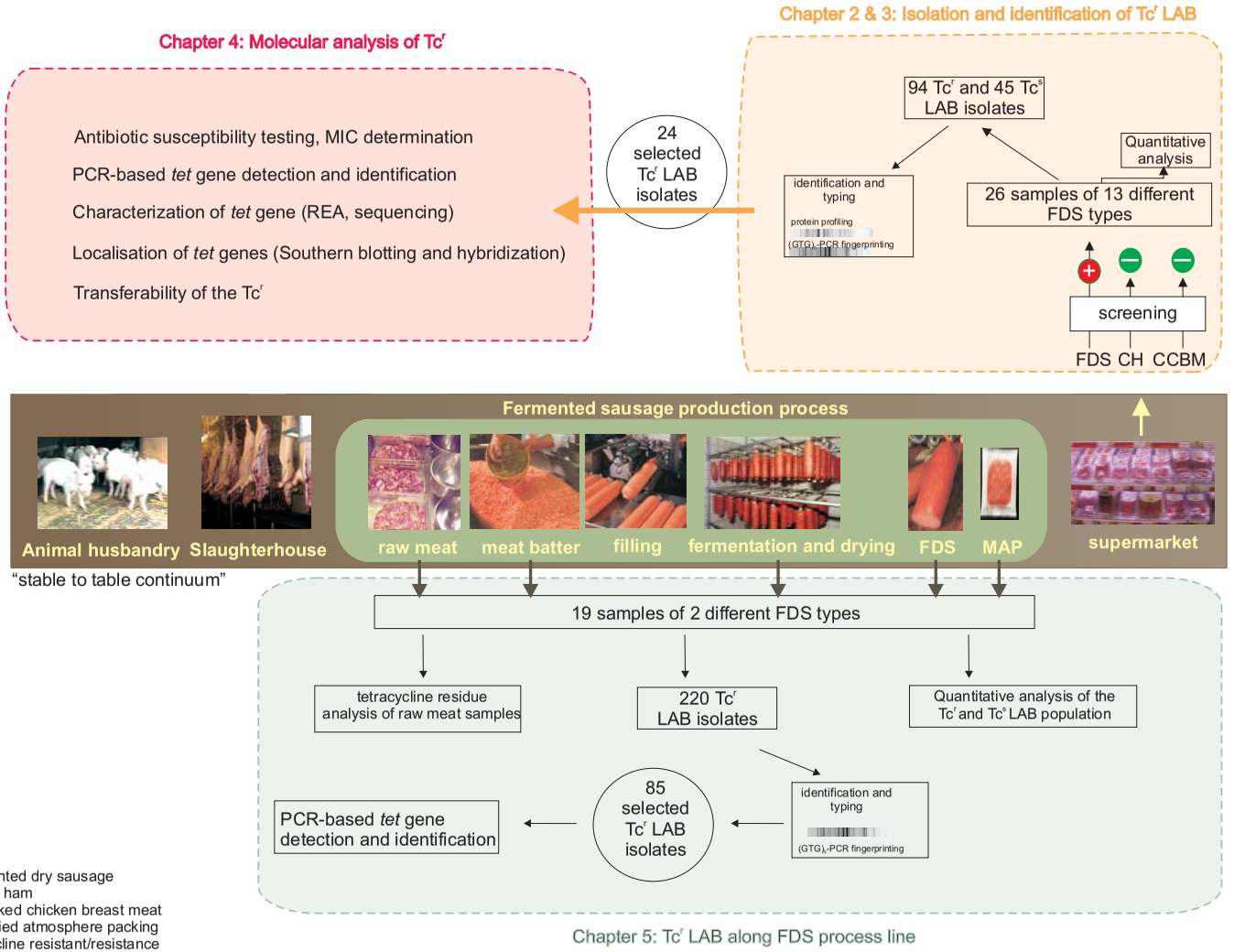
Schematic overview of experimental studies



Legend

FDS: fermented dry sausage
CH: cooked ham
CCBM: cooked chicken breast meat
MAP: modified atmosphere packing
Tcr: tetracycline resistant/resistance
LAB: lactic acid bacteria
REA: restriction enzyme analysis

2

ISOLATION AND IDENTIFICATION OF TETRACYCLINE RESISTANT LACTIC ACID BACTERIA FROM MODIFIED ATMOSPHERE PACKED READY-TO-EAT MEAT PRODUCTS

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SUMMARY

In recent years, the food chain, and meat products in particular, has been recognised as one of the main routes for transmission of antibiotic resistant bacteria between the animal and human population. In this regard, the current study aimed to investigate if tetracycline resistant (Tc^r) lactic acid bacteria (LAB) are present in modified atmosphere packed (MAP) meat products including fermented dry sausage, cooked chicken breast meat and cooked ham. From breakpoint experiments, only fermented dry sausage (FDS) was shown to contain a high-level Tc^r LAB population. Further, 26 samples of 13 different types of FDS end products were analysed for the presence of Tc^r LAB, of which 14 (54%) were positive. From these positive samples, a total of 139 strains were isolated on MRS-S agar without tetracycline (n = 45) and on MRS-S agar supplemented with tetracycline in a breakpoint concentration of 64 μg ml^{-1} (n = 94). By antibiotic susceptibility testing it was shown that isolates from the non-selective plates were Tc^s, and from the selective plates Tc^r. Identification using protein profiling revealed that all Tc^r LAB belonged to the genus *Lactobacillus*, including the species *Lb. plantarum*, *Lb. sakei*, and *Lb. curvatus*, whereas the Tc^s isolates were either *Pediococcus pentosaceus* or *Lb. sakei* subsp. *carnosus*. Protein profiling was found to be limited in resolving power, and in order to study the intraspecies diversity the use of a genotypic fingerprinting method is certainly warranted. Different batches of the same FDS type were found to be variable in the presence of Tc^r LAB and composition of the Tc^r LAB subpopulation. This study has clearly demonstrated that various Tc^r lactobacilli species occur in MAP FDS sold in Belgian retail shops, and yielded an interesting set of isolates for an in-depth molecular analysis of the acquired resistance genes and their transferability.

INTRODUCTION

The emergence and epidemiological spread of antimicrobial resistance genes throughout the human environment represents a major public health problem in developed and developing countries (Levy, 1992). So far, research on the horizontal transfer of drug resistance determinants has mainly focussed on opportunistic and primary pathogenic bacteria and less attention has been drawn to the possible role of human and animal commensal bacteria as reservoir organisms for drug resistance genes (Salyers, 1995). Such reservoir organisms could possibly be found in various foods and food products containing high densities of non-pathogenic bacteria as a result of their natural production process. The food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human population (Witte, 1997). More specifically, raw milk dairy products and fermented meats that are not heat-treated before consumption, provide a vehicle for antibiotic resistant bacteria with a direct link between the animal indigenous microflora and the human gastrointestinal tract.

At present, modified atmosphere packing (MAP) of ready-to-eat meat products has become common practice as food manufacturers have attempted to meet consumer demands for fresh, refrigerated foods with extended shelf life (Farber, 1991). In MAP meat products, the aerobic spoilage organisms are significantly suppressed by the presence of CO_2 which results in an autochthonous microflora that is largely dominated by lactic acid bacteria (LAB) (Borch *et al.*, 1996). Although most food-associated LAB have acquired the 'Generally Regarded As Safe' (GRAS) status, the potential health risk due to the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract and hence to pathogenic bacteria has not been fully addressed.

During the past decade, the incidence of antibiotic resistant LAB in food and food products has been reported. As such, Perreten and co-workers isolated a multi-resistant *Lactococcus lactis* subsp. *lactis* strain, carrying a conjugative plasmid, from a raw milk soft cheese (Perreten *et al.*, 1997). Antibiotic-resistant LAB isolates have also been recovered from raw meat, including mainly enterococci (Knudtson and Hartman, 1993; Wegener *et al.*, 1997; Klein *et al.*, 1998; Quednau *et al.*, 1998) and lactobacilli (Vidal and Collins-Thompson, 1987; Tannock *et al.*, 1994; Lin *et al.*, 1996). The horizontal transfer of antibiotic resistance genes located on conjugative transposons and plasmids by LAB has been

reported in literature and is reviewed by Teuber et al. (1999). The incidence of resistance to the broad-spectrum antibiotic tetracycline is high in the above-mentioned literature. In fact, the emergence of tetracycline resistant strains has nowadays limited its widely use of the past decades (Chopra and Roberts, 2001). Because of this widespread prevalence and because the molecular basis of tetracycline resistance is relatively well studied and documented (Chopra and Roberts, 2001), this agent was chosen as a model for the purpose of this study.

The current study was undertaken to document the incidence of tetracycline resistant LAB in ready-to-eat MAP meat products including fermented dry sausage, cooked ham and cooked chicken breast meat.

MATERIALS AND METHODS

Determination of the tetracycline breakpoint concentration: The tetracycline breakpoint concentration is defined here as the minimal concentration of tetracycline that has to be supplemented to de Man, Rogosa and Sharpe-Sorbic acid agar plates (MRS-S agar, 0882210, BD, Franklin Lakes, US) for the preparation of an isolation medium selective for high-level Tc^r LAB, and was determined as follows. Modified atmosphere packed sliced meat products including fermented dry sausage (7 batches of 2 types), cooked ham (4 batches of 2 types), and cooked chicken breast meat (3 batches of 2 types) investigated in this study, were purchased from local supermarkets and stored at 4 °C until further research. At the end of its indicated shelf life, a 25 g sample was taken from each meat product, added to 225 ml sterile peptone physiological saline solution (PPS) (8.5 g/l NaCl and 1 g/l neutralised bacteriological peptone [LP0034, Oxoid, Basingstoke, UK]) and homogenised in a Stomacher® (Seward, London, UK). Serial decimal dilutions (10^{-1}–10^{-8}) in PPS were prepared and 1 ml samples of appropriate dilutions were poured in a series of MRS-S agar plates supplemented with tetracycline (T-3383, Sigma, Bornem, Belgium) in doubling concentrations ranging between 0 and 256 µg/ml. After incubation for five days at 30 °C under microaerophilic conditions (3.75% CO_2, 5% O_2, 7.5% H_2, 83.75% N_2), counts were performed manually. From these results, the breakpoint concentration was deduced.

Susceptibility testing: A modified version of the Kirby-Bauer disc diffusion method (Kirby et al., 1966), in which Meuller-Hinton medium was replaced by MRS-S agar, was used for determination of antibiograms. Oxoid susceptibility test discs of tetracycline (30 µg) were applied on inoculated MRS-S agar plates using the Oxoid Disc Dispenser. Diameters of the respective inhibition zones were measured using a digital calliper (Mauser digital 2, Ludwigsburg, Germany) following a 16-18 h incubation of the antibiograms at 30°C and recorded. Classification of the isolates into sensitive and resistant groups was based on resistance histograms (*i.e.* number of strains *versus* size of the inhibition zone). Cut-off values to differentiate among resistant and susceptible groups were defined on the basis of the bimodal distribution of the population in the resistance histograms.

Screening of fermented dry sausage end products. In this study, a total of 26 samples of 13 different types of modified atmosphere packed FDS end products were purchased from local supermarkets in Belgium and analysed for the presence of a Tc^r LAB subpopulation. At the end of its indicated shelf life, appropriate dilutions of meat homogenates were prepared as described above. These dilutions were poured in MRS-S agar plates supplemented with or without a breakpoint concentration (64 μg/ml) of tetracycline. Plates were incubated as described above.

Selection and storage of strains: Colonies were randomly selected from non-selective MRS-S plates (without antibiotics) and from selective MRS-S plates (supplemented with 64 μg ml^{-1} tetracycline) and further purified under aerobic conditions on non-selective MRS-S plates. Isolates were stored in a bead storage system (Microbank system, Pro-Lab Diagnostics, Wirral, UK) at –80°C.

Protein profiling: Sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell bacterial proteins was done as described previously (Pot *et al.*, 1994). Identification of the isolates was performed by comparison of their protein patterns with a laboratory database containing over 6000 reference strains encompassing all known LAB species. Pattern storage and database comparisons were performed using the software package BioNumerics v2.5 (Applied Maths, Sint-Martens Latem, Belgium).

RESULTS AND DISCUSSION

Three kinds of MAP sliced meat products, *i.e.* fermented dry sausage, cooked chicken breast meat and cooked ham, were tested for the presence of high-level Tc^r LAB using breakpoint experiments. As shown in Fig. 2.1, fermented dry sausage clearly contains a high-level Tc^r LAB population. Concentrations of tetracycline up to 32 µg/ml have a moderate influence on the number of CFU that grow under standard conditions. A concentration of tetracycline of 64 µg/ml diminishes the number of CFU with 5 log units. For tetracycline concentrations as high as 256 µg/ml (the upper limit of tested range), a significant number (2 to 3 log CFU/g) of LAB was observed after 5 days of incubation. These results indicate a tetracycline breakpoint concentration for the LAB population in fermented dry sausage between 32 and 64 µg/ml. This finding was confirmed when screening other fermented dry sausages. Recently, tetracycline, erythromycin, lincomycin and penicillin resistant *Enterococcus faecalis* and *E. casseliflavus* strains were found in FDS end products (Teuber *et al.*, 1999). Moreover, the authors speculated that fermented foods made from raw milk and meat may contain antibiotic resistant LAB from the originating animals including enterococci, lactobacilli and lactococci.

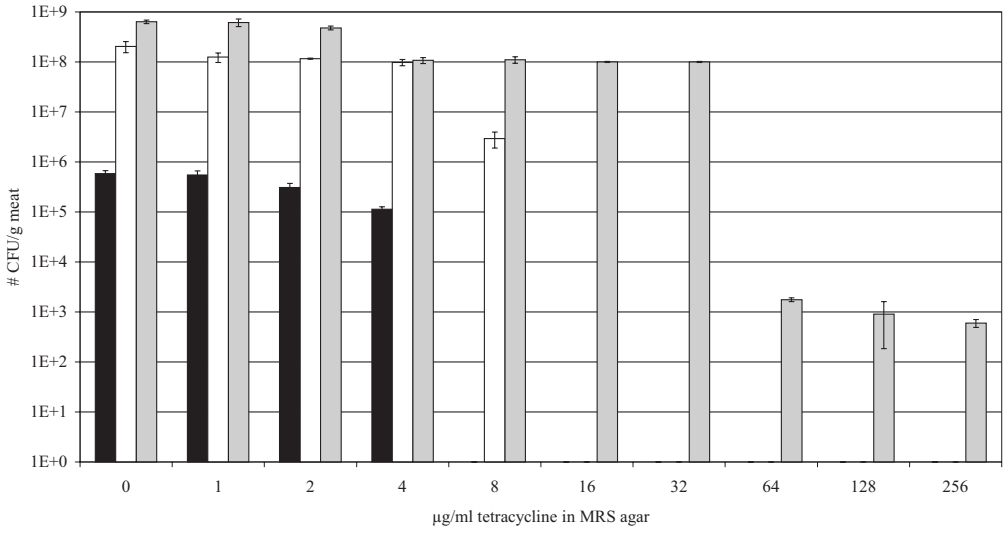

Fig. 2.1. Determination of the tetracycline breakpoint concentration for the lactic acid bacteria populations in cooked chicken breast meat (black), cooked ham (white) and fermented dry sausage (grey). Standard deviations are based on counts in triplicate.

Cooked ham and cooked chicken breast meat samples, on the other hand, did not contain a high-level Tc^r LAB population at the end of the shelf life (Fig. 2.1). In fact, a concentration of 8 and 16 µg/ml tetracycline, respectively, was sufficient to inhibit growth of LAB on MRS-S agar. A possible explanation for the lack of high-level resistant LAB in cooked ham and cooked chicken breast meat might be related to the heat treatment during the production process that eliminates most of the viable bacteria naturally present on the raw meat. In fact, the main cause of spoilage of these products lies with the environmental microflora recontaminating the products after cooking, during slicing and packaging (Bjorkroth and Korkeala, 1997; Samelis *et al.*, 1998).

In order to verify the breakpoint concentration as determined by the two-fold dilution technique, colonies were isolated from different MRS-S plates (poured with meat homogenates of FDS-01 and FDS-08) supplemented with 0, 32 and 64 µg/ml of tetracycline, and subjected to susceptibility testing using the disc diffusion method (Fig. 2.2). Strains isolated from non-selective MRS-S plates (*i.e.* 0 µg/ml tetracycline) were all tetracycline sensitive (Tc^s), whereas the isolates recovered from plates supplemented with 32 µg/ml of tetracycline were divided in Tc^r and Tc^s strains. A concentration of 64 µg/ml of tetracycline was chosen as the breakpoint concentration to prepare the selective isolation medium, because 100% of the strains isolated from plates with this concentration of tetracycline are Tc^r.

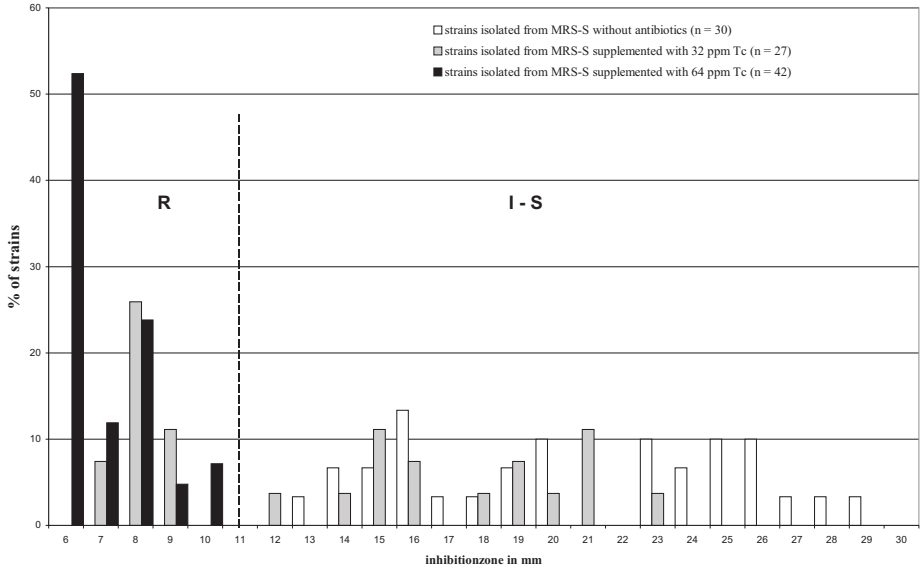

Fig 2.2. Resistance histogram for tetracycline (Tc). The histogram displays a bimodal distribution of the population enabling the differentiation among a resistant (R) and an intermediate (I) to susceptible (S) group.

These experiments has provided evidence for the possible presence of Tc^r LAB in MAP ready-to-eat FDS and the absence in cooked chicken breast meat and cooked ham sold in Belgian retail shops. Based on these findings, further research focus on Tc^r LAB in FDS.

Using a tetracycline breakpoint concentration set at 64 µg/ml, a total of 26 samples of 13 different types of fermented dry sausage were analysed for the presence of Tc^r LAB in two separate periods. In a first isolation round, 10 types of FDS were tested. The total number of LAB in the examined sausages typically ranged between 6 and 9 log CFU/g. Four out of these 10 samples contained Tc^r LAB in different concentrations ranging between 1.70 and 4.35 log CFU/g (Table 2.1). A total of 97 colonies were randomly isolated from both non-selective (n = 45) and selective plates (n = 52) and stored in a bead storage system. All strains from the non-selective plates were found to be susceptible to tetracycline; whereas all strains from the selective plates were Tc^r. These results clearly confirm the selectivity of the primary isolation medium supplemented with 64 µg/ml tetracycline for high-level Tc^r LAB. These isolates were subjected to protein profiling and could be unequivocally allocated to a specific (sub)species by comparison of their unknown profiles with the protein profiles of reference strains in the laboratory database (Table 2.2; Fig. 2.3). Among the Tc^r isolates only *Lactobacillus* species were found, including *Lb. sakei* subsp. *carnosus* (FDS-07A, -08A, -11A), *Lb. plantarum* (FDS-01A) and *Lb. curvatus* (FDS-07A), whereas the Tc^s isolates were identified as *Pediococcus pentosaceus* (FDS-01A, -07A, -08A) or *Lb. sakei* subsp. *carnosus* (FDS-08A, -11A). These four species are known to be very well adapted to the specific conditions of fermented sausage (low pH and a_w) and are therefore readily used as meat starter cultures (Jessen, 1995). When comparing per FDS the protein profiles of the Tc^r and Tc^s isolates, either a difference at the genus level (as is the case for FDS-01A and FDS-07A) or at the intra-species level (FDS-08A and FDS-11A) was found (Fig. 2.4). It is reasonable to assume that the dominating microflora that is recovered from FDS on non-selective plates are strains that were added in high densities to the FDS as starter cultures; consequently, the differences in protein profiles between the strains from the non-selective plates and the selective plates suggest that the Tc^r LAB isolates are no members of the starter culture or are starter cultures that acquired Tc^r determinants during fermentation. However, this observation needs further research in order to obtain indisputable proof.

Table 2.1. Quantitative analysis of the LAB population and the Tc^r LAB subpopulation in different samples of FDS end products

		First isolation round (batch A)						Second isolation round (batch B)				
FDS No	Brand	Total # of LAB (log CFU/g)		# of Tc^r LAB (log CFU/g)		# of Tc^s isolates	# of Tc^r isolates	Total # of LAB (log CFU/g)		# of Tc^r LAB (log CFU/g)		# Tc^r isolates
1	I	8.58	7.93	2.05	1.67	4	16	7.85	6.93	0		
2	II	ND		0				7.66	6.79	2.47	1.40	4
3	II	ND		0				6.49	5.22	0		
4	II	ND		0				7.37	5.86	0		
5	II	7.49	6.27	0				ND		0		
6	II	Not included in first round						8.12	6.91	2.04	1	2
7	III	8.31	7.30	1.70	1.30	17	3	8.16	7.13	4.61	3.61	4
8*	IV	8.37	7.02	4.12	2.85	12	9	8.16	7.15	0		
								8.17	7.11 (C)	5.05	4.02	5
								8.13	7.16 (D)	2.94	1.93	2
								8.15	7.10 (E)	3.13	2.29	5
9	IV	ND		0				8.58	7.32	3.12	2.18	5
11	V	8.51	7.18	4.35	2.83	12	24	8.93	8.15	3.24	1.96	5
12	VI	6.94	5.86	0				7.36	6.66	2.87	1.93	5
13	IV	Not included in first round						8.75	7.88	0		
14	VII	Not included in first round						8.39	6.51	3.03	1.62	5

ND: not determined; standard deviations are based on counts in triplicate; the detection limit of the used isolation procedure is 50 CFU/g; * For FDS-08 an extra of three batches were included in this study, these batches are indicated with the letters (C), (D), and (E).

Cluster analysis combined with visual inspection of digitised protein fingerprints clearly shows that several isolates originating from the same sausage and belonging to the same species can display highly similar if not identical patterns (Fig. 2.3). In order to verify the intraspecies diversity of these isolates, the use of a genotypic fingerprinting technique with a higher discriminatory power is certainly warranted.

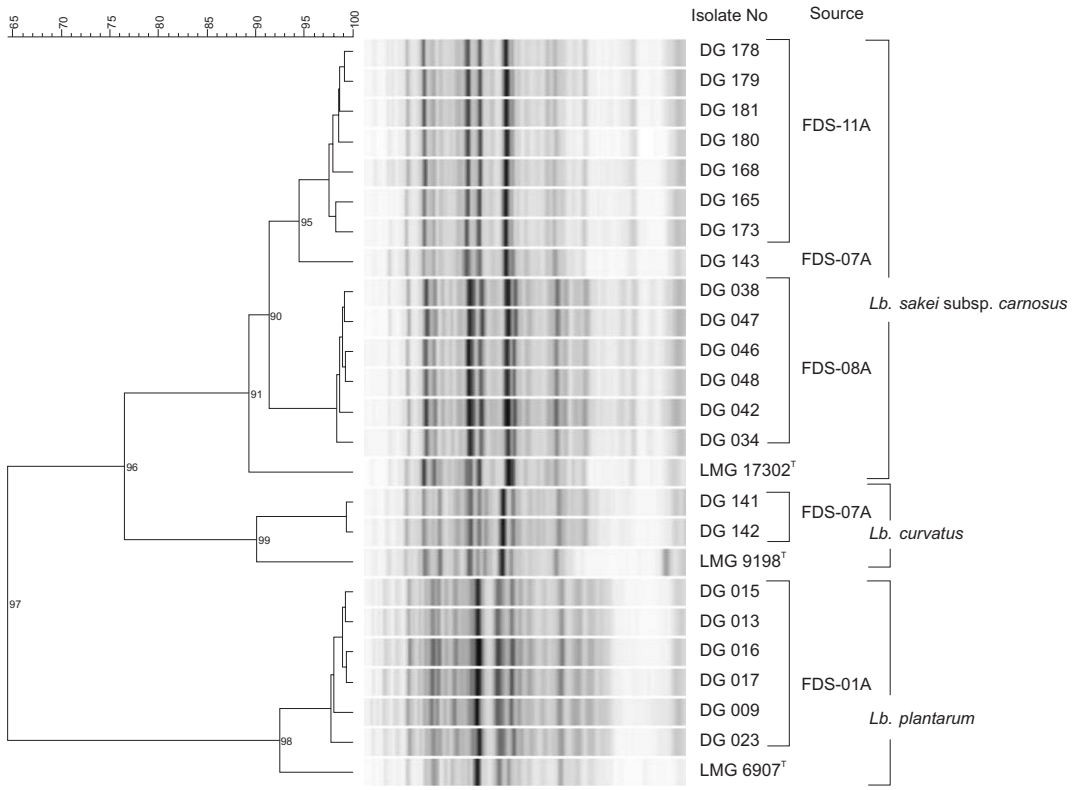

Fig. 2.3. Dendrogram based on the cluster analysis of the digitized protein profiles of a subset of the Tc^r *Lactobacillus* strains isolated from four different types of fermented dry sausage (FDS-01A, -07A, -08A, and -11A). The dendrogram was constructed using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with correlation levels expressed as % values of the pearson correlation coefficient. Cophenetic correlations (shown on the branch of the dendrogram) indicate how faithfully the dendrogram represents the similarity matrix.

Table 2.2. Comparison between the Tc^r and Tc^s population from four different sa

	FDS No		
	1	7	8
No of strains isolated and identified	20	20	21
Tc^r/Tc^s			
P. pentosaceus	-/4	-/17	-/3
Lb. plantarum	16/-	-/-	-/-
Lb. sakei subsp. *carnosus*	-/-	1/-	9/9
Lb. curvatus	-/-	2/-	-/-

Distinction between tetracycline susceptible (Tc^s) and resistant (Tc^r) is based on susceptibility testing using the disc diffusion method. Identification was based o protein profiling.

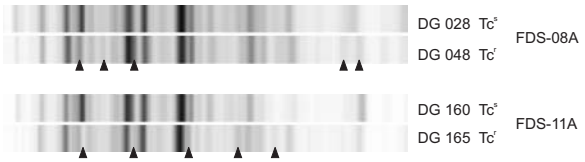

Fig. 2.4. Comparison between the protein profiles of representatives of Tc^r and Tc^s isolates from FDS-08A and FDS-11A. Differences in profiles are marked with a triangle.

In a second isolation round, new batches of the same 10 types as in round one and three additional types (FDS-06, -13, -14) were examined for the presence of Tc^r LAB (Table 2.1). For one particular FDS (FDS-08), a more extensive analysis was performed and four new batches (B to E) were examined in this round. This resulted in a total of 16 batches that were investigated in this second round, of which ten samples contained a high-level Tc^r LAB subpopulation in different concentrations ranging between 2.04 and 5.05 log CFU/g. One type that originally exhibited high-level Tc^r LAB (FDS-01) did not contain any Tc^r LAB following a second analysis. Three types previously lacking Tc^r LAB were now clearly positive (FDS-02, -09, -12). Of the four new batches of FDS-08, three were positive (FDS-08 batches C, D and E). These data indicate that the presence of Tc^r LAB in different batches of a given fermented dry sausage is subject to variation. To explain this instability observed with the presence of Tc^r LAB an analysis encompassing the complete process line of a

fermented dry sausage will be necessary. This is to determine the origin of Tc^r LAB in the fermented meat product, which could be the cause of the instability itself, and to study the ecology of the microorganisms (*i.e.* natural or contaminating flora and the added starter culture) in relation to their environment (*i.e.* from the raw meat until the fermented product). The collection of Tc^r LAB isolates of the first round was extended with 42 new Tc^r LAB isolates recovered similarly from the ten positive batches out of this second isolation round.

In conclusion, this study has provided evidence for the presence of a subpopulation of Tc^r LAB in 54% of the samples of MAP ready-to-eat FDS and the absence in cooked chicken breast meat and cooked ham. Isolation and identification of Tc^r LAB revealed that only Tc^r lactobacilli could be recovered and that the Tc^r microflora is clearly different from the Tc^s microflora. Identification using protein profiling was found to be rather laborious and had an inadequate discriminatory power, therefore the use of a genotypic fingerprinting technique to verify the intraspecies diversity of the isolates is certainly warranted. Variability in the presence of Tc^r LAB was found when comparing different batches of the same FDS type, suggesting a variable source. Further research will focus on the confirmation of the phenotypically determined resistance to tetracycline by molecular analysis of the tetracycline resistance genes and on the study of the transferability of these resistances to other bacterial hosts.

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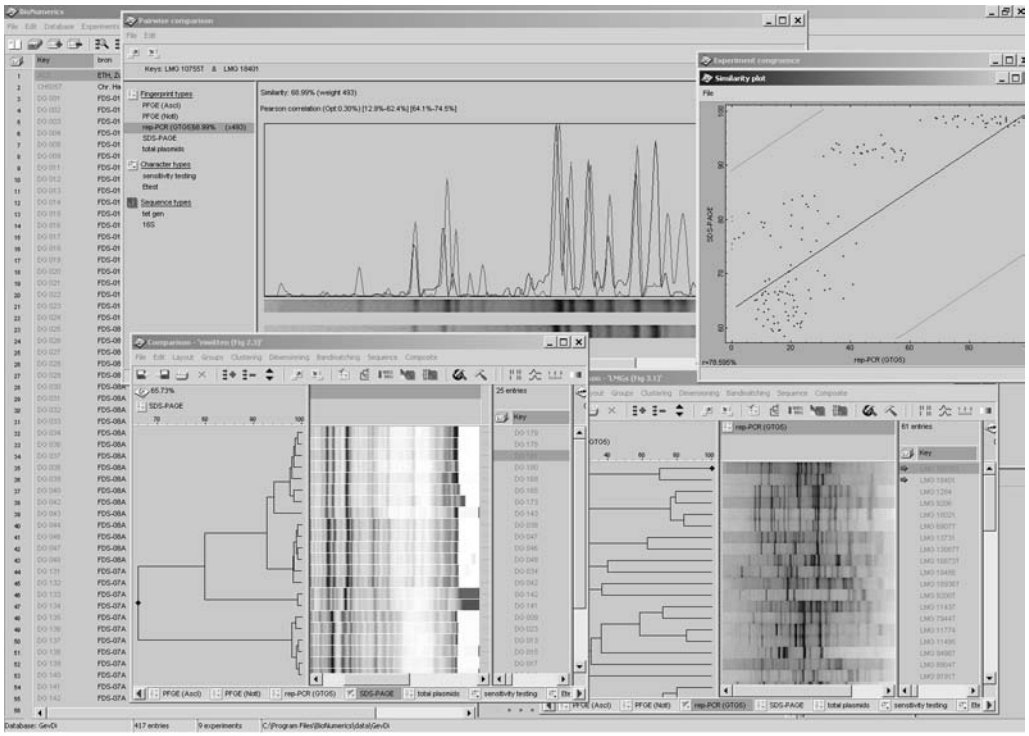
3

IDENTIFICATION AND TYPING OF *LACTOBACILLUS* SPECIES USING $(GTG)_5$-PCR FINGERPRINTING



3.1.

Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species

Published as:

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SUMMARY

The rep-PCR fingerprinting technique using the $(GTG)_5$ primer ($(GTG)_5$-PCR fingerprinting) was proven to be useful for differentiation of a wide range of lactobacilli (*i.e.* 26 different (sub)species) at the species, subspecies and potentially up to the strain level. Using this rapid and reproducible genotypic technique new *Lactobacillus* isolates recovered from different types of fermented dry sausage could be reliable identified at the (sub)species level. In conclusion, $(GTG)_5$-PCR was found to be a promising genotypic tool for rapid and reliable speciation and typing of lactobacilli and other lactic acid bacteria important in food fermentations.

INTRODUCTION

Lactic acid bacteria (LAB) are of great economical importance to the dairy industry and for the production of other fermented foods and food supplements. Next to their functional characteristics, reliable identification and high-resolution typing of LAB strains is essential in nutritional sciences and fundamental food research. Traditionally, LAB have been classified on the basis of phenotypic properties including physiological parameters and sugar fermentation patterns (Pot *et al.*, 1994a; Vandamme *et al.*, 1996). However, as witnessed within the *Lactobacillus acidophilus* complex and the *Lactobacillus casei* complex, a correct classification and identification of LAB is difficult without the support of genotypic techniques (Gancheva *et al.*, 1999; Tynkkynen *et al.*, 1999; Kandler and Weiss, 1986). The currently used methods for the study of LAB such as protein profiling (Pot *et al.*, 1994b), 16S rRNA sequencing (Collins *et al.*, 1991), ribotyping (Zhong *et al.*, 1998), and pulsed-field gel electrophoresis (Tenover *et al.*, 1995) are either too laborious, are limited in their resolving power or require a species-specific methodology. Therefore, a method that is universally suitable for the LAB with a high-resolving power both on species and intraspecies level would be a highly valuable tool. In this regard, PCR-based genomic fingerprinting techniques are believed to have the most potential, and are easy-to-perform (Olive and Bean, 1999).

So far, randomly amplified polymorphic DNA (RAPD) fingerprinting is by far the most used PCR-based genomic technique for identification of LAB (Daud Khaled *et al.*, 1997; Bjorkroth *et al.*, 1996; Johansson *et al.*, 1995; Cocconcelli *et al.*, 1997; Du Plessis and Dicks, 1995). However, primers with a high discriminatory power and a broad applicability within a large group of LAB species have not been described. Moreover, because RAPD primers are not directed against a particular genetic locus, the resulting band patterns often exhibit a poor reproducibility (Olive and Bean, 1999; Meunier and Grimont, 1993). Alternatively, PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the following characteristics: (i) a high discriminatory power, (ii) low cost, (iii) suitable for a high-throughput of strains, and (iv) considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Versalovic *et al.*, 1994; Olive and Bean, 1999). To our knowledge, the use of the rep-PCR fingerprinting technique on lactic acid bacteria has been

described in three papers. De Urraza and co-workers (De Urraza *et al.*, 2000) demonstrated the usefulness of the BOXA1R primer for typing thermophilic LAB associated with dairy products. Hyytiä-Trees and co-workers (Hyytiä-Trees *et al.*, 1999) suggest that an adequate level of discrimination among *Lactobacillus sakei* strains can be achieved by using a combination of rep-PCR using BOX and REP primers and RAPD. Sohier and co-workers (Sohier *et al.*, 1999) reported on the applicability of rep-PCR to differentiate the species *Lb. hilgardii* and *Lb. brevis*.

The aim of the current study was to assess the applicability of rep-PCR fingerprinting for the genotypic differentiation of a broad range of *Lactobacillus* species. For this purpose, a set of oligonucleotide primers targeting various repetitive DNA elements was evaluated. The method was tested on both reference strains and newly isolated lactobacilli from different types of fermented dry sausage.

MATERIALS AND METHODS

Strains and growth conditions. The taxonomical framework of reference strains consisted of facultatively heterofermentative lactobacilli (39 strains representing 20 species), obligately homo- and heterofermentative lactobacilli (15 strains representing 6 species), other LAB (7 strains including *Enterococcus faecalis*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Weissella viridescens*, *Weissella halotolerans*). All reference strains were obtained from the BCCM™/LMG bacteria collection (http://www.belspo.be/bccm/lmg) (Fig. 3.1). Two sets of *Lactobacillus* isolates, recovered from different types of fermented dry sausages, were investigated in this study. For the first set of isolates (n = 52), the isolation and the identification at the (sub)species level by means of protein profiling were described in a previous paper (Gevers *et al.*, 2000; Chapter 2). The isolates of the second set (n = 42) were obtained in a similar way, but were not identified prior to this study. All LAB strains were grown overnight at 30°C on MRS agar (0882210, BD, Franklin Lakes, US), except for the *Lactococcus lactis* strains, which were grown overnight at 30°C on M17 agar (CM785, Oxoid, Basingstoke, UK).

Protein profiling. Both reference strains and the first set of isolates were identified using sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell bacterial proteins. Preparation of protein extracts, SDS-PAGE of whole-cell bacterial proteins and computer processing were done as described previously (Pot *et al.*, 1994*b*).

Total DNA preparation. Total DNA was extracted from 10 ml of cultures harvested in the exponential phase (A_{600} of 0.5 – 1). Cells were collected by centrifugation (3000 *g*, 4 °C, 10 min) and frozen for at least 1 hour at –20 °C. The thawed pellet was washed in 1 ml TES buffer (6.7% sucrose, 50 mM Tris/HCl pH 8.0, 1 mM EDTA) and resuspended in 300 µl STET buffer (8% sucrose, 5% Triton-X-100, 50 mM Tris/HCl pH 8.0, 50 mM EDTA). Seventy-five microliters of Lysis buffer (TES containing 1330 U ml^{-1} mutanolysine and 40 mg ml^{-1} lysozyme) was added and the suspension incubated at 37 °C for 1 hour. After addition of 40 µl preheated (37 °C) 20% SDS in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and glass beads, cells were vortexed for 60 s and incubated at 37 °C for 10 min followed by 10 min incubation at 65 °C. One hundred microliters of TE-buffer were added

and the lysate was extracted with 1 volume phenol/chloroform/isoamylalcohol (49:49:1). Phases were separated by centrifugation (18000 *g,* 5 min) using Phase Lock Gel™ tubes (Eppendorf, Hamburg, Germany). The aqueous phase was carefully mixed with 70 µl 5 M NaCl and 1 ml isopropanol and DNA precipitated on ice for at least 15 min. DNA was collected by centrifugation (20000 *g*, 4 °C, 30 min) and the pellet washed in ice-cold 70% ethanol. DNA was dried by vacuum centrifugation and resuspended in 100 µl TE. One microliter RNAse (10 mg ml^{-1}) was added and the solution was incubated at 37 °C for 10 min and stored at 4 °C.

rep-PCR genomic fingerprinting. The rep-PCR oligonucleotide primers evaluated in this study were REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I (5'-IIICGNCGNCATCNGGC-3'), BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'), and $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3') each with its own optimal PCR program (Versalovic *et al.*, 1994). PCR amplifications were performed with a DNA thermal cycler Perkin Elmer 9600 as described before (Versalovic *et al.*, 1994), using Goldstar DNA polymerase (Eurogentec, Seraing, Belgium).

The PCR products were electrophorized in a 1.5% agarose gel (15 cm by 20 cm) for 16 hours at a constant voltage of 2 V cm^{-1} in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 4 °C. The rep-PCR profiles were visualised after staining with ethidium bromide under ultraviolet light, followed by digital image capturing using a CCD camera. The resulting fingerprints were analysed by the BioNumerics V2.5 software package (Applied Maths, Sint-Martens Latem, Belgium). A dendrogram was constructed from the digitised profiles using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with correlation levels expressed as % values of the Pearson correlation coefficient.

RESULTS AND DISCUSSION

EVALUATION OF DIFFERENT PRIMER SETS

For the evaluation of the rep-PCR fingerprinting technique, two single oligonucleotide primers (BOXA1R and $(GTG)_5$) and one primer pair (REP1R-I and REP2-I) were initially tested for their ability to type a subset of 30 LAB isolates that had already been identified using protein profiling (results not shown). In comparison with the BOXA1R primer and the REP1R-I and REP2-I primer set, the $(GTG)_5$ primer clearly generated banding patterns with the highest complexity. The use of BOX and REP primers resulted in a banding pattern containing approximately 0 to 6, and 1 to 10 visualised PCR products, respectively, while the $(GTG)_5$ primer generated fingerprints containing between 10 and 20 visualised PCR products with an average of 16.5 bands. The size of the DNA fragments obtained after amplification using the $(GTG)_5$ primer ranged between 300 and 4000bp. We found that the discriminatory power did not seem to be significantly enhanced when combining BOX, REP and $(GTG)_5$ banding patterns compared to the increase in amount of work. Because it was intended to optimise an easy-to-perform, rapid and reproducible method for cost-efficient speciation and typing of unknown LAB isolates, the use of the $(GTG)_5$ primer was preferred above a PCR assay combining multiple primers. To date, very few studies are available on the use of the $(GTG)_5$ primer for rep-PCR fingerprinting (Versalovic *et al.*, 1994). In this regard, Nick and co-workers (Nick *et al.*, 1999) recently demonstrated the usefulness of rep-PCR fingerprinting with the $(GTG)_5$ primer in combination with REP, ERIC and BOX primers for typing of rhizobial strains. A combined REP+ERIC+BOX+$(GTG)_5$ dendrogram was generated because a maximized specificity of the patterns was preferred.

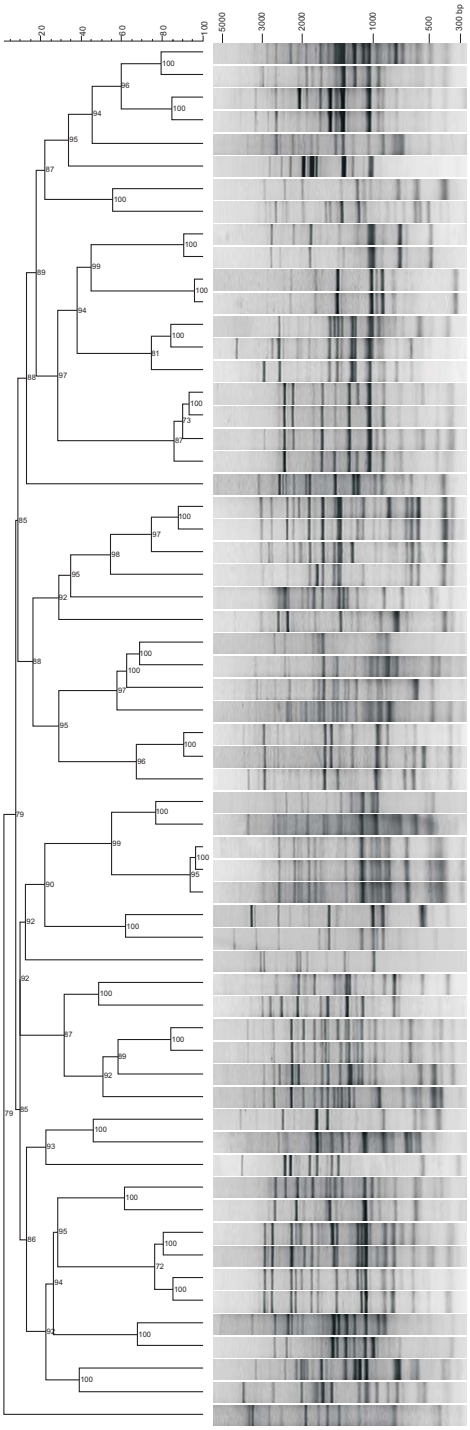
REPRODUCIBILITY OF REP-PCR FINGERPRINTING

A selection of 16 isolates and 5 reference strains was used to assess the reproducibility of the banding pattern with rep-PCR fingerprinting using the $(GTG)_5$ primer ($(GTG)_5$-PCR). PCR amplification and electrophoresis were performed in three separate trials starting from the same DNA preparation and using the same PCR reagents. None of the strains tested

showed qualitative differences in banding patterns, *i.e.* presence *versus* absence of a band. On the other hand, minor quantitative variations in band intensity were occasionally found, but with no pronounced effect on the stability of cluster analysis. The similarity index between three separately obtained banding patterns of the same strain ranged between 91 and 97%. In a second reproducibility test, we investigated the influence of the DNA preparation, and again, no qualitative differences in banding patterns could be detected. Moreover, each PCR reaction was controlled for reproducibility by inclusion of the reference strains LMG 6907^{T}and LMG 17302^T. In a series of ten PCR reactions, an average of 93% similarity between the corresponding banding patterns of each reference strain was found, and no qualitative variations were noticed. In order to maintain a high reproducibility in $(GTG)_5$-PCR fingerprinting we strongly recommend the use of filter tips, small aliquots of PCR reagents, and the same thermal cycler for all PCR reactions. From our experience, and in contrast to the original protocol of Versalovic and co-workers (Versalovic *et al.*, 1994), working on ice to prepare the PCR reaction mixture was preferred above room temperature performance.

IDENTIFICATION AND TYPING OF LACTOBACILLI WITH $(GTG)_5$-PCR

A total of 61 reference strains were subjected to $(GTG)_5$-PCR fingerprinting. The results of numerical analysis of the generated $(GTG)_5$-PCR banding patterns are shown in a dendrogram (Fig. 3.1). All reference strains clearly grouped in separate clusters according to their respective taxonomic designations, except for representatives of *Lb. brevis*, which were dispersed in two clusters. *Lb. brevis* is known to be a phenotypically and genotypically heterogeneous species, as observed from protein profiling (D. Gevers, unpublished data) and DNA-DNA hybridisation data (Kandler and Weiss, 1986). The newly obtained $(GTG)_5$-PCR fingerprinting results confirm this previous finding, hereby indicating the need for a revision of the species status of *Lb. brevis*. In a number of cases, it was also noted that the taxonomic resolution of the $(GTG)_5$-PCR method was higher than that of protein profiling. The closely related species *Lb. pentosus*, *Lb. plantarum* and *Lb. paraplantarum* cannot be differentiated using protein profiling (data not shown).



LMG No[a]	Group[b]	Taxon
LMG 11437	C	*Lb. brevis*
LMG 7944^T	C	*Lb. brevis*
LMG 11774	C	*Lb. brevis*
LMG 11495	C	*Lb. brevis*
LMG 6904^T	B	*Lb. casei*
LMG 9496^T	A	*Lb. amylovorus*
LMG 19460	LAB	*Lc. lactis* subsp. *lactis* BU2-60
LMG 9191^T	B	*Lb. paracasei* subsp. tolerans
LMG 11384^T	LAB	*P. acidilactici*
LMG 10739	LAB	*P. acidilactici*
LMG 11462	B	*Lb. intestinalis*
LMG 14196^T	B	*Lb. intestinalis*
LMG 11998	C	*Lb. brevis*
LMG 11992	C	*Lb. brevis*
LMG 11969	C	*Lb. brevis*
LMG 17299	B	*Lb. curvatus*
LMG 12008	B	*Lb. curvatus*
LMG 9198^T	B	*Lb. curvatus*
LMG 12007	B	*Lb. curvatus*
LMG 11457^T	C	*Lb. parabuchneri*
LMG 17306	B	*Lb. sakei* subsp. *carnosus*
LMG 17304	B	*Lb. sakei* subsp. *carnosus*
LMG 17302^T	B	*Lb. sakei* subsp. *carnosus*
LMG 17305	B	*Lb. sakei* subsp. *carnosus*
LMG 9825^T	B	*Lb. graminis*
LMG 13558^T	B	*Lb. sakei* subsp. sakei
LMG 17551	C	*Lb. fermentum*
LMG 18026	C	*Lb. fermentum*
LMG 8896	C	*Lb. fermentum*
LMG 6902^T	C	*Lb. fermentum*
LMG 11398	B	*Lb. agilis*
LMG 11399	B	*Lb. agilis*
LMG 9186^T	B	*Lb. agilis*
LMG 9197^T	B	*Lb. coryniformis* subsp. *torquens*
LMG 9196^T	B	*Lb. coryniformis* subsp. *coryniformis*
LMG 11432	B	*Lb. bifermentans*
LMG 9845^T	B	*Lb. bifermentans*
LMG 11431	B	*Lb. bifermentans*
LMG 9187^T	B	*Lb. alimentarius*
LMG 3507^T	LAB	*W. viridescens*
LMG 14189^T	B	*Lb. murinus*
LMG 6892^T	C	*Lb. buchneri*
LMG 19152^T	B	*Lb. paralimentarius*
LMG 6400^T	B	*Lb. rhamnosus*
LMG 8153	B	*Lb. rhamnosus*
LMG 10768	B	*Lb. rhamnosus*
LMG 18030	B	*Lb. rhamnosus*
LMG 9200^T	A	*Lb. farciminis*
LMG 18936^T	B	*Lb. perolens*
LMG 11385	LAB	*P. pentosaceus*
LMG 13731	B	*Lb. paracasei*
LMG 13087^T	B	*Lb. paracasei* subsp. *paracasei*
LMG 1284	B	*Lb. plantarum*
LMG 9206	B	*Lb. plantarum*
LMG 6907^T	B	*Lb. plantarum*
LMG 18021	B	*Lb. plantarum*
LMG 10755^T	B	*Lb. pentosus*
LMG 18401	B	*Lb. pentosus*
LMG 16673^T	B	*Lb. paraplantarum*
LMG 19456	LAB	*E. faecalis*
LMG 9469^T	LAB	*W. halotolerans*

Fig. 3.1 Dendrogram generated after cluster analysis of the digitised (GTG)5-PCR fingerprints of the reference strains. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient. [a] LMG: BCCM™/LMG bacteria collection (Laboratory of Microbiology, Ghent University, Belgium), T: type strain[b] LAB: lactic acid bacteria, Group A: obligately homofermentative lactobacilli, Group B: facultatively heterofermentative lactobacilli, Group C: obligately heterofermentative lactobacilli.

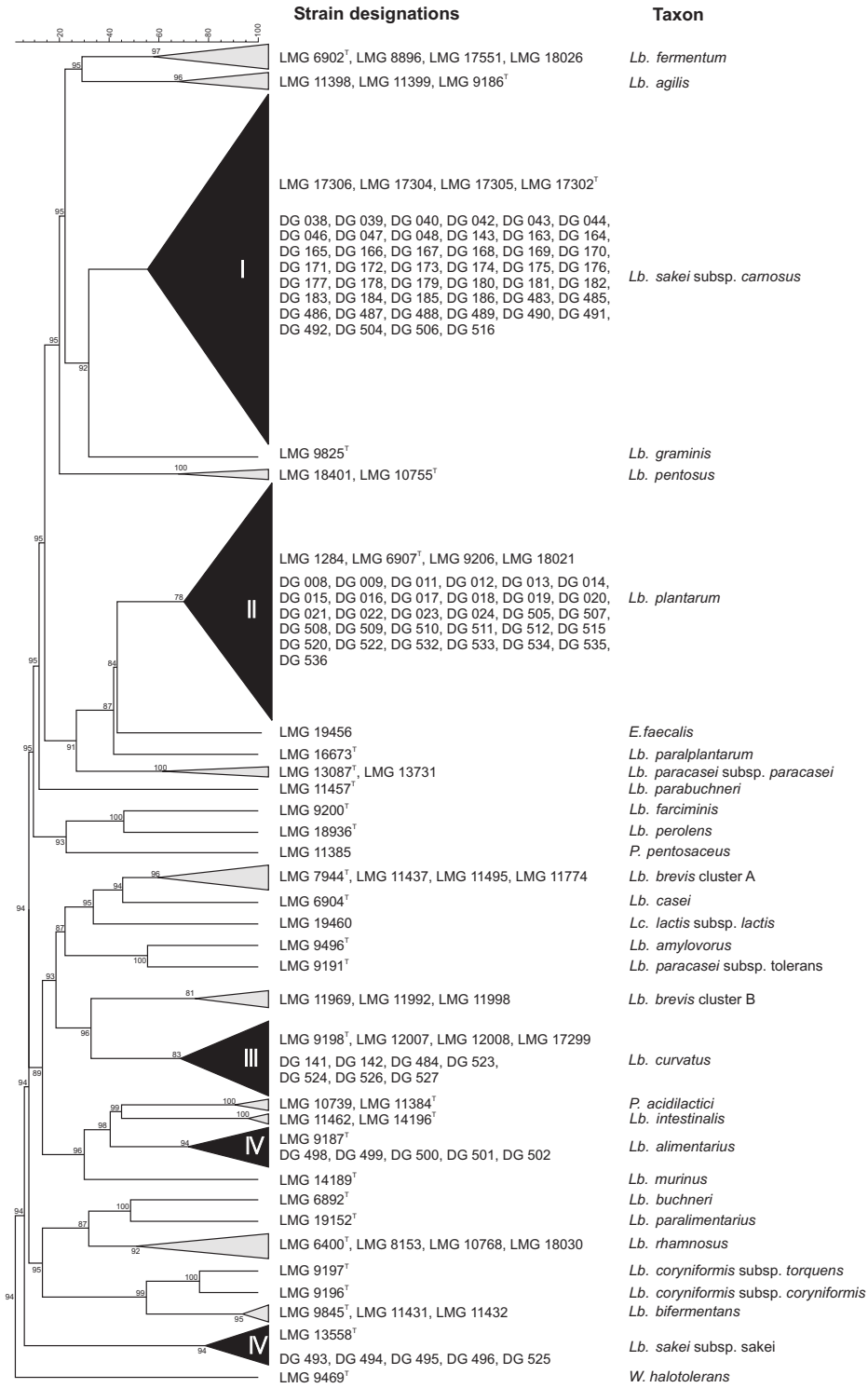


Fig. 3.2. Dendrogram generated after cluster analysis of the digitised (GTG)5-PCR fingerprints of the 61 reference strains and 94 isolates. The dendrogram was constructed using the unweighted pair-group method using arithmetic averages with correlation levels expressed as percentage values of the Pearson correlation coefficient. Block clusters I to IV contain reference strains and isolates.

As shown in Fig. 3.1, this was not a problem in numerical analysis of $(GTG)_5$-PCR fingerprints. For each of the investigated taxa, group-specific bands could be observed that allowed visual verification of the clusters obtained with numerical analysis. It was found that the complexity of the $(GTG)_5$-PCR band pattern was not the same for all species. Generally, the number of bands ranged between 10 and 20, with an average of 16.5 bands. For *Lb. alimentarius* and *Lb. intestinalis*, however, the number of bands was lower, *i.e.* six and five respectively. The banding patterns of seven strains belonging to other LAB genera (*i.e.* *Enterococcus*, *Lactococcus*, *Pediococcus* and *Weissella*) displayed a comparable complexity as seen among the majority of *Lactobacillus* fingerprints. On the other hand, the grouping obtained in Fig. 3.1 clearly did not reflect classification in different genera as lactobacilli were not clearly separated from strains of other Gram-positive genera. A fairly high discriminatory power up to the strain level was found for the set of 61 reference strains as all of these strains could be differentiated from each other on the basis of at least one band difference in their respective $(GTG)_5$-PCR fingerprints (Fig. 3.1)

In order to evaluate the applicability of $(GTG)_5$-PCR for identification of unknown isolates, two different sets of isolates were subjected to $(GTG)_5$-PCR fingerprinting (Fig. 3.2). The first set contained 52 isolates that were previously identified at the (sub)species level by means of protein profiling (Gevers *et al.*, 2000; chapter 2). The results of numerical analysis of the generated $(GTG)_5$-PCR banding patterns confirms identification and the clustering based on the protein profiles for all 52 isolates. For the second set of 42 previously unidentified isolates, the $(GTG)_5$-PCR banding patterns were clustered together with the reference strains and isolates of the first set. The previously unidentified LAB isolates were assigned to the clusters representing *Lactobacillus alimentarius*, *Lb. curvatus*, *Lb. plantarum*, *Lb.* sakei subsp. *carnosus* and *Lb. sakei* subsp. *sakei*. Clearly, the addition of these isolates had no pronounced effect on the stability of cluster analysis based on the $(GTG)_5$-PCR banding patterns of the reference strains. Overall, $(GTG)_5$-PCR banding patterns displayed a much higher heterogeneity among isolates, compared to the corresponding protein profiles (data not shown). In this way, $(GTG)_5$-PCR analysis revealed that a given species was represented by different strains within the same sample of fermented dry sausage. On the other hand, isolates with highly similar or even identical $(GTG)_5$-PCR fingerprints were frequently found within the set of isolates recovered from the same sample of fermented dry sausage. From our experience, $(GTG)_5$-PCR fingerprinting can be used for identification and possibly for intraspecies differentiation and is especially useful for screening a large number of strains. In specific cases, however, it may be necessary to

further subtype a cluster of similar $(GTG)_5$-PCR fingerprints using pulsed field gel electrophoresis (PFGE). Further research will include more strains of a broader group of lactic acid bacteria and investigate the usefulness of $(GTG)_5$-PCR for high-resolution typing.

In conclusion, the rep-PCR fingerprinting technique using the $(GTG)_5$ primer ($(GTG)_5$-PCR) is a rapid, easy-to-perform, and reproducible tool for differentiation of a wide range of food-associated lactobacilli at the species, subspecies and potentially up to the strain level with a single-performance protocol. In our hands, this technique is a promising genotypic tool for rapid and reliable speciation and typing of lactobacilli and other lactic acid bacteria in food fermentations.

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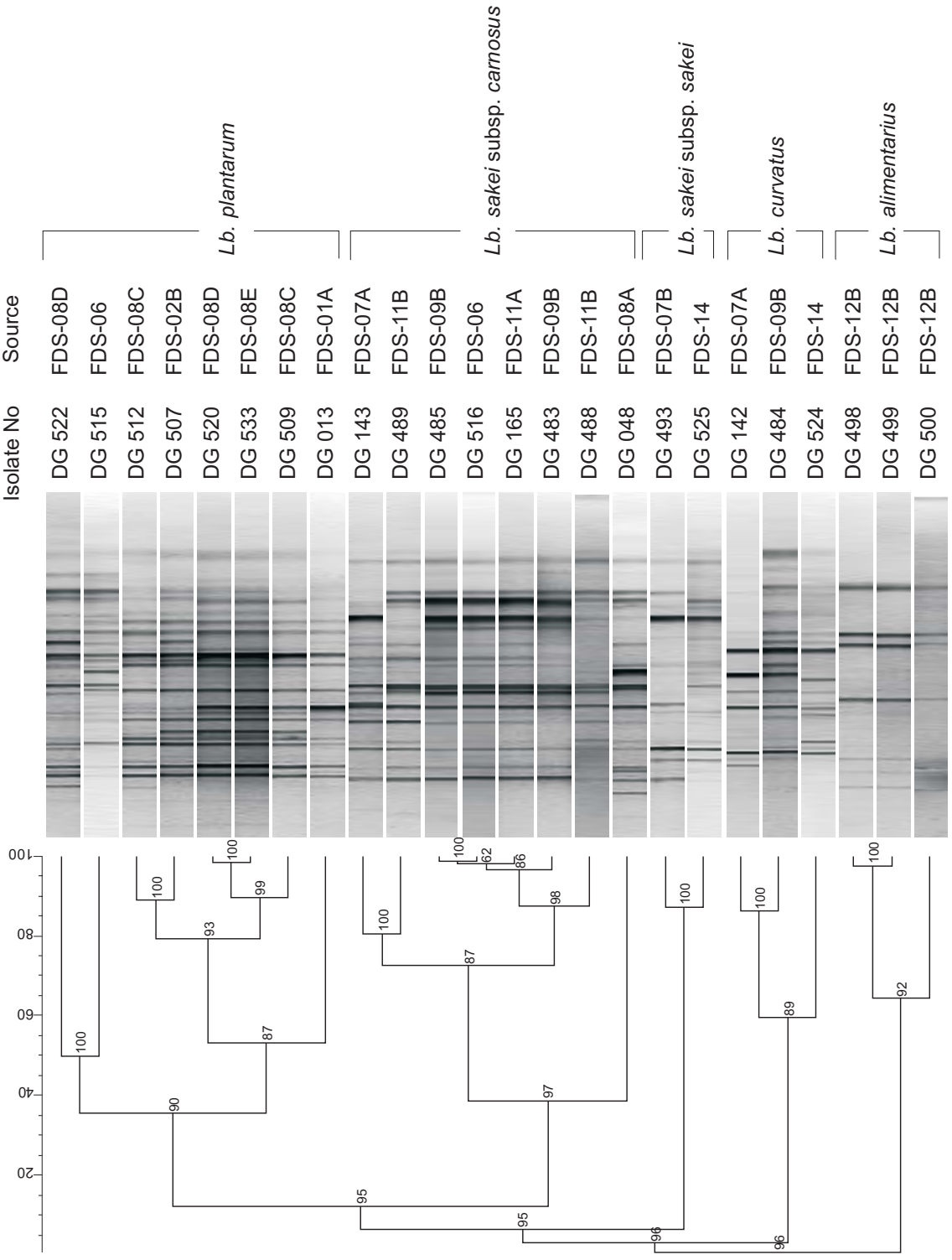
3.2.

ADDITIONAL REMARKS

In section 3.1, the $(GTG)_5$-PCR fingerprinting results of the Tc^r LAB isolates were interpreted towards the applicability of the method. In this paragraph, further interpretation and conclusions are made concerning the diversity of the Tc^r LAB subpopulation in FDS end products.

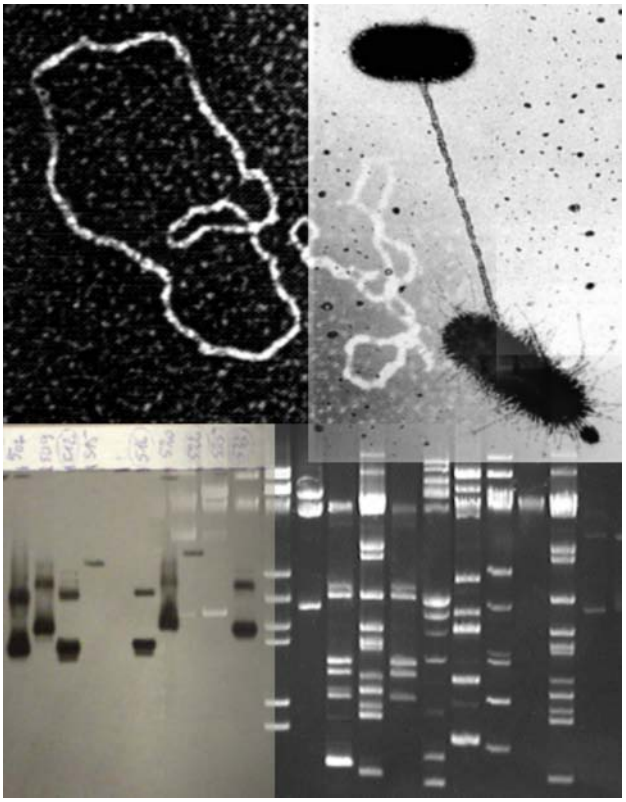
In five batches (FDS-08C, -08D, -9B, -11B, and -12B) $(GTG)_5$-PCR fingerprinting revealed an intraspecies diversity among the isolates. Different batches of a particular FDS type were not found to contain identical $(GTG)_5$-PCR fingerprints, except for FDS-08C (DG 520) and FDS-08D (DG 533), but the two isolates were shown to have different plasmid profiles (chapter 4). In some cases even different species compositions were obtained in the different batches, indicating that the source of Tc^r lactobacilli is highly variable. Interestingly, isolates DG 165, DG 485 and DG 516 originating from three different FDS types (FDS-06, -09B and -11B) displayed identical fingerprints, suggesting a possible common source of Tc^r *Lactobacillus* contamination. Numerical analysis of the $(GTG)_5$-PCR fingerprints showed that isolates originating from the same batch often displayed identical banding patterns (results not shown). In order not to select multiple isogenic strains, one isolate per unique $(GTG)_5$-PCR fingerprint type was selected, resulting in a collection of 24 Tc^r *Lactobacillus* isolates for further research (Fig. 3.3).

Fig. 3.3. (opposite page) Dendrogram based on the cluster analysis of the digitized (GTG)5-PCR fingerprints of 24 representative Tc^r *Lactobacillus* isolates from 14 different FDS end products. The dendrogram was constructed using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with correlation levels expressed as % values of the pearson correlation coefficient. Cophenetic correlations (shown on each branch of the dendrogram) indicate how faithfully the dendrogram represents the similarity matrix.


Isolate No
Source
DG 522 FDS-08D
DG 515 FDS-06
DG 512 FDS-08C
DG 507 FDS-02B
DG 520 FDS-08D
DG 533 FDS-08E
DG 509 FDS-08C
DG 013 FDS-01A
DG 143 FDS-07A
DG 489 FDS-11B
DG 485 FDS-09B
DG 516 FDS-06
DG 165 FDS-11A
DG 483 FDS-09B
DG 488 FDS-11B
DG 048 FDS-08A
DG 493 FDS-07B
DG 525 FDS-14
DG 142 FDS-07A
DG 484 FDS-09B
DG 524 FDS-14
DG 498 FDS-12B
DG 499 FDS-12B
DG 500 FDS-12B
Lb. plantarum
Lb. sakei subsp. carnosus
Lb. sakei subsp. sakei
Lb. curvatus
Lb. alimentarius
100
80
60
40
20

4

MOLECULAR ANALYSIS OF THE TETRACYCLINE RESISTANCE IN *LACTOBACILLUS* ISOLATES FROM DIFFERENT TYPES OF FERMENTED DRY SAUSAGE END PRODUCTS



SUMMARY

Little is known about the genetics of the acquired antibiotic resistances in lactobacilli from food products. Therefore a set of 24 tetracycline resistance (Tc^r) lactobacilli recovered from nine different fermented dry sausage types was subjected to a polyphasic molecular study with the aim to detect, identify and localise the genes conferring Tc^r. The *tet* genes were determined by means of PCR, and only *tet*(M) was detected. Restriction enzyme analysis with *Acc*I and *Sca*I revealed two different *tet*(M) allele types. This grouping was confirmed by partial sequencing of the *tet*(M) ORF, which indicated that the two allele types displayed high sequence similarities with *tet*(M) genes previously reported in two pathogenic species. Southern hybridisation of the plasmid profiles with a *tet*(M) probe revealed that most of the detected *tet*(M) genes were located on plasmids. One isolate harboured, in addition to the *tet*(M) gene, an *erm*(B) gene on a different plasmid than the one coding for the Tc^r. It was also shown by PCR that none of the *tet*(M) genes in the 24 Tc^r isolates were located on a transposon of the Tn*916*/Tn*1545* family. In the second part of the molecular analysis of the Tc^r, the ability of the Tc^r lactobacilli to transfer their resistance by conjugation was examined by using filter mating experiments. Out of these 24 Tc^r lactobacilli, seven were able to transfer their Tc^r to *Enterococcus faecalis* at relatively high frequencies (10^{-4} – 10^{-5}). Two of them could also transfer their R-plasmid to *Lc. lactis* subsp. *lactis*. These data suggest that lactobacilli may be reservoir organisms for acquired resistance genes that can be spread to other bacteria.

4.1.

MOLECULAR CHARACTERIZATION OF *TET*(M) GENES IN *LACTOBACILLUS* ISOLATES FROM DIFFERENT TYPES OF FERMENTED DRY SAUSAGE

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INTRODUCTION

For several decades, studies on the selection and dissemination of antibiotic resistance have mainly focussed on clinically relevant bacterial species. More recently many investigators speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens (Salyers, 1995; Levy and Salyers, 1998). Such commensal reservoir bacteria can be present in the intestines of farmed animals exposed to antibiotics (Witte, 1998; Aminov *et al.*, 2001) and may subsequently contaminate the raw meat produced from these animals even when hygiene regulations are respected. Several examples of antibiotic resistant lactic acid bacteria (LAB) isolated from raw meat exist (Klein *et al.*, 1998; Quednau *et al.*, 1998; Vidal and Collins-Thompson, 1987; Pavia *et al.*, 2000). Fermented foods prepared from raw meat or milk can therefore be considered as potential vehicles for the spread of antibiotic resistant LAB along the food chain to the consumer (Teuber and Perreten, 2000). Genes conferring resistance to tetracycline, chloramphenicol, erythromycin, and vancomycin, have been detected and characterized in *Lactococcus lactis* (Perreten *et al.*, 1997) and enterococci (Teuber and Perreten, 2000; Giraffa and Sisto, 1997) isolated from fermented meat and milk products. In contrast, no molecular data are available on the occurrence of antibiotic resistance genes in lactobacilli present in fermented food products. Members of the genus *Lactobacillus* also constitute an important part of the natural microflora associated with fermented products and are indigenous to the animal and human gastro-intestinal tract. Along with enterococci, these properties make lactobacilli interesting indicator organisms to study the molecular ecology of antibiotic resistance determinants in food fermentations.

Foregoing, we have isolated tetracycline resistant (Tc^r) strains of various *Lactobacillus* species from different types of modified atmosphere packed fermented dry sausage sold in Belgian retail shops (Gevers *et al.*, 2000; chapter 2). All *Lactobacillus* isolates collected in this way were resistant to at least 64 μg/ml of tetracycline as determined with the MRS agar dilution method (Gevers *et al.*, 2000; chapter 2). The aim of this study was to perform a molecular characterization of the *tet* genes conferring the high-level phenotypic resistance to tetracycline in these isolates.

MATERIALS AND METHODS

Bacterial isolates. In this study a total of 24 Tc^r LAB isolates recovered from 14 batches of 9 different fermented dry sausage types were included. A type is defined here as a specific variety distributed under a specific commercial brand and each type is designated a number (*i.e.* FDS-01, FDS-02, FDS-06, FDS-07, FDS-08, FDS-09, FDS-11, FDS-12, and FDS-14); whereas a batch (*i.e.* a group of the same product made at one time) is indicated with a letter from A to E. Isolates were recovered on the basis of colony morphology rather than relative abundance in order to obtain a set of isolates with the highest diversity. All these FDS were prepared using a starter culture. The isolation and identification of the Tc^r LAB isolates are described in Chapter 2 and 3, respectively. All isolates were stored in a bead storage system (Microbank system, Pro-LAB Diagnostics, Wirral, UK) at –80°C and grown in MRS at 30°C under microaerophilic conditions (3.75% CO_2, 5% O_2, 7.5% H_2, and 83.75% N_2).

Antibiotic susceptibility testing and MIC determination. A modified version of the Kirby-Bauer disc diffusion method (Kirby et al., 1966), in which Meuller-Hinton medium was replaced by MRS agar, was used for antibiotic sensitivity testing. Oxoid (Basingstoke, UK) susceptibility test discs of ampicillin (25 µg), chloramphenicol (30 µg), clindamycin (10 µg), erythromycin (10 µg), penicillin G (10 U), rifampicin (30 µg) and tetracycline (30 µg) were applied on inoculated MRS plates using the Oxoid Disc Dispenser. Diameters of the respective inhibition zones were measured using a digital callipers (Mauser digital 2, Ludwigsburg, Germany) following a 16-18 h incubation of the plates at 30°C. For each of the antibiotics tested, classification of the isolates into sensitive and resistant groups was based on resistance histograms (*i.e.* number of strains *versus* size of the inhibition zone). Cut-off values to differentiate among resistant and susceptible groups were defined on the basis of the bimodal distribution of the population in the resistance histograms. The MIC of tetracycline was determined by applying an Etest® strip (AB Biodisk, Solna, Sweden) on an inoculated MRS plate according to manufacturer's instructions. The Etest strip was read following 16-18 h incubation at 30°C.

DNA preparation and manipulations. Total genomic DNA of each isolate was extracted and purified as described previously (Gevers *et al.*, 2001; chapter 3). Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay (Anderson and McKay, 1983). Restriction endonuclease digestions of the *tet*(M) gene, agarose gel electrophoresis and Southern blotting were carried out following standard procedures (Sambrook *et al.*, 1989). The labelling of DNA probes with horseradish peroxidase using the ECL Direct Nucleic Acid Labelling kit (RPN3000, Amersham Biosciences,Uppsala, Sweden), was performed according to the manufacturer's instructions.

PCR detection of *tet*, *erm* and *int* genes. PCR assays (total volume, 50 µl) contained 20 pmol of each primer (Table 4.1), 1 x PCR buffer (Applied Biosystems, Warrington, UK), each dNTPs at a concentration of 200 µM, and 1 U of AmpliTaq® DNA Polymerase (N808-0160, Applied Biosystems, Warrington, UK). A 50 ng portion of purified total DNA was used as a template. In a first PCR assay, *tet* genes encoding ribosomal protection proteins (RPP) were detected using degenerate primers DI and DII (Clermont *et al.*, 1997). If positive for RPP genes, additional PCR assays were performed using specific primers for *tet*(M), *tet*(O), and *tet*(S) (4.1). Next to RPP *tet* genes, isolates were also tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L), and for transposon integrase (*int*) gene of the Tn*916*/Tn*1545* family (Table 4.1). One strain that expressed an erythromycin resistance was analysed with *erm*(B) specific primers as described previously (Jensen *et al.*, 1999). All PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer, Wellesley, US) using the following temperature program: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, for 1 min at the annealing temperature (T_{an}) as indicated in Table 4.1, and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR products (5 µl) were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Sequencing of PCR products. For *tet*(M) positive isolates, the purified PCR products (obtained with DI and *tet*(M)-R primers) were directly sequenced using the primers DI, DII and *tet*(M)-R (Table 4.1). Sequencing was performed using a BigDye® Terminator v2 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems). On-line similarity searches were performed by using the BLAST (Basic Local Alignment Search Tool) family of programs in GenBank (National Center for Biotechnology Information, Bethesda, US).

Nucleotide sequence accession numbers. Sequences of *tet*(M) genes described in this paper have been assigned GenBank Accession numbers AY149574 to AY149597.

Table 4.1. Primers for PCR detection of *tet, erm,* and *int* genes

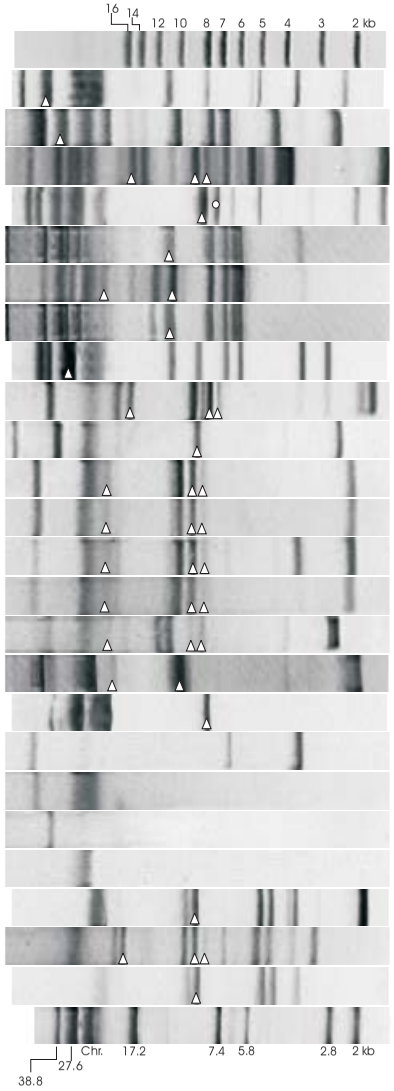
Primer	Class targeted	Sequence	T_{an}	Amplicon size (bp)	Positive control strain and reference	Reference or source of primers
DI DII	RPP	5'-GAYACNCCNGGNCAYRTNGAYTT-3' 5'-GCCCARWANGGRTTNGGNGGNACYTC-3'	45°C	1083	pJI3 (Morse *et al.*, 1986)	Clermont *et al.* (1997)
DI TetM-R	*tet* (M)	5'-GAYACNCCNGGNCAYRTNGAYTT-3' 5'-CACCGAGCAGGGATTTCTCCAC-3'	55°C	1513	pJI3 (Morse *et al.*, 1986)	Clermont *et al.* (1997)
TetS-FWT 1 TetS-RVT 2	*tet* (S)	5'-ATCAAGATATTAAGGAC-3' 5'-TTCTCTATGTGGTAATC-3'	55°C	573	pVP2 (Perreten *et al.*, 1997)	Charpentier *et al.* (1993)
TetO-FW 1 TetO-RV 1	*tet* (O)	5'- AATGAAGATTCCGACAATTT-3' 5'- CTCATGCGTTGTAGTATTCCA-3'	55°C	781	pAT121 (Collard J.M.)	Sougakoff *et al.* (1987)
TetK-FW 1 TetK-RV 1	*tet* (K)	5'-TTATGGTGGTTGTAGCTAGAAA-3' 5'-AAAGGGTTAGAAACTCTTGAAA-3'	55°C	348	pAT102 (Courvalin P.)	Collard J.M.
TetL-FW 3 TetL-RV 3	*tet* (L)	5'-GTMGTTGCGCGCTATATTCC-3' 5'-GTGAAMGRWAGCCCACCTAA-3'	55°C	696	pAT103 (Courvalin P.)	Collard J.M.
Int-FW Int-RV	*int*	5'-GCGTGATTGTATCTCACT-3' 5'-GACGCTCCTGTTGCTTCT-3'	50°C	1028	Tn*1545* (Courvalin P.)	Doherty *et al.* (2000)
ErmB-FW ErmB-RV	*erm* (B)	5'-CATTTAACGACGAAACTGGC-3' 5'-GGAACATCTGTGGTATGGCG-3'	55°C	405	Tn*1545* (Courvalin P.)	Jensen *et al.* (1999)

RESULTS

Phenotypic characterization of resistance. Etests revealed that the MIC of tetracycline ranged between 32 and > 256 µg/ml (upper limit of test), and the MIC_{50} is > 256 µg/ml (Fig. 4.1). Next to a high-level tetracycline resistance found among the 24 selected isolates, some strains showed in disc diffusion susceptibility testing an additional phenotypic resistance towards erythromycin (n = 1), rifampicin (n = 4) and penicillin (n = 1).

Detection, characterization and localisation of *tet* genes. Total genomic DNA preparations from all 24 Tc^r *Lactobacillus* isolates were subjected to PCR amplification with the RPP set of primers, with class-specific primers for *tet*(M), *tet*(O), *tet*(S), *tet*(K) and *tet*(L), and with primers for detection of *int* genes of the Tn*916*/Tn*1545* family of transposons, all using appropriate positive controls as indicated in Table 4.1. In all 24 isolates, only *tet*(M) was detected, and no *int* genes were found. Positive controls were included and results were conclusive. Restriction enzyme analysis (REA) of the *tet*(M) PCR product (74% of ORF) with *Acc*I and *Sca*I revealed two different *tet*(M) allele types, *i.e. tet*(M)-*1* and *tet*(M)-*2* respectively (Fig. 4.1). Most batches contained strains belonging to one allele type, except for batches FDS-02B, FDS-07A and FDS-14 that contained strains displaying both allele types (Fig. 4.1). In order to characterise the *tet*(M) genes more profoundly, the ORF was partially (74%) sequenced from approximately position 280 (DI primer) to position 1700 (TetM-R primer). Sequence alignments revealed two sequence homology groups and one mosaic gene (Fig. 4.2). Between the homology groups a difference of at least 25 bases was found. A first group comprised 9 isolates in which the sequenced part of the *tet*(M) gene showed maximum five base differences with the *tet*(M) gene of *Neisseria meningitidis* (GenBank Accession No X75073) (Gascoyne-Binzi *et al.*, 1994). This group corresponds to *tet*(M)-*1* found by REA. A second group of 14 isolates with maximum 3 base differences with the *tet*(M) gene of *Staphylococcus aureus* MRSA 101 (GenBank Accession No M21136) (Nesin *et al.*, 1990) corresponded to *tet*(M)-*2* defined by REA. The *tet*(M) gene of isolate DG 013 exhibited a mosaic structure combining partial sequences of the two foregoing homology groups. The sequence up to position 1508 of the ORF displayed 1 base difference with the *tet*(M) gene of *N. meningitidis* and is identical to the *tet*(M) gene of *S. aureus* MRSA 101 in the remaining part. REA classified the *tet*(M) gene of this strain as a *tet*(M)-*2*. The one isolate expressing a phenotypic erythromycin resistance (DG 507) was

shown to contain an *erm*(B) gene, by PCR and partial sequencing of the PCR product. Plasmid profiling of the 24 Tc^r *Lactobacillus* isolates showed that 23 isolates (excluding DG 524) contained at least one and usually more than one plasmid (Fig. 4.1). Using Southern blotting and hybridisation with a specific *tet*(M) and/ or *erm*(B) probe, the *tet*(M) genes of 20 isolates and the *erm*(B) gene in isolate DG 507 could be localised on a plasmid. Most of these R-plasmids had a size of approximately 10 kb, and in a few cases the R-plasmid was larger than 25 kb. Most plasmid profiles showed more than one band (up to three) that hybridised with the *tet*(M) probe (Fig. 4.1), due to hybridisation with (low concentrations of) open circular and/or linear plasmid forms. For the remaining four Tc^r *Lactobacillus* isolates that did not carry the *tet*(M) gene on a plasmid (*i.e.* DG 142, DG 484, DG 524 and DG 525), Southern blots of *Eco*RI digested DNA hybridised to the *tet*(M) probe with fragments between 8 and 12 kb (results not shown).



Isolate No	Source	Taxon	*tet*(M) allele number (REA)	*tet*(M) localisation	MIC of tetracyclin	Other resistances
supercoiled DNA ladder, Gibco BRL (plasmid size marker)						
DG 522	FDS-08D	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	
DG 515	FDS-06	*Lb. plantarum*	*tet*(M)-*2*	plasmid	>256	
DG 512	FDS-08C	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	
DG 507	FDS-02B	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	*erm*(B)
DG 520	FDS-08D	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	
DG 533	FDS-08E	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	
DG 509	FDS-08C	*Lb. plantarum*	*tet*(M)-*1*	plasmid	>256	Pen^r
DG 013	FDS-01A	*Lb. plantarum*	*tet*(M)-*2*	plasmid	>256	
DG 143	FDS-07A	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*1*	plasmid	192	Rif^r
DG 489	FDS-11B	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	96	
DG 485	FDS-09B	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	192	
DG 516	FDS-06	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	>256	
DG 165	FDS-11A	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	192	
DG 483	FDS-09B	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	192	
DG 488	FDS-11B	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*2*	plasmid	32	
DG 048	FDS-08A	*Lb. sakei* subsp. *carnosus*	*tet*(M)-*1*	plasmid	>256	
DG 493	FDS-07B	*Lb. sakei* subsp. *sakei*	*tet*(M)-*1*	plasmid	192	Rif^r
DG 525	FDS-14	*Lb. sakei* subsp. *sakei*	*tet*(M)-*2*	chromosomal	48	Rif^r
DG 142	FDS-07A	*Lb. curvatus*	*tet*(M)-*2*	chromosomal	48	
DG 484	FDS-09B	*Lb. curvatus*	*tet*(M)-*2*	chromosomal	32	
DG 524	FDS-14	*Lb. curvatus*	*tet*(M)-*2*	chromosomal	48	
DG 498	FDS-12B	*Lb. alimentarius*	*tet*(M)-*2*	plasmid	>256	
DG 499	FDS-12B	*Lb. alimentarius*	*tet*(M)-*2*	plasmid	>256	
DG 500	FDS-12B	*Lb. alimentarius*	*tet*(M)-*2*	plasmid	>256	
Lc. lactis subsp. *cremoris* AC1 (plasmid size reference)						

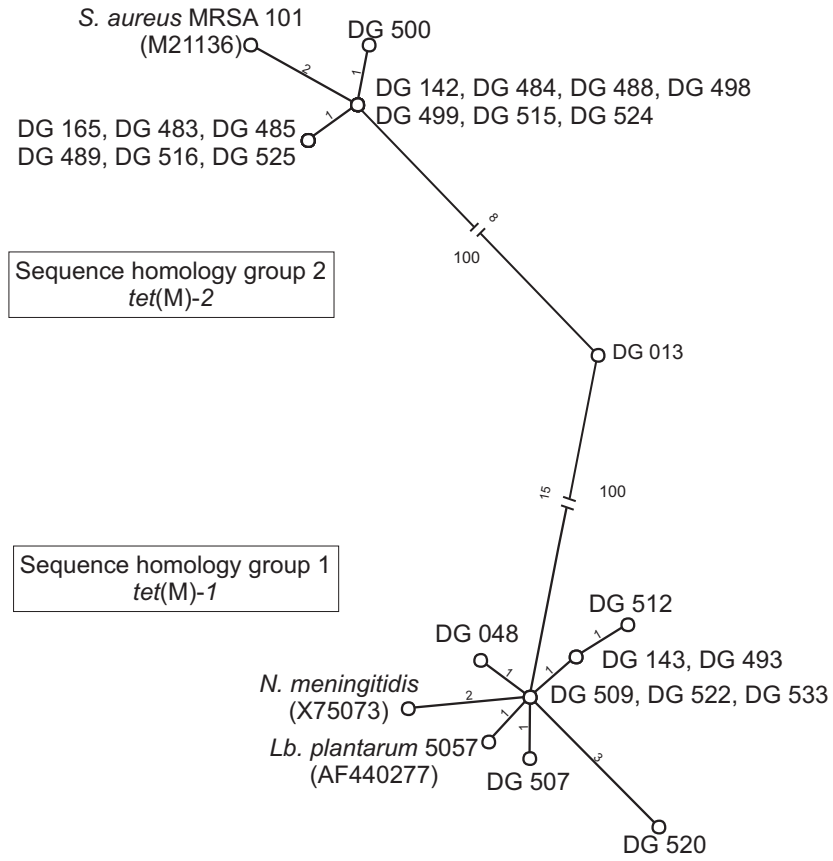


Fig. 4.2. Single most parsimonious tree (unrooted) for *tet*(M) gene relationships of the 24 Tc^r LAB isolates and two reference strains [*Neisseria meningitidis* (X75073) and *Staphylococcus aureus* MRSA 101 (M21136)]. The recently published *tet*(M) gene of *Lb. plantarum* 5057 (AF440277) was included (Danielsen, 2002). The numbers of nucleotide changes are indicated on each branch. The bootstrap percentages (500 replicates) are indicated for the separation between the two homology groups

Fig. 4.1. (opposite page) Inverted plasmid profiles of the 24 Tc^r *Lactobacillus* isolates from fermented dry sausage (FDS) end products. A small triangle or circle indicates the position where the *tet*(M) or *erm*(B) probe, respectively, hybridized on the Southern blot of the plasmid DNA. *Lactococcus lactis* subsp. *cremoris* strain AC1 was used as a plasmid size marker (Neve *et al.*, 1984). *Tet*(M)-*1* and *tet*(M)-*2* allele types correspond with the *tet*(M) genes found in *Neisseria meningitidis* (X75073) and *Staphylococcus aureus* MRSA 101 (M21136), respectively, based on restriction enzyme analysis (REA) using *Acc*I and *Sca*I. *Tet*(M) localisation: as was determined by Southern hybridisation. MIC of tetracycline: minimum inhibitory concentration as was determined by Etest. Chr.: chromosomal band

DISCUSSION

To our knowledge, this is the first detailed molecular study of antibiotic resistance genes in *Lactobacillus* species isolated from fermented dry sausages (FDS). The presence of antibiotic resistant *Lactobacillus* species has been documented in wine, cheese (Teuber *et al.*, 1999; Herrero *et al.*, 1996), poultry, calf, swine (Lin *et al.*, 1996; Tannock *et al.*, 1994; Frei *et al.*, 2001; Vescovo *et al.*, 1982), pig faeces (Fons *et al.*, 1997; Axelsson *et al.*, 1988; Rinckel and Savage, 1990), faeces of healthy humans (Ishiwa and Iwata, 1980) and maize silage (Danielsen, 2002). As described in chapter 2 and 3, a total of 94 Tc^r LAB isolates was recovered from 14 batches representing nine different FDS types and identified by $(GTG)_5$-PCR fingerprinting. All strains could be allocated to five different *Lactobacillus* taxa commonly associated with fermented meat products, *i.e. Lb. plantarum*, *Lb. sakei* subsp. *carnosus*, *Lb. sakei* subsp. *sakei*, *Lb. curvatus* and *Lb. alimentarius* (Hammes *et al.*, 1990). To avoid selection of multiple isogenic strains, one strain of each of the 24 unique $(GTG)_5$-PCR fingerprint type was selected for further molecular research.

Given the fact that the set of Tc^r *Lactobacillus* isolates was heterogeneous composed, it was somewhat surprising that in all isolates only *tet*(M) out of the 5 *tet* genes tested, was detected. According to current insights, *tet*(M) is the most widely distributed *tet* gene being detected in at least eight Gram-negative and 18 Gram-positive genera including *Enterococcus*, *Streptococcus*, and *Bifidobacterium* (Chopra and Roberts, 2001). It was suggested that the origin of *tet*(M) is most probably the tetracycline-producing species of *Streptomyces*, and that its integration into mobile genetic elements (plasmids and transposons) has led to its widespread distribution (Oggioni *et al.*, 1996; Chopra and Roberts, 2001). At the moment of discovery, only *tet*(O) and *tet*(Q) have been reported in members of the genus *Lactobacillus* (Chopra and Roberts, 2001), but recently also a *tet*(M) gene was found in a *Lb. plantarum* strain (Danielsen, 2002). Partial sequencing revealed that the *tet*(M) genes in the Tc^r *Lactobacillus* isolates belonged to two homology groups and one individual. The two homology groups corresponds to sequences that were published before, *i.e.* group I correspond with the *tet*(M) found in *Neisseria meningitidis* (Gascoyne-Binzi *et al.*, 1994), and group II corresponds with *tet*(M) of *Staphylococcus aureus* MRSA 101 (Nesin *et al.*, 1990). Isolate DG 13 represents a new allelic variation, showing high partial similarities with both the *N. meningitidis* and *S. aureus tet*(M) genes. This group may have arisen from homologous recombination and corresponds to the mosaic structures exhibited by the *tet*(M)

gene, as previously described (Oggioni *et al.*, 1996). The *tet*(M) genes found in these *Lactobacillus* isolates differ from those found in other lactic acid bacteria including *Enterococcus faecalis* (X56353; M85225; X92947; X04388) with a base difference ranging between 12 and 115 bases; and *Streptococcus pneumoniae* (X90939) with a base difference ranging between 69 and 86 bases.

The *tet*(M) genes of *N. meningitidis* and *S. aureus* MRSA 101 are located on a plasmid and on the chromosome, respectively (Gascoyne-Binzi *et al.*, 1994; Nesin *et al.*, 1990). Within the set of Tc^r *Lactobacillus* isolates, the *tet*(M) genes of sequence homology group I and isolate DG13 were exclusively found on plasmids (n = 10), whereas for sequence homology group II *tet*(M) genes were localised on the chromosome (n = 4) or on a plasmid (n = 10). R-plasmids encoding tetracycline, chloramphenicol, gentamicin, or macrolide-lincosamide-streptogramine (MLS) resistance have been reported previously in *Lb. reuteri* (Axelsson *et al.*, 1988; Lin *et al.*, 1996; Tannock *et al.*, 1994; Vescovo *et al.*, 1982), *Lb. fermentum* (Fons *et al.*, 1997; Ishiwa and Iwata, 1980), *Lb. acidophilus* (Vescovo *et al.*, 1982), and *Lb. plantarum* (Ahn *et al.*, 1992) isolated from raw meat and faeces. Most of these R-plasmids had a size smaller than 10 kb. The Tc^r *Lactobacillus* isolates with a chromosomal *tet* gene of this study show a significant lower MIC compared to the plasmid-encoded tetracycline resistance, *i.e.* 32 - 48 µg/ml and > 192 µg/ml respectively, with exception of two *Lactobacillus sakei* subsp. *carnosus* isolates (DG 488 and DG 489). More research is needed to investigate to what extent this marked difference in phenotypic resistance levels is linked to the location of the *tet*(M) gene.

In conclusion, the results of the current study indicate that *Lactobacillus* species from fermented meat products can harbour acquired Tc^r encoded by a *tet*(M) gene, most of which were located on plasmids and displayed very high genotypic similarities with *tet*(M) genes previously reported in two pathogenic species. Further research may focus on the diversity and transferability of these *Lactobacillus* plasmids into other commensal bacteria, on the source of Tc^r lactobacilli in the production process of FDS and can include *in vivo* transfer experiments in fermented dry sausage and/or gastro-intestinal tract.

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4.2.

CONJUGAL TRANSFER OF TETRACYCLINE RESISTANCE FROM *LACTOBACILLUS* ISOLATES RECOVERED FROM FERMENTED DRY SAUSAGE TO OTHER LACTIC ACID BACTERIA

INTRODUCTION

Lactobacilli are common in foods and are members of the resident microflora of the gastrointestinal tracts of humans and animals. Because of their broad environmental distribution, these commensal bacteria may function as vectors for the dissemination of antibiotic resistance determinants via the food chain to the consumer (Teuber *et al.*, 1999). In addition, this normal flora might be capable of supplying drug resistance genes to foodborne or enteric pathogens (Salyers, 1995). Although plasmids are very common in lactobacilli (Wang and Lee, 1997), and even plasmid located antibiotic resistance determinants have been reported in lactobacilli (Ahn *et al.*, 1992; Lin *et al.*, 1996; Tannock *et al.*, 1994; Danielsen, 2002), the literature on the conjugal transfer of native *Lactobacillus* plasmids is limited. So far, only the conjugal transfer of plasmid-encoded lactose metabolism from *Lb. casei* (Chassy and Rokaw, 1981) and of plasmid-encoded bacteriocin production and resistance from *Lb. acidophilus* (Klaenhammer, 1988) have been reported before. Therefore, this study was performed to analyse the possibility of Tc^r *Lactobacillus* isolates recovered from fermented dry sausage end products to transfer their *tet* genes to other lactic acid bacteria, including *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis*.

MATERIALS AND METHODS

Bacterial strains. The cultures used in this work are listed in Table 4.2. The Tc^r lactobacilli, used as donor strains for mating experiments, were isolated from fermented dry sausage end products as described in chapter 2, and grown on MRS at 30 °C. The recipient strains: (i) *Enterococcus faecalis* JH2-2 (Jacob and Hobbs, 1974) was grown in brain heart infusion medium (BHI, BD, Franklin Lakes, US) at 37 °C and (ii) for the cultivation of the lactose-negative *Lactococcus lactis* subsp. *lactis* Bu2-60 (Neve *et al.*, 1984), M17 broth medium (CM0817, Oxoid, Basingstoke, UK) was used in which lactose was replaced by glucose (GM17), and incubated at 30 °C. Antibiotics (Sigma, Bornem, Belgium) were used in the following concentrations to maintain the resistance genes or for the selection of transconjugants: tetracycline 10 µg/ml; rifampicin 50 µg/ml. All strains were stored in a bead storage system (Microbank system, Pro-LAB Diagnostics, Wirral, UK) at –80°C.

Table 4.2. Bacterial strains used in this study

Strain	Relevant properties	Remarks	References
Lactobacillus spp.			This study (chapter 2)
DG 013, 048, 143, 165, 483, 485, 488, 489, 493, 498, 499, 500, 507, 509, 512, 515, 516, 520, 522, 533	Plasmid located *tet* (M) gene	Donor strains, Source: fermented dry sausage end products	
DG 142, 484, 524, 525	Chromosomal located *tet* (M) gene		
DG 507	Plasmid located *tet* (M) and *erm* (B) gene (2 different plasmids)		
Enterococcus faecalis			
JH2-2	Fus^r, Rif^r, plasmid free	Recipient	Jacob and Hobbs (1974)
Lactococcus lactis subsp. *lactis*			
Bu2-60	Str^r, Rif^r, plasmid free	Recipient	Neve *et al.* (1984)
Lactococcus lactis subsp. *cremoris*			
AC1	Used as plasmid size marker		Neve *et al.* (1984)

Fus.: fusidic acid; Rif.: rifampicin; Str.: streptomycin

Mating procedure. Transferability of resistance genes was examined by using filter mating experiments. Donor and recipient strains were grown in non-selective broth medium to the mid-logarithmic phase of growth (approx. 4 h). The donor culture (1 ml) was added to the recipient culture (1 ml) and the mixture was filtrated through a sterile mixed

cellulose esters filter (0.45 µm) (MF-Millipore membrane filter, HAWP 02500, Millipore, Bedford, US) using the Swinnex® filter holders (SX00 025 00, Millipore). After donor and recipient cells were filtrated, sterilized pepton physiological saline solution (PPS) (8.5 g/l NaCl and 1 g/l neutralised bacteriological peptone [LP0034, Oxoid] was passed through the filter to trap the cells more tightly into the membrane, according to Sasaki *et al.* (1988). The filters were incubated overnight on non-selective agar medium corresponding with the growth medium and conditions of the recipient strain. The bacteria were washed from the filters with 2 ml PPS. Dilutions of the mating mixtures were spread onto agar plates containing appropriate selective antibiotics (double selective medium) and incubated for 24 to 48 h. Control cultures of donor and recipient strains alone were also plated on the double selective agar plates.

Antibiotic susceptibility testing and MIC determination. Possible transconjugants were screened for their antibiotic resistance pattern, using a modified version of the Kirby-Bauer disc diffusion method, in which Meuller-Hinton medium was replaced by MRS agar, as described in chapter 2. The MIC of tetracycline was determined by the Etest® (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions with slight modifications as previously described (Gevers *et al.*, 2002, chapter 4.1).

Typing of transconjugants. The fingerprints of transconjugants, obtained by high-resolution $(GTG)_5$-PCR fingerprinting as described in chapter 3, were compared to the fingerprints of recipient strains for confirmation purposes.

DNA preparation and manipulations. Total genomic DNA of each isolate was extracted and purified as described in chapter 3. Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay (1983). Restriction endonuclease digestions of the *tet*(M) gene, agarose gel electrophoresis and Southern blotting were carried out following standard procedures (Sambrook *et al.*, 1989). Labelling of DNA probes with horseradish peroxidase using the ECL Direct Nucleic Acid Labelling kit (RPN3000, Amersham Biosciences, Uppsala, Sweden) was performed according to the manufacturer's instructions.

PCR detection of *tet* genes. PCR assays are as described in section 4.1.

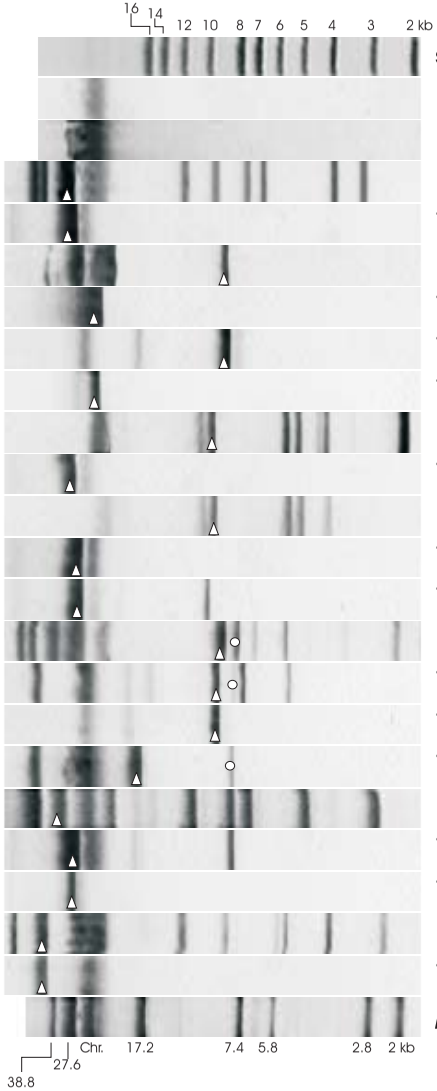
RESULTS

A total of 24 Tc^r *Lactobacillus* isolates (Table 4.2) all containing a *tet*(M) gene, was used to test their ability to transfer tetracycline resistance genes to *Enterococcus faecalis* JH2-2 by conjugation. Several attempts to transfer the R-plasmids by filter mating using a 0.2 µm pore size filter were ineffective (data not shown), whereas the use of a 0.45 µm membrane with a sponge-like structure was more successful. Tetracycline resistant transconjugants were obtained from matings with seven isolates, including four *Lb. plantarum* strains (DG 013, DG 507, DG 515 and DG 522), two *Lb. alimentarius* strains (DG 498 and DG 500), and one *Lb. sakei* subsp. *sakei* strain (DG 493) at frequencies ranging between 10^{-4} and 10^{-6} transconjugants per recipient. Higher transfer frequencies were found when cells were grown until the mid-logarithmic phase (4–6 h) in comparison to overnight cultures (data not shown). For two out of these seven Tc^r *Lactobacillus* isolates (DG 493 and DG 515) transfer of Tc^r was also shown when using *Lactococcus lactis* subsp. *lactis* Bu2-60 as a recipient at frequencies ranging between 10^{-5} and 10^{-7} transconjugants per recipient. Potential transconjugant colonies (approx. 5 per experiment) were isolated from the double selective medium at the end of the filter mating experiment and checked for coccoid cell morphology using standard phase-contrast microscopy. Using disc diffusion testing, susceptibility to tetracycline and rifampicin was compared between donor (Tc^r/Rif^s), recipient (Tc^s/Rif^r) and a selection of transconjugants (Tc^r/Rif^r). All selected Tc^r cocci had the Tc^r/Rif^r pattern. Further confirmation of transconjugants identity was obtained by comparing the $(GTG)_5$-PCR fingerprints of donor, recipient and transconjugants, and by checking the presence of the *tet*(M) gene by PCR. On the basis of these criteria, all Tc^r cocci that were isolated from the double selective medium were confirmed as true transconjugants.

Genotypic characterization of the transferred plasmids was obtained by plasmid profiling in combination with Southern blotting and hybridisation. In most cases, all transconjugants resulting from a particular donor/recipient combination exhibited the same plasmid profile, and from those, only one transconjugant was selected for blotting and hybridisation experiments. Among transconjugants obtained from mating of donor strains DG 493, DG 500 and DG 507 with the *E. faecalis* JH2-2 recipient strain, however, more than one different plasmid profile per combination was found. In these cases, one strain for each different plasmid profile was selected. A total of 13 transconjugants from 9 different

donor/recipient combinations was selected for blotting and hybridisation experiments (Fig. 4.3). In six transconjugants, the plasmid band that hybridised with the *tet*(M) probe was different in size compared to the original R-plasmid of the donor strain. Next to the plasmid of approx. 10 kb encoding the tetracycline resistance, two out of three transconjugants from the matings with DG 507 as donor strain, also received a second plasmid (approx. 8.5 kb) containing an *erm*(B) gene. This was also reflected in the MICs for erythromycin, that increased from 1 µg/ml for the erythromycin susceptible transconjugants to > 256 µg/ml for those that received the plasmid containing the *erm*(B) gene (Fig. 4.3). The MICs for tetracycline of the *E. faecalis* JH2-2 transconjugants were more than 3 times lower than the MIC of the corresponding donor strain, whereas the MICs of the *Lc. lactis* Bu2-60 transconjugants were comparable to those of the corresponding donor strain.

Fig. 4.3. (opposite page) Southern hybridisation analysis of the plasmid profiles of the donor (DG), recipient (LMG) and transconjugants (TC). Small triangle/circle indicates the position where the *tet*(M)/*erm*(B) probe, respectively, hybridised on the Southern blot of the plasmid DNA. *Lactococcus lactis* subsp. *cremoris* strain AC1 was used as a plasmid size marker (Neve *et al.*, 1984). MIC: minimum inhibitory concentration as was determined by Etest; Chr.: chromosomal band



Strain No	Taxon	MIC of tetracyclin	MIC of erythromycin
supercoiled DNA ladder, Gibco BRL (plasmid size marker)			
LMG 19456	*E. faecalis* JH2-2 (recipient)	0.5	1
LMG 19460	*Lc. lactis* Bu2-60 (recipient)	0.19	
DG 013	*Lb. plantarum*	>256	
TC 013 - 1	*E. faecalis* JH2-2	96	
DG 493	*Lb. sakei* subsp. *sakei*	192	
TC 493 - 1	*E. faecalis* JH2-2	64	
TC 493 - 4	*E. faecalis* JH2-2	64	
TC 493 - 21	*Lc. lactis* Bu2-60	192	
DG 498	*Lb. alimentarius*	>256	
TC 498 - 1	*E. faecalis* JH2-2	96	
DG 500	*Lb. alimentarius*	>256	
TC 500 - 1	*E. faecalis* JH2-2	64	
TC 500 - 3	*E. faecalis* JH2-2	64	
DG 507	*Lb. plantarum*	>256	>256
TC 507 - 1	*E. faecalis* JH2-2	64	>256
TC 507 - 2	*E. faecalis* JH2-2	64	1
TC 507 - 4	*E. faecalis* JH2-2	64	>256
DG 515	*Lb. plantarum*	>256	
TC 515 - 1	*E. faecalis* JH2-2	64	
TC 515 - 21	*Lc. lactis* Bu2-60	>256	
DG 522	*Lb. plantarum*	>256	
TC 522 - 1	*E. faecalis* JH2-2	64	
Lc. lactis subsp. *cremoris* AC1 (plasmid size reference)			

DISCUSSION

The mating experiments described here demonstrate that intergeneric transfer of R-plasmids from *Lactobacillus* spp. to other LAB can occur at a relatively high frequency under laboratory conditions of intimate cell-to-cell contact. From the practical point of view, two factors seemed to significantly affect the transfer frequency, namely the type of membrane filter (type, pore size and side of membrane) and the age of donor and recipient cultures. Similar filter dependent transfer frequencies were reported before by Sasaki and co-workers (1988), who indicated that the use of a sponge-like membrane with a pore size of 0.45 μm and front side up, resulted in the highest transfer frequencies. Moreover, they indicated that these frequencies could be increased when cells were trapped more tightly in the spongy structure of the membrane by passing sterile water or buffer through the filter.

The host range of the transferable R-plasmids was clearly variable, as not all plasmids that could be transferred to *E. faecalis* could also be transferred to *Lc. lactis*. In a few transconjugants (TC 500-3, TC 507-1, TC 507-4 and TC 515-1) additional plasmids other than the plasmid that was selected for, *i.e.* the R-plasmid coding for the tetracycline resistance, seemed to have co-transferred spontaneously. This resulted for example in a co-transfer of the erythromycin resistance determinant from DG 507 into *E. faecalis*. Remarkably, in six of the investigated transconjugants the band that hybridised with the *tet*(M) probe displayed a different size than the R-plasmid of the donor strain. These bands were two (TC 507-4) to three (TC 493-1, TC 493-21, TC 498-1, TC 500-1 and TC 500-3) times the size of the R-plasmid of the donor strain. So far, no further research has been undertaken to elucidate this finding. In the transconjugants TC 493-1 and TC 493-21, the band that hybridises with the *tet*(M) probe coincides with the chromosomal band, which might suggest a chromosomal integration of the resistance determinant. However, location on a plasmid that migrates at the same height as the chromosomal band cannot be excluded as yet.

This is the first report on conjugal transfer of native *Lactobacillus* plasmids encoding an antibiotic resistance determinant. A few studies have shown the transfer of an introduced plasmid, such as pAMβ1 (encoding an erythromycin resistance) from *Lb. reuteri* and *Lb. plantarum* to other Gram-positive bacteria *in vitro* (Tannock, 1987; West and Warner, 1985) and *in vivo* (Morelli *et al.*, 1988). Conjugation of the broad host range plasmid pAMβ1 into different *Lactobacillus* spp. has been reported in the framework of optimising recombinant DNA technologies to improve strain properties and has been reviewed by

Wang and Lee (1997). The mobilization of a non-conjugative, native plasmid encoding chloramphenicol resistance from *Lb. plantarum* to *Carnobacterium piscicola* was achieved by co-mobilization with the conjugative plasmid pAMβ1 (Ahn *et al.*, 1992).

In conclusion, our data suggest that *Lactobacillus* spp. may be reservoir organisms for acquired resistance genes that can be spread to other bacteria, a possibility that so far was not fully addressed. Further research may elaborate on the host range of these R-plasmids by transferring to a broader range of bacteria including the characterization of the R-plasmids.

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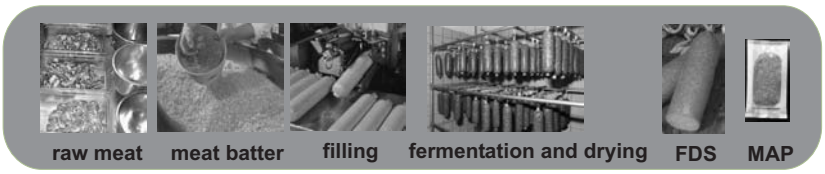
5

PREVALENCE AND DIVERSITY OF TETRACYCLINE RESISTANT LACTIC ACID BACTERIA AND THEIR *TET* GENES ALONG THE PROCESS LINE OF FERMENTED DRY SAUSAGES



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SUMMARY

In order to study the prevalence and diversity of tetracycline resistant lactic acid bacteria (Tc^r LAB) along the process line of two different fermented dry sausage (FDS) types, samples from the raw meat, the meat batter and the fermented end product were analysed quantitatively and qualitatively by using a culture-dependent approach. Both the diversity of the *tet* genes and their bacterial hosts in the different stages of FDS production were determined. Quantitative analysis showed that all raw meat components of both FDS types (FDS-01 and FDS-08) contained a subpopulation of Tc^r LAB, and that for FDS-01 no Tc^r LAB could be recovered from the samples after fermentation. Qualitative analysis of the Tc^r LAB subpopulation in FDS-08 included identification and typing of Tc^r LAB isolates by $(GTG)_5$-PCR fingerprinting, plasmid profiling, protein profiling and a characterization of the resistance by PCR detection of *tet* genes. Two remarks can be made when the results of this analysis for the different samples are compared. (i) The taxonomic diversity of Tc^r LAB varies along the process line, with a higher diversity in the raw meat (lactococci, lactobacilli, streptococci, and enterococci), and a decrease after fermentation (only lactobacilli). (ii) Also the genetic diversity of the *tet* genes varies along the process line. Both *tet*(M) and *tet*(S) were found in the raw meat, whereas only *tet*(M) was found after fermentation. A possible relationship was found between the disappearing of species other than lactobacilli and *tet*(S), because *tet*(S) was only found in lactococci, enterococci, and streptococci. These data suggest that fermented dry sausages are among those food products that can serve as vehicles for Tc^r LAB and that the raw meat already contains a subpopulation of these bacteria. Whether these results reflect the transfer of resistant bacteria or of bacterial resistance genes from animals to man via the food chain is difficult to ascertain and may require a combination of cultivation-dependant and cultivation-independent approaches.

INTRODUCTION

In recent years, the selection of antibiotic resistance genes by antimicrobial use in food animals has been of great public concern, especially with regard to the prevalence of antibiotic therapy failures in humans (Gustafson and Bowen, 1997). Clinical practices and animal husbandry are powerful foci of selective antibiotic pressure, and reservoirs of antibiotic resistance determinants in humans and animals have been shown to interact via various ecological routes, including the human food chain (Witte, 2000). Enteric bacteria can be readily transmitted through foods, as are antibiotic resistant pathogens and commensals. Although well documented for zoonotic pathogens (Threlfall *et al.*, 2000), very few attempts have been made to study the spread of antibiotic resistance genes by commensal bacteria.

During the past decade, it has become clear that commensal bacteria can act as reservoirs for resistance genes, and are thus important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations. The high incidence of antibiotic resistant commensals is clearly illustrated by the fact that the majority of human individuals is known to carry oral tetracycline-resistant (Tc^r) viridans streptococci regardless of tetracycline therapy history or age, whereas Tc^r pathogenic streptococci are significantly less common in most human populations (Luna and Roberts, 1998). Although tetracyclines are still important agents in both human and animal (veterinary and aquacultural) medicine, the emergence of Tc^r pathogens, opportunistic microbes and members of the normal flora has certainly limited their effectiveness (Chopra and Roberts, 2001). However, our current understanding of the bacterial hosts and environmental dissemination of tetracycline resistance genes (*tet* genes) and Tc^r plasmids has clearly demonstrated that Tc^r is one of the model markers to monitor the molecular ecology of antibiotic resistance genes. The widespread distribution of specific *tet* genes like *tet*(M) in Gram-negative and Gram-positive hosts supports the hypothesis that *tet* genes are exchanged by bacteria from many different ecosystems and between humans and animals. Previously, we reported on the presence of Tc^r lactic acid bacteria (LAB) in fermented dry sausage (FDS) end products (Gevers *et al.*, 2000, 2002; chapter 2 and 3). In the latter paper, it was shown that the Tc^r LAB flora in FDS sold in Belgian retail markets was dominated by *Lactobacillus* isolates that carried plasmid-encoded *tet*(M) genes. The current study was undertaken to analyse the prevalence of Tc^r LAB and their *tet* genes along the process line of different FDS types,

from the raw meat components to the end products. By using a culture-dependent approach, we determined the diversity of *tet* genes and their bacterial hosts in the different stages of FDS production.

MATERIALS AND METHODS

Fermented dry sausages (FDS). Two types of FDS that were previously found to contain lactobacilli exhibiting high-level resistance to tetracycline (Gevers *et al.*, 2000; chapter 2) were investigated in this study: one batch of FDS-01 composed of 1/3 beef, 1/3 pork, and 1/3 lard, and two batches (I and II) of FDS-08 composed of 2/3 pork, and 1/3 lard.

Processing of meat samples. To study the process line of FDS-01, samples of lard (1A), fresh pork (1C), fresh beef (1D), the meat batter (2A), the meat batter after addition of the starter culture (2B), the fermented sausage (3A), and the dry end product (3B) were obtained from a local FDS production facility. Samples that were obtained from the process line of FDS-08 included frozen lard (1A), frozen pork (1B), fresh pork (1C), the meat batter after addition of the starter culture and spices (2B), the fermented sausage (3A) and the sliced and packed dry end product (4). Of the latter process line, two batches with a time interval of one week were sampled. A 25 g sample was taken, added to 225 ml sterile peptone physiological saline solution (PPS) (8.5 g/l NaCl and 1 g/l neutralised bacteriological peptone [LP0034, Oxoid, Basingstoke, UK]) and homogenised in a Stomacher® (Seward, London, UK). Serial decimal dilutions (10^{-1} - 10^{-8}) in PPS were prepared and 1 ml samples of appropriate dilutions were poured in triplicate on de Man, Rogosa and Sharpe-Sorbic acid agar (MRS-S agar, 0882210, BD, Franklin Lakes, US) supplemented with or without 64 µg/ml tetracycline (T-3383, Sigma, Bornem, Belgium). Plates were incubated for five days at a temperature of 30°C under microaerophilic conditions (3.75% CO_2, 5% O_2, 7.5% H_2, and 83.75% N_2).

Selection and storage of strains. For both batches of FDS-08, colonies were randomly selected from MRS-S plates supplemented with 64 µg/ml tetracycline and further purified on non-selective MRS-S plates. Isolates were stored in a bead storage system (Microbank system, Pro-Lab Diagnostics, Wirral, UK) at –80°C.

Identification and typing of isolates. All Tc^r LAB isolates were subjected to rep-PCR fingerprinting with the $(GTG)_5$ primer as previously described (Gevers *et al.*, 2000; chapter 3). Some clusters of digitised profiles remained unidentified after comparison with the limited $(GTG)_5$-PCR database of reference strains (Gevers *et al.*, 2000; chapter 3). Representatives of these clusters were further identified with protein profiling as described before (Gevers *et al.*, 2000; chapter 2).

DNA preparation and manipulations. Total genomic DNA of each isolate was extracted and purified as described previously (Gevers *et al.*, 2000; chapter 3). Isolation of plasmid DNA was based on the alkaline lysis method of Anderson and McKay (Anderson and McKay, 1983). Restriction endonuclease digestions of the *tet*(M) gene, agarose gel electrophoresis and Southern blotting were carried out following standard procedures (Sambrook *et al.*, 1989). Labelling of DNA probes with horseradish peroxidase using the ECL Direct Nucleic Acid Labelling kit (RPN3000, Amersham Biosciences, Uppsala, Sweden) was performed according to the manufacturer's instructions.

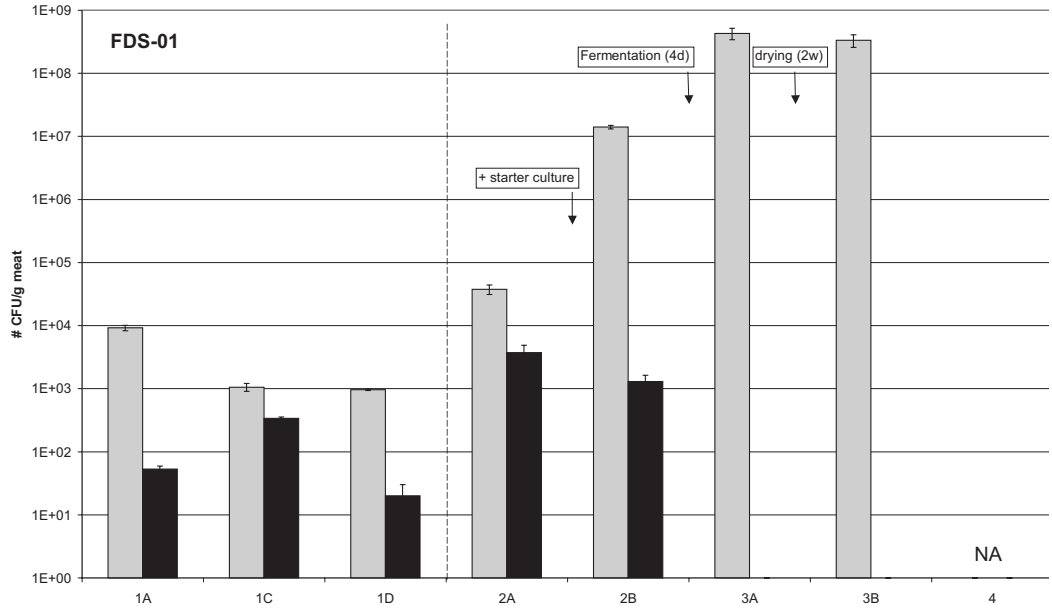
PCR detection of *tet* genes. PCR reaction mixes (total volume, 50 µl) contained 20 pmol of each primer, 1 x PCR buffer (Applied Biosystems, Warrington, UK), each of the four dNTPs at a concentration of 200 µM, and 1 U of AmpliTaq$^{(R)}$ DNA Polymerase (N808-0160, Applied Biosystems, Warrington, UK). A 50 ng portion of purified total genomic DNA was used as a template. In a first PCR assay, *tet* genes encoding ribosomal protection proteins (RPP) were detected using degenerate primers DI and DII (Clermont *et al.*, 1997). If positive for RPP genes, additional PCR assays were performed using specific primers for *tet*(M), *tet*(O), and *tet*(S) as described before (Gevers *et al.*, 2002). Next to RPP *tet* genes, isolates were also tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L) (Gevers *et al.*, 2002). All PCR amplifications were performed in a GeneAmp 9600 PCR system (Perkin-Elmer) using the following temperature program: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, for 1 min at the appropriate annealing temperature (T_{an}), and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR products (5 µl) were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

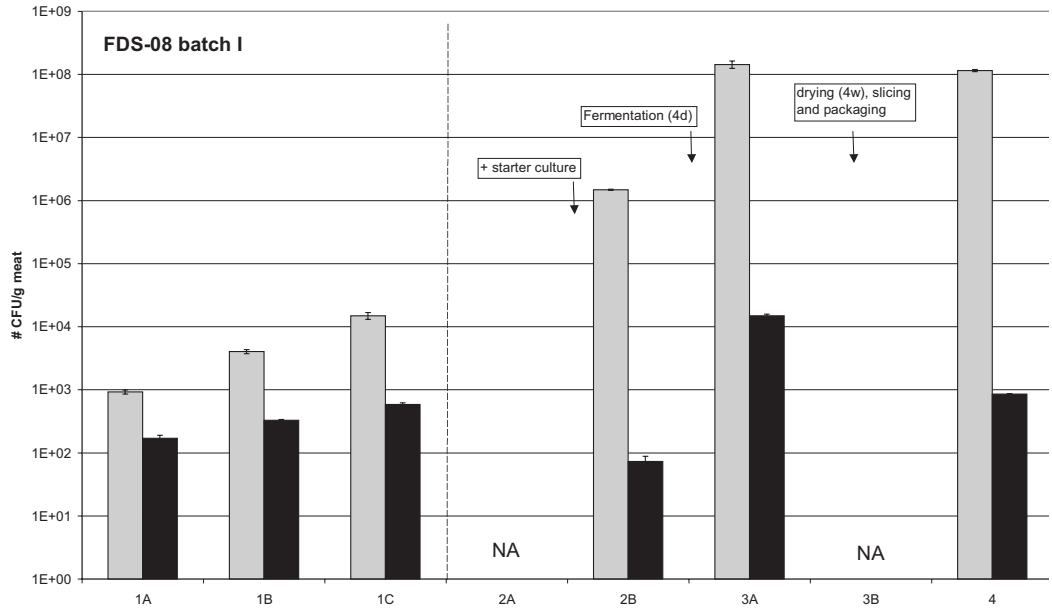
Analysis of meat samples for tetracycline residues. The presence of tetracycline residues was analysed as described before (Croubels *et al.*, 1997). Essentially, animal tissue was homogenised in sodium succinate buffer and methanol, followed by centrifugation and clean-up of tetracycline residues using a metal chelate affinity chromatography with further concentration of tetracyclines using cation exchange membrane. The final extract was analysed by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. The detection limit of the method was estimated at 0.42 ng/g and the measured limits of quantification were 2 ng/g for oxytetracycline.

RESULTS

Quantitative analysis. In order to study the prevalence of Tc^r LAB along the process line of FDS, samples of the raw meat ingredients (lard, pork, beef), the meat batter (before and after addition of the starter culture), the fermented sausage and the dry end product were analysed quantitatively. The quantitative analysis of the total number of LAB and the number of Tc^r LAB for FDS-01 and FDS-08 batch I is shown in Fig. 5.1. Analysis of the second batch (II) of FDS-08 showed comparable quantitative results as obtained with batch I (data not shown). All raw meat components (1A, 1B, 1C and 1D) of both FDS types contained a subpopulation of Tc^r LAB ranging between 1 and 3 log CFU per g of meat. In FDS-01, the prevalence of the Tc^r LAB in the pork sample was somewhat higher (1 log unit) compared to the beef sample. For both sausage types, samples of the meat batter (2B) showed an increase in the number of LAB (approximately 2 log units) compared to previous samples, which reflects the addition of the starter culture. The number of Tc^r LAB in samples of the meat batter after addition of the starter culture (2B) does not show a similar increase. The quantitative results of the samples after fermentation were remarkably different between the two sausage types. In case of FDS-01, no Tc^r LAB could be recovered from the samples after fermentation, whereas for FDS-08 a comparable increase in number of LAB and Tc^r LAB was found after fermentation (Fig. 5.1). The total number of LAB in both FDS types remained relatively constant after the drying process of four weeks. In comparison, the number of Tc^r LAB in FDS-08 decreased with one log unit throughout the drying process.

Fig. 5.1. (opposite page) Quantitative analysis of the total LAB population (white) and the Tc^r LAB population (black) along the process line of two fermented dry sausages (FDS-01 and FDS-08). 1A: (frozen) lard; 1B: frozen raw pork; 1C: fresh raw pork; 1D: fresh raw beef; 2A: meat batter; 2B: meat batter after addition of the starter culture and spices; 3A: the fermented sausage; 3B: the dry end product and 4: the sliced and packed end product. The plating was done in triplicate and values shown are the average of three counts ± standard deviation. NA: not analysed because no samples could be obtained from the plant.


FDS-01
CFU/g meat
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
Fermentation (4d)
drying (2w)
+ starter culture
NA
1A
1C
1D
2A
2B
3A
3B
4


FDS-08 batch I
CFU/g meat
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
Fermentation (4d)
drying (4w), slicing and packaging
+ starter culture
NA
NA
1A
1B
1C
2A
2B
3A
3B
4

Identification and typing of isolates. For identification purposes, only the two batches of FDS-08 were considered. A total of 220 Tc^r LAB isolates, *i.e.* 136 and 84 for batch I and II, respectively, was taxonomically characterized using a polyphasic approach including $(GTG)_5$-PCR fingerprinting, plasmid profiling, and protein profiling. Based on the combined profile of $(GTG)_5$-PCR fingerprinting and plasmid profiling, isolates that displayed an unique genotypic profile were selected from each sample for further research. In this way, the original set of 220 Tc^r LAB isolates was reduced to 85 unique strains originating from batch I (n=53) and batch II (n=32) (Table 5.1 and 5.2). The results obtained from both batches indicated a clear shift in species and strain diversity along the process line. A relatively high taxonomic diversity was observed in the strain set originating from raw meat components (samples 1A, 1B, 1C) which was dominated by *Lactococcus* spp. (> 60%), followed by strains of *Streptococcus parauberis*, *Enterococcus* sp., *Leuconostoc citreum*, *Pediococcus pentosaceus* and different *Lactobacillus* spp. In the samples collected after fermentation (samples 3 and 4), however, only *Lactobacillus* spp. were recovered. In the freshly fermented sausage (sample 3), *Lactobacillus plantarum* was the main species found.

Table 5.1. Diversity among the Tc^r LAB isolates (n = 53) along the process line of fermented dry sausage FDS-08 (batch I)

	Process line sample[b]					
Species[a]	**1A**	**1B**	**1C**	**2B**	**3A**	**4**
Lc. garvieae		8	7			
Lc. lactis subsp. *lactis*	6		2	1		
Lc. lactis subsp. *cremoris*	1	2				
S. parauberis			1			
Enterococcus sp.		1				
P. pentosaceus				1		
Lb. curvatus	1	4	1			2
Lb. reuteri			1			
Lb. plantarum			1		3	4
Lb. sakei subsp. *sakei*		1				1
Lb. sakei subsp. *carnosus*						1
Lb. paracasei						1
Lb. brevis-like			2			

[a] *Lc.*: *Lactococcus*; *S.*: *Streptococcus*; *P.*: *Pediococcus*; *Lb.*: *Lactobacillus*; *Leuc.*: *Leuconostoc*

[b] For each sample the number of isolates with a unique combined profile of $(GTG)_5$-PCR fingerprinting and plasmid profiling is shown. Numbers as in Fig. 5.1.

Table 5.2. Diversity among the Tc^r LAB isolates (n = 32) along the process line of fermented dry sausage FDS-08 (batch II)

	Process line sample[b]					
Species[a]	**1A**	**1B**	**1C**	**2B**	**3A**	**4**
Lc. garvieae	2		7	2		
Lc. lactis subsp. *lactis*			1			
Lc. lactis subsp. *cremoris*	1					
S. parauberis	1					
Leuc. citreum		1				
P. pentosaceus				2		
Lb. plantarum			1		7	
Lb. sakei subsp. *sakei*		1		1		
Lb. sakei subsp. *carnosus*						1
Lb. brevis -like			1		1	2

Abbreviations and numbers as in Table 5.1.

Molecular analysis of *tet* genes. Total genomic DNA preparations from 86 selected Tc^r LAB isolates were subjected to PCR screening for the presence of RPP genes with degenerated primers DI and DII, and with primers specific for *tet*(M), *tet*(O), and *tet*(S), and for the presence of efflux genes with primers specific for *tet*(K) and *tet*(L) (Table 5.3). For both batches, it was found that the majority of the isolates recovered from the raw meat components (sample 1A, 1B and 1C) contained a *tet*(S) gene, *i.e.* 64% and 68% in batch I and II, respectively. Furthermore, in approximately 30% of the isolates originating from the raw meat samples, a *tet*(M) gene was detected. In three lactococci recovered from batch I – sample 1A (n = 2) and from batch II – sample 2B (n = 1), both *tet*(M) and *tet*(S) were detected. None of the isolates were found to contain *tet*(K), *tet*(L), or *tet*(O), whereas one isolate from the raw meat components of batch I possessed an RPP gene other than *tet*(M), *tet*(O) or *tet*(S). In all isolates recovered from samples 3 and 4 obtained after fermentation only *tet*(M) was found.

The *tet*(M) genes of 11 isolates from batch I were localised on the genome and characterized with restriction enzyme analysis (REA) using *Acc*I and *Sca*I. REA of the *tet*(M) PCR product (74% of ORF) revealed two different *tet*(M) allele types that appeared mixed along the process line. Using Southern blotting of plasmid profiles and chromosomal *Eco*RI digested DNA and hybridisation with a *tet*(M) probe, 9 of the *tet*(M) genes were found to be located on plasmids.

Table 5.3. The *tet* gene diversity among the Tc^r LAB isolates along the process line of a fermented dry sausage

	Species	*tet* gene	Process line sample[a] 1	2B	3A	4
Batch I (n = 53)	Cocci	*tet* (M)	1			
		tet (S)	25	1		
		tet (M) and *tet* (S)	2			
		ND[b]		1		
	Lactobacilli	*tet* (M)	10		3	9
		tet (S)				
		RPP	1			
Batch II (n = 32)	Cocci	*tet* (M)	2	1		
		tet (S)	11	2		
		tet (M) and *tet* (S)		1		
	Lactobacilli	*tet* (M)	3	1	8	3
		tet (S)				

a/ Numbers as in Fig. 5.1. b/ ND: not defined gene other than RPP, *tet* (K), *tet* (L), RPP: determinants encoding a ribosomal protection protein

Tetracycline residues in meat samples. The three raw meat components (1A, 1B and 1C) of the two batches of FDS-08 were analysed for the presence of tetracycline residues (Table 5.4). In one fresh pork sample used in batch II, a residual concentration of 368.1 ng/g was detected. Based on EEC regulation N° 2377/90, this value clearly exceeds the imposed maximum residue limit (MRL) of 100 ng/g muscle tissue for tetracycline residues allowed in foodstuffs of animal origin. In addition, four other raw meat components contained trace amounts (< 100 ng/g) of tetracyclines. All residues could be identified as oxytetracycline. Because of the high value in a raw meat component of batch II, also the end product (sample 4) of this batch was analysed. In this sample, only a trace amount of oxytetracycline (11.5 ng/g) was found.

Table 5.4. Concentration of oxytetracycline residues in meat samples used for production of fermented dry sausage FDS-08

Batch	Process line sample[a] 1A	1B	1C	4
I	21.6	< 10	<10	NT
II	< DL	14.8	368.1	11.5

[a] Values expressed in ng/g; NT: not tested; DL: detection limit (0,42 ng/g); Numbers as in Fig. 5.1.

DISCUSSION

In the course of previous studies, we have demonstrated that several types of sliced modified atmosphere packed fermented dry sausage (FDS) end products contain a subpopulation of Tc^r lactobacilli carrying plasmid-located *tet*(M) genes (Gevers *et al.*, 2000, 2002; chapter 2 and 3). From these data, the question arose at what stage these Tc^r bacteria actually enter the production process. The results of the quantitative analyses in this study suggest that Tc^r LAB are introduced into the FDS process line along with the raw meat components. Although these data indicate that the raw meat may be a major source of antibiotic resistant bacteria in FDS fermentation, it is currently unclear what the origin of these bacteria is. Amongst other sources, it cannot be excluded that human handling and the production environment introduce Tc^r bacteria in the FDS fermentation process. Most likely Tc^r LAB originate from contamination with animal bacteria when considering that faecal contamination of raw meat during slaughtering cannot be completely avoided in spite of the fairly high hygienic standards in most developed countries. In addition, the fact that a significant part of the European livestock is being treated with or exposed to tetracyclines (FEDESA) is likely to exert a selective pressure stimulating the selection and dissemination of Tc^r bacteria in the animal gut flora (Aarestrup, 1999). De Wasch and co-workers (1998) reported that >5% of pork meat samples purchased from Belgian retail outlets contained residues of tetracyclines in the range of 50-1000 µg/kg. High concentrations of antibiotic residues could be of concern, because the (local) inhibition of the starter culture during fermentation might result in a (local) fermentation failure and growth of spoilage or pathogenic bacteria (Holley and Blaszyk, 1997). For the human consumer, any of the potentially hazardous effects due to presence of tetracycline residues in the final product as reported in the present study are probably negligible because of the very low concentrations. Tancrede and Bacaret (1989) titrated oxytetracycline in human volunteers and reported that a slight shift in antibiotic susceptibility of faecal anaerobes could be seen at 20 mg/d, but not at 2 mg/d. Theoretically, an amount of 2000 kg of the end product of FDS-08 batch II would be necessary to reach a dose of 20 mg.

Since most meat products are heat treated before consumption, no viable (resistant) bacteria are expected to be present in the final product. During manufacturing and ripening of FDS, however, no proper heat treatment is performed and antibiotic resistant bacteria originating from the raw meat may end up in the final ready-to-eat product (Teuber and Perreten,

2000). Until now, very few data are available on the survival of resistant bacteria during meat fermentation processes. In the current study, Tc^r LAB were recovered from post-fermentation samples of both batches of FDS-08 whereas the end product of FDS-01 was negative for the presence of Tc^r LAB although the raw meat used for fermentation of the latter type was contaminated with Tc^r LAB (Fig. 5.1). In a previous study (Gevers *et al.*, 2000; chapter 2), one out of two batches and four out of five batches of FDS-01 and FDS-08 were found to contain Tc^r LAB, respectively. These findings point to the fact that the prevalence of Tc^r LAB in batches from different production periods of a given FDS type can be variable. The differences between batches in the composition of the Tc^r microflora on the raw meat and, as a consequence, its variable ability to compete with the starter culture can be put forward as possible explanations for this variability.

The composition of the Tc^r LAB microflora isolated from the raw meat components used for production of the FDS-08 type appeared to be predominated mainly by lactococci and lactobacilli (Table 5.1-2). In contrast, only Tc^r lactobacilli could be recovered from the end products. From all LAB species recovered in this study, the species *Lb. reuteri*, *Lb. plantarum*, *Lb. brevis* and *Lc. garvieae* have been found in the porcine gut before (Leser *et al.*, 2002). The relative dominance of lactobacilli in post-fermentation samples may be explained by the fact that lactobacilli and especially those species that are also used as meat starters are better adapted to the physico-chemical conditions created after fermentation, *i.e.* an increased lactic acid concentration, a lowered pH and water activity, and the possible presence of bacteriocins (Hammes *et al.*, 1990). During the drying of the fermented sausage, the water activity drops further which may cause shifts in the composition of the natural microflora.

As a result of pronounced survival of lactobacilli towards the end of fermentation, only *tet*(M) genes were found at this stage of the process line. In contrast, *tet*(S) genes appeared to be confined to LAB cocci in the raw meat components (Table 5.3),but were no longer found in the end product, which is congruent with the fact that lactococci, pediococci, enterococci or streptococci could not be recovered in post-fermentation samples. In a previous study on end products of nine different FDS types (Gevers *et al.*, 2002), only *tet*(M) was detected in Tc^r lactobacilli. The majority of these *tet*(M) genes was located on 10 kb plasmids and displayed very high genotypic similarities with *tet*(M) genes previously reported in *Neisseria meningitidis* and *Staphylococcus aureus* MRSA 101. Based on restriction enzyme analysis of the *tet*(M) genes reported in this study, similar allele types are found in isolates along the process line compared to isolates from the end products. Likewise, Teuber

and co-workers (1999) reported only *tet*(M) in Tc^r enterococci from fermented sausage end products. Recently, a culture-independent survey of the pig intestinal contents and swine feed demonstrated the presence of various RPP *tet* genes including *tet*(M), *tet*(O), *tet*(Q), *tet*(S) and *tet*(W) (Aminov *et al.*, 2001). Compared to the current insights on the taxonomic distribution of *tet* genes (Chopra and Roberts, 2001), this study revealed new host organisms for *tet*(M) and *tet*(S). To our knowledge, *tet*(M) genes have not been reported in lactococci, and neither has *tet*(S) been detected in *Leuconostoc* and *Pediococcus*.

From the quantitative data shown in Fig. 5.1, it was clear that the addition of the meat starter culture was not linked to an increase in Tc^r LAB numbers. Together with the fact that a panel of commercially available European meat starter cultures were all found to be susceptible to tetracyclines (D. Gevers, unpublished data), it is very likely that the starters should not be regarded as sources of Tc^r bacteria in FDS. Previously, other researchers have reported on the absence of antibiotic resistances in meat starter cultures (Holley and Blaszyk, 1997; Raccach *et al.*, 1985). However, to our knowledge no regulations or guidelines have been officially accepted on the presence of transferable resistance genes in starter cultures for human food production. To some extent this is due to the lack of conformity in methodologies and breakpoint values for susceptibility testing of non-pathogenic LAB. Recently, antimicrobial breakpoints were proposed for resistance screening of different *Lactobacillus* spp. (Danielsen and Wind, 2002).

In conclusion, the present study has shown that fermented dry sausages are among those food products that can serve as vehicles for Tc^r LAB and that the raw meat already contains a subpopulation of these bacteria. Furthermore, it is clear that the prevalence and diversity of Tc^r LAB along the process line changes significantly towards a dominance of *tet*(M)-carrying lactobacilli although previously also *tet*(M) containing enterococci have been reported in FDS end products (Teuber *et al.*, 1999). Whether these results reflect the transfer of either resistant bacteria or of bacterial resistance genes from animals to humans via the food chain is difficult to ascertain and definitely requires more research. In this regard, the combined efforts of conventional cultivation and identification techniques and of cultivation-independent methods such as DGGE may be the optimal route to follow (Aminov *et al.*, 2001).

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6

CONCLUSIONS AND PERSPECTIVES

In conclusion, this work has demonstrated that (i) acquired antibiotic resistance genes can be present in lactobacilli associated with ready-to-eat fermented dry sausage (FDS) end products, (ii) these resistance genes are highly similar to genes found in pathogenic species, (iii) most of them are plasmid located and some of them are transferable by conjugation, and (iv) similar genes and host organisms can be found along the process line of FDS from the end product to the raw meat components. Taken together, this study is an elaborated example of the role that the normal bacterial flora may play in the maintenance and spread of antibiotic resistance via the food chain, a topic that has long been largely underestimated but which is nowadays growing in interest of food microbiologists. According to Teuber (1999), the resistance problem in human medicine will not be solved if there is a constant influx of resistance genes into the human microflora via the food chain. With the established genetic mechanisms for exchange of DNA between bacteria, the normal flora is capable of supplying drug resistance genes to their pathogenic counterpart. Therefore, an important preventive action against antibiotic resistance in bacteria causing infections is to keep the level of antibiotic resistant bacteria in the normal flora at a low level.

Although this study has identified a potential hazard with the finding that ready-to-eat food products contain transferable antibiotic resistance genes, the magnitude of the risk is yet to be established. The risk represents the theoretical frequency and severity of an adverse effect due to the hazard. In order to be able to perform a risk analysis, not only more data but also more knowledge on acquired antibiotic resistance in non-pathogenic bacteria is required, mainly on two topics: (1) from a safety point of view, it is crucial to be able to differentiate between intrinsic and acquired resistance and (2) the *in vitro* and *in vivo* transferability of the acquired resistance genes to pathogenic bacteria needs an in-depth analysis to be able to model a dose-response reaction. In this regard, the set of isolates obtained in this study can be subjected to further characterization of the R-plasmids and to *in vivo* transfer experiments. Risk analysis within the field of food safety is a strongly evolving activity and is mainly applied for pathogens-food combinations, but in the case of antibiotic resistance as a hazard, a risk analysis is still lacking. Up to now, decisions by the authorities in regard to antibiotic resistance are made on the basis of the 'precautionary principle' to protect public health, e.g. the ban of use of most antibiotics as growth promoters by the European Commission.

Our findings points out that in fermented meat products, next to enterococci (Teuber *et al.*, 1999), also lactobacilli can be a host for acquired resistance genes and may spread their resistances through bacterial populations. However, the basic scientific knowledge of the mechanisms of antibiotic resistance in LAB and its transmissibility still remain very limited. Because LAB are extensively used for food and feed, including starter cultures for fermentation, probiotic cultures as food and feed ingredients, and protective cultures to inhibit specific spoilage organisms, this knowledge is becoming increasingly important with regard to food safety issues. LAB added in traditional foods have a 'long history of safe use' and are considered as GRAS (Generally Regarded As Safe) organisms. But the use of a newly developed LAB in a food lacks this history of safe use, which leads to the need for the evaluation of its safety prior to its market approval. In order to gain an authorization for a microorganism as a feed additive, the following safety issues must be addressed: genetic stability, toxins and virulence factors, antibiotic production and antibiotic resistance, tolerance in target species, effect of the microflora in the digestive tract, genotoxicity, oral toxicity, worker safety and environmental risk assessment. In a recent report by the Scientific Committee on Animal Nutrition (SCAN, 2002), criteria for assessing the safety of microorganisms resistant to antibiotics are given. If a high level of phenotypical expressed antibiotic resistance is found, they request that transferability is examined and the genetic basis of the resistance (intrinsic or acquired) is determined. The guidelines for feed additives are far more stringent than those currently applied to live microorganisms used in foods and consumed directly by humans. Therefore, SCAN urges the European Commission to adopt a consistent approach to all microbial products entering the food chain. The current large attention on food safety and future legislative perspectives insist on more basic scientific knowledge on antibiotic resistance in LAB and its transferability, in order to be able to perform reliable antibiotic susceptibility tests.

Realizing their practical significance in fermentation, bioprocessing, agriculture, food, and more recently medicine, the LAB have been the subject of considerable research and commercial development over the past decade. Contributing to this increased interest have been the recent efforts to determine the genome sequences of a representative collection of LAB species. Currently, one LAB genome is completely sequenced, annotated and publicly available, and 27 projects are ongoing of which up to 15 LAB genomes are expected to be available in the public domain by the end of 2003 (Klaenhammer *et al.*, 2002). Comparative and functional genomic approaches of multiple LAB species will provide a better understanding of core functions such as production of lactic acid, proteolytic and peptidase

activities, survival at low pH, stress tolerance, production of antimicrobials, transport systems, and cell signalling, which will create the ability to improve LAB strains used in the industry. Genomics are also a tool to detect unwanted genes, e.g. antibiotic resistance genes, potential virulence genes, and metabolic pathways that could lead to hazardous or undesirable metabolites. The whole genome sequence enables to screen for such unwanted genes and reveal whether or not they are located on potential mobile genetic elements, but provides also the data to design DNA hybridisation assays (DNA-microarray). DNA-microarrays, in their turn, are useful tools to perform a high-throughput sequence comparison of a large set of strains to document the prevalence of unwanted genes in the environment. In addition, DNA-microarrays can be used for transcriptome analysis to create a view on the abundance of all mRNA in a cell and document the expression of (unwanted) genes under different conditions.

Antibiotic resistance in food-associated bacteria reflects the resistance situation in bacteria from all the various environments from where food for human consumption originates. Efforts towards keeping antibiotics effective for medical treatment of infections for the coming years should penetrate to all parts of this 'food web'. The debate about which part of the food web has the greater impact on the development of antibiotic resistance in the human flora is still unresolved. One important fact that cannot be ignored is that a large number of studies point to an ever increasing level of antibiotic resistance in food-associated bacteria. The obvious way to act against this emergence of antibiotic resistance is to think globally and act locally.

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SUMMARY - SAMENVATTING

Summary

In this study, the possible role of commensal microorganisms associated with ready-to-eat meat products, in the spread of antibiotic resistance determinants was investigated. For this purpose, we have chosen to focus on tetracycline resistant (Tc^r) lactic acid bacteria (LAB) in modified atmosphere packed pre-sliced fermented dry sausage, cooked chicken breast meat and cooked ham. A first screening of these three types of meat products by tetracycline breakpoint experiments clearly indicated that some types of fermented dry sausage contained a high-level Tc^r LAB population. Cooked ham and cooked chicken breast meat samples, on the other hand, were not found to contain a Tc^r LAB subpopulation, although high densities of susceptible LAB (5–8 log CFU/g of meat) were found. A possible explanation for the lack of high-level resistant LAB in the latter two meat types might be related to a fundamental difference in the manufacturing of these products, namely whether or not a heat treatment step is applied during the production process. During their production, fermented sausages are not heat treated before consumption, and the microflora of the end product might, at least partially, originate from the raw meat components. In contrast, most viable bacteria naturally present on raw ham and chicken breast meat are eliminated by a heat treatment, leaving the main source of bacteria on the cooked end products with the environmental microflora re-contaminating the products after cooking, during slicing and packaging. By packaging under modified atmosphere, the aerobic spoilage organisms are significantly suppressed by the presence of CO_2 which results in an autochthonous microflora that is largely dominated by LAB. The presence of LAB improves the microbiological safety of these cooked meat products by inhibiting the spoilage and pathogenic microorganisms, and our findings suggest that these organisms do not contain any high-level Tc^r subpopulations. For this reason, subsequent research was focussed on fermented dry sausage (FDS). Based on the results of the breakpoint experiments (agar dilution), a concentration of 64 µg/ml of tetracycline was chosen as the concentration to prepare a selective medium for the isolation of Tc^r LAB from FDS, *i.e.* the breakpoint concentration.

Using the newly defined selective isolation medium, a total of 26 samples, *i.e.* different batches of 13 different types of FDS, was analysed for the presence of Tc^r LAB. The total number of LAB in the examined sausages counted on non-selective MRS ranged between 6 and 9 log CFU/g of meat, which are typical densities found in FDS. Fourteen samples (54%) contained Tc^r LAB in different concentrations ranging between 1.7 and 5.1 log CFU/g of meat. Our data indicate that the presence of Tc^r LAB in a given type of FDS is subject to variation. From the 10 FDS types of which more than one batch was sampled, three were always negative, two were always positive and five were variable for the presence of Tc^r LAB. In order to explain this variation, an analysis encompassing the complete process line of a FDS was performed at a later stage of this study.

Out of the fourteen positive samples, a total of 94 Tc^r LAB was randomly isolated and stored for further research. These isolates were all identified as members of the genus *Lactobacillus*, including *Lb. sakei* subsp. *carnosus* (49%), *Lb. plantarum* (33%), *Lb. curvatus* (8%), *Lb. sakei* subsp. *sakei* (5%), and *Lb. alimentarius* (5%). All these species have been associated with fermented meat and, except for *Lb. alimentarius*, these species are particularly well adopted to conditions created in FDS and are therefore frequently used in meat starter cultures. At the start of the project, the most obvious technique for identification seemed protein profiling, because the Laboratory of Microbiology has set up an up-to-date and extended database of digitised and normalized protein profiles of all known (sub)species of LAB and the technique has been shown to give reliable identifications at the (sub)species level in most cases. But the identification of a first subset of isolates showed that the discriminatory power of this technique was insufficient within the framework of this study. Isolates originating from the same sausage and belonging to the same species displayed highly similar, if not identical, protein profiles, and no information was obtained on the intra-species diversity. Therefore a technique with a higher taxonomical resolution was chosen. From our data, it was concluded that the rep-PCR fingerprinting technique using the $(GTG)_5$ primer is a rapid, easy-to-perform, and reproducible tool for differentiation of a wide range of food-associated lactobacilli at the (sub)species and potentially up to the strain level, with a single protocol. So far, rep-PCR fingerprinting was only limited used for LAB and no reports on the use of the $(GTG)_5$ primer were found. Overall, $(GTG)_5$-PCR banding patterns displayed a much higher heterogeneity among isolates, compared to the corresponding protein profiles. In this way, $(GTG)_5$-PCR analysis revealed that among the Tc^r LAB population of a particular FDS sample not only different species occur, but that a given species can be represented by different strains. Different batches of one FDS type

were not found to contain identical DNA fingerprints, indicating that the source of Tc^r lactobacilli is variable. The diversity within a batch and between different batches suggests that the starter culture is not likely to be a source of Tc^r LAB. On the other hand, isolates with highly similar or even identical $(GTG)_5$-PCR fingerprints were frequently found within the set of isolates recovered from the same sample of FDS. To avoid the selection of multiple isogenic strains, one strain of each of the 24 unique $(GTG)_5$-PCR fingerprint types was selected.

In this work it was shown that in the 24 selected Tc^r *Lactobacillus* isolates from nine different FDS types, representing five different species, only *tet*(M) was detected. These *tet*(M) genes could be localised mainly on plasmids, except in four strains which had a chromosomal resistance. Most of these R-plasmids ranged in size of approximately 10 kb, and in three cases the R-plasmid was larger than 25 kb. Using PCR detection, no transposons of the Tn*916*/Tn*1545*-family were found in either one of the isolates. One isolate (DG 507) contained a second R-plasmid with an *erm*(B) gene. Further characterization of the *tet*(M) genes by REA and sequencing revealed high sequence homology with the previously reported *tet*(M) gene of either *Neisseria meningitidis* or *Staphylococcus aureus* MRSA 101, and significant differences with *tet*(M) genes found in the closest related species *Enterococcus faecalis* and *Streptococcus pneumoniae*.

Although plasmids are very common in lactobacilli, and even plasmid located antibiotic resistance determinants have been reported in lactobacilli, almost no literature is available on the conjugal transfer of native lactobacilli plasmids. We found that seven out of 24 Tc^r *Lactobacillus* isolates could transfer their *tet*(M) gene to *Enterococcus faecalis* at frequencies ranging between 10^{-4} and 10^{-5} transconjugants per recipient. Further, two of them were able to transfer their resistance also to *Lactococcus lactis* subsp. *lactis*. In a few transconjugants spontaneous co-transfer of native plasmids other than the plasmid selected for, *i.e.* the R-plasmid encoding the tetracycline resistance, was found. This resulted for example in a spontaneous co-transfer of the erythromycin resistance determinant from DG 507 into *E. faecalis*.

In order to better understand the source of the Tc^r LAB subpopulation in FDS end products, the prevalence and diversity of Tc^r LAB and their *tet* genes along the process line of two different FDS types, from the raw meat components to the end product, were determined in a culture-dependent approach. Based on the findings of this study, it was concluded that Tc^r LAB enter the FDS process line, at least partially, via the raw meat components and that the starter culture is not a source of the Tc^r determinants. Subpopulations of

Tc^r LAB are most likely to originate from contamination of the carcass with animal faecal bacteria during slaughtering, even when fairly high hygienic standards are applied. The fact that a significant part of the European livestock is being treated with or exposed to tetracyclines (FEDESA), is likely to exert a selective pressure stimulating the selection and dissemination of Tc^r bacteria in animal gut flora. According to data of the European federation of animal health (FEDESA), in 1997 an amount of 1,646 tons of tetracycline was used in Europe. The frequent use is also reflected in the finding that at least three out of six tested raw meat components contained trace amounts of oxytetracycline, suggesting a history of treatment with this agent. Amongst other sources, it cannot be excluded that human handling and the environment introduce Tc^r bacteria into the process line. The composition of the Tc^r LAB subpopulation as well as the diversity of the *tet* genes are changing along the process line: the raw meat components are predominated mainly by lactococci (containing *tet*(S) or *tet*(M)) and to a lesser extent by lactobacilli (*tet*(M)), whereas from the samples after fermentation only Tc^r lactobacilli containing *tet*(M) could be recovered.

Taking into account the data on the Tc^r LAB prevalence and diversity along the process line of two different FDS types and in the 26 samples of FDS end products, the variable presence of Tc^r LAB in end products between batches from different production periods of a given FDS type, can be explained as follows. The contaminating microflora of the raw meat batter used to prepare fermented sausages will be variable between different production batches as a consequence of its variable composition. Consequently, also the Tc^r LAB subpopulation will be variable in composition as well as its ability to compete with the starter culture, that is added in high densities (6 log CFU/g of meat) before fermentation. The presence of Tc^r LAB in the end product is determined by factors such as the density of the Tc^r subpopulation before fermentation, the competitiveness with the starter culture, and the adaptability to the fermented meat conditions, *i.e.* an increased lactic acid concentration and lowered pH and a_w value, and the possible presence of bacteriocins. The competition with the starter culture and the adaptability of the Tc^r LAB subpopulation to the conditions created after fermentation is also an explanation for the shift towards lactobacilli after fermentation. Lactobacilli and especially *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum* are better adapted to these conditions.

Samenvatting

Het ontstaan en de snelle verspreiding van antibiotica-resistenties binnen en tussen verschillende populaties van onze samenleving behoort ongetwijfeld tot de meest actuele problemen in de publieke gezondheidssector. Hospitalen zijn daarbij ongetwijfeld een belangrijke kern van ontwikkeling en verspreiding van antibiotica resistentie. Daarnaast echter, draagt het (overmatig) gebruik van antibiotica in de veeteelt hieraan bij, en wordt er gesuggereerd dat levensmiddelen van dierlijke oorsprong, en dan vnl. vleesproducten, een belangrijk vector vertegenwoordigen voor de verspreiding van resistente bacteriën en/of hun resistentiegenen tussen de dierlijke en menselijke populatie. Onze huidige kennis beperkt zich hoofdzakelijk tot de aanwezigheid van antibiotica-resistenties in voedselpathogene bacteriën zoals *Salmonella*, *Campylobacter*, *Listeria*, *Staphylococcus* en *Clostridium*. Weinig of geen informatie is echter beschikbaar over de mogelijke rol van de niet-pathogene 'commensale' microflora in en op levensmiddelen als reservoir organismen voor resistentiegenen. Het potentieel gevaar schuilt in de mogelijkheid van directe of indirecte transfer van antibiotica resistentiegenen naar pathogene bacteriën. In dit proefschrift wordt het onderzoek naar de aanwezigheid van niet-pathogene antibiotica-resistente bacteriën op de menselijke voeding en de capaciteit om hun resistenties te verspreiden, gepresenteerd. Er werd gefocusseerd op tetracycline-resistente (Tc^r) melkzuurbacteriën (MZB) in gemodificeerde atmosfeer verpakte fijne vleeswaren zoals gefermenteerde droge worst, gekookte kippewit en gekookte ham.

Een screening van deze drie verschillende vleeswaren door middel van tetracycline breekpuntexperimenten, zoals die beschreven staat in hoofdstuk 2, toonde aan dat sommige stalen van gefermenteerde droge worst een Tc^r MZB subpopulatie bevatten. De gekookte vleeswaren daarentegen, vertoonde geen aanwezigheid van een Tc^r subpopulatie, hoewel hoge aantallen van MZB (5-8 log KVE/g vlees) gevonden werden. Een mogelijke verklaring hiervoor kan te vinden zijn in de fundamentele verschillen in de bereidingswijze van deze vleeswaren, nl. het al dan niet toepassen van een hittebehandeling in het productieproces.

Gefermenteerde droge worst wordt op geen enkel moment verhit, en de microflora van het eindproduct kan, op zijn minst gedeeltelijk, afkomstig zijn van de rauwe grondstoffen. De meeste micro-organismen op rauwe ham en kipvlees worden daarentegen geëlimineerd door de kookstap. Bijgevolg is de belangrijkste bron van bacteriën op het gekookte eindproduct toe te wijzen aan postcontaminatie van het product tijdens het versnijden en verpakken. Omwille van deze resultaten, werd in het hierop volgende onderzoek gefocusseerd op gefermenteerde droge worst (GDW). Gebaseerd op de resultaten van de breekpuntexperimenten werd een concentratie van 64 µg/ml tetracycline gekozen als de breekpuntconcentratie voor het aanmaken van een selectief medium voor de isolatie van Tc^r MZB uit GDW.

Gebruik makend van dit selectieve isolatiemedium, werd een totaal van 26 stalen van 13 verschillende GDW types geanalyseerd op de aanwezigheid van Tc^r MZB. Het totaal aantal KVE geteld op een niet selectief medium varieerde tussen 6 en 9 log KVE/g vlees, wat normale aantallen zijn voor GDW. Veertien stalen (54%) bevatten een Tc^r MZB subpopulatie in verschillende concentraties variërend tussen 1,7 en 5,1 log KVE/g vlees. Onze data suggereren dat de aanwezigheid van een Tc^r subpopulatie in een bepaald type GDW onderhevig is aan variatie. Van de tien GDW types waarvan meer dan één batch werd onderzocht, waren er drie steeds negatief, twee steeds positief en vijf types waren variabel voor de aanwezigheid van Tc^r MZB. Om deze variatie te kunnen verklaren, dringt een analyse van een volledige GDW proceslijn zich op.

Uit de veertien positieve stalen, werd een totaal van 94 Tc^r MZB geïsoleerd en bewaard voor verder onderzoek. Deze isolaten werden geïdentificeerd en behoren allen tot het genus *Lactobacillus*: *Lb. sakei* subsp. *sakei* (49%), *Lb. plantarum* (33%), *Lb. curvatus* (8%), *Lb. sakei* subsp. *sakei* (5%) en *Lb. alimentarius* (5%). Bij de aanvang van dit project leek eiwitprofilering de meest voor de hand liggende identificatie techniek, en dit omdat reeds werd aangetoond dat deze techniek in staat is om in de meeste gevallen een betrouwbare (sub)species identificatie te bekomen. Bovendien beschikt het Laboratorium voor Microbiologie over een up-to-date en uitgebreide databank van gedigitaliseerde en genormaliseerde eiwitprofielen van alle gekende (sub)species behorend tot de MZB. De identificatie van een eerste subset van isolaten toonde echter aan dat het discriminerend vermogen van deze techniek onvoldoende was in het kader van deze studie. Isolaten afkomstig uit hetzelfde staal en behorend tot hetzelfde species vertoonde namelijk zeer gelijke of zelfs identieke eiwitprofielen, zodat geen informatie omtrent de intraspecies diversiteit kon worden bekomen. Daartoe werd een techniek met een hogere taxonomische resolutie gekozen. Uit

de resultaten zoals beschreven in hoofdstuk 3, kon worden besloten dat rep-PCR fingerprinting met de $(GTG)_5$-primer een snelle, eenvoudige en reproduceerbare methode is ter onderscheiding van een breed spectrum van voedselgeassocieerde lactobacilli op het (sub)species en intraspecies niveau, en dit met één enkel protocol. In het algemeen vertoonde $(GTG)_5$-PCR patronen een grotere heterogeniteit bij de isolaten in vergelijking met de overeenkomstige eiwitprofielen. Zo kon worden aangetoond dat een Tc^r MZB subpopulatie niet enkel meerdere species kan bevatten, maar ook dat een bepaald species kan worden vertegenwoordigd door verschillende stammen. Hiertegenover staat wel dat isolaten met identieke $(GTG)_5$-PCR profielen frequent werden gevonden binnen een staal. Voor verder onderzoek werd daarom per staal een set van representatieve stammen geselecteerd, met één stam voor elke $(GTG)_5$-PCR fingerprint type, resulterend in een set van 24 Tc^r MZB stammen.

In hoofdstuk 4 word beschreven dat in de 24 geselecteerde Tc^r *Lactobacillus* isolaten afkomstig uit 14 verschillende stalen en behorend tot vijf verschillende species, enkel *tet*(M) werd gevonden. Het merendeel van deze *tet*(M) genen kon worden gelokaliseerd op plasmiden, behalve bij vier stammen die een chromosomaal *tet*(M) gen hebben. De meeste van deze R-plasmiden hebben een grootte van ongeveer 10 kb, en drie stammen hebben een R-plasmide van met een grootte van meer dan 25 kb. Door middel van PCR detectie werd aangetoond dat deze *tet*(M) genen niet op een transposon van de Tn*916*/Tn*1545*-familie gelegen zijn, hoewel dit gen hiermee vaak wordt geassocieerd. Eén isolaat (DG 507) bevat naast het *tet*(M) gen, ook een plasmide gelokaliseerd *erm*(B) gen. Door een verdere karakterisering van de *tet*(M) genen met restrictie-enzym analyse (REA) en DNA sequenering kon een hoge homologie worden aangetoond met de *tet*(M) genen voorheen gevonden in *Neisseria meningitidis*, of *Staphylococcus aureus* MRSA 101, en werden significante verschillen gevonden met de *tet*(M) genen gevonden in de meest nauw verwante species *Enterococcus faecalis* en *Streptococcus pneumoniae*.

Hoewel plasmiden frequent voorkomen in lactobacilli, en zelfs plasmide gelokaliseerde antibiotica-resistentie determinanten werden gerapporteerd, is de literatuur omtrent de conjugatieve transfer van natuurlijke plasmiden bij lactobacilli beperkt. Wij vonden dat zeven van de 24 Tc^r *Lactobacillus* isolaten in staat zijn om hun plasmide gelokaliseerd *tet*(M) gen te transfereren naar *Enterococcus faecalis*. Verder, waren twee van deze zeven isolaten eveneens in staat om hun resistentie te transfereren naar *Lactococcus lactis* subsp. *lactis*. In een aantal transconjuganten werd spontane co-transfer van andere natuurlijke plasmide, dan diegene waarvoor werd geselecteerd (d.i. R-plasmide coderend voor de Tc^r),

vastgesteld. Dit resulteerde bijvoorbeeld in een spontane co-transfer van de erythromycine-resistentie determinant vanuit *Lb. plantarum* DG 507 in *E. faecalis*.

Met het oog op een beter inzicht in de bron van de Tc^r MZB subpopulatie in GDW eindproducten, werd de mate van voorkomen en diversiteit van Tc^r MZB en hun *tet* genen langsheen het productieproces van twee verschillende GDW types, van het rauwe vlees tot aan het eindproduct, bepaald door middel van een cultuur-afhankelijke benadering. In hoofdstuk 5 werd geconcludeerd dat Tc^r MZB op zijn minst gedeeltelijk via het rauwe vlees worden geïntroduceerd in het productieproces van GDW en dat de startercultuur niet de bron is van de Tc^r determinanten. Subpopulaties van Tc^r MZB zijn hoogst waarschijnlijk afkomstig van karkassen gecontamineerd met dierlijke fecale bacteriën tijdens het slachten wat zelfs onder zeer strenge hygiënische condities niet te vermijden is. Het voorkomen van Tc^r bacteriën in de dierlijke fecale flora is eveneens zeer waarschijnlijk, aangezien dat tetracyclines de meest frequent gebruikte therapeutische antibiotica in de veeteelt zijn. Volgens gegevens van de Europese Federatie voor Dierenwelzijn (FEDESA), werd in het jaar 1997 een hoeveelheid van 1.646 ton tetracycline gebruikt in Europa, d.i. 66% van de totale antibiotica consumptie in dat jaar. Dit frequent gebruik wordt nog benadrukt door onze bevinding dat minstens drie van de zes stalen van het rauwe vlees sporen van oxytetracycline bevatten, wat een behandeling met dit antibioticum doet vermoeden. Het kan echter niet worden uitgesloten dat menselijke handelingen en productie-omgeving Tc^r bacteriën introduceren in de proceslijn. De samenstelling van de Tc^r MZB subpopulatie en de diversiteit van de *tet* genen wijzigde tijdens het productieproces: de stalen van het rauwe vlees bevatten in hoofdzaak lactococci (met *tet*(S) en *tet*(M) genen) en in mindere mate lactobacilli (met *tet*(M)), waar de stalen na fermentatie enkel Tc^r lactobacilli (met *tet*(M)) bevatten.

Indien alle resultaten bekomen in onze studie wordt samengenomen, kan de variatie in de aanwezigheid van Tc^r MZB tussen verschillende batches van GDW eindproducten als volgt verklaard worden. De contaminerende microflora van het gemalen vlees dat wordt gebruikt om darmen af te vullen is verschillend tussen verschillende batches als gevolg van de variabele samenstelling van dit gemalen vlees. Bijgevolg zal de samenstelling van de eventueel aanwezige Tc^r MZB subpopulatie verschillen, en met de samenstelling ook het competitief vermogen van de subpopulatie ten opzichte van de startercultuur, die in overmaat wordt toegevoegd (6 log KVE/g vlees). De aanwezigheid van Tc^r MZB in het eindproduct wordt bepaald door factoren zoals de densiteit van de Tc^r subpopulatie voor fermentatie, het competitief vermogen t.o.v. de startercultuur, de leefbaarheid onder de condities van GDW,

zijnde een verhoogde melkzuur concentratie, een verlaagde pH en a_w waarde, en de mogelijke aanwezigheid van bacteriocines. Het competitief vermogen en de leefbaarheid in GDW condities zijn tevens een verklaring voor de verschuiving naar een dominerende *Lactobacillus* flora na fermentatie. Lactobacilli, en *Lb. sakei*, *Lb. curvatus* en *Lb. plantarum* in het bijzonder, zijn goed aangepast aan deze condities.

Er kan geconcludeerd worden dat dit werk heeft aangetoond dat (i) verworven antibiotica-resistentie genen aanwezig kunnen zijn in lactobacilli geassocieerd met GDW eindproducten, (ii) dat deze resistentie genen zeer hoge sequentiegelijkenissen vertonen met genen van pathogene species, (iii) dat deze resistentie genen voornamelijk op plasmide zijn gelegen, waarvan een aantal kon worden getransfereerd via conjugatie, en (iv) dat gelijke genen en gastheer organismen kunnen worden teruggevonden langsheen het productieproces van GDW. Bijgevolg is dit een gedetailleerde uitwerking van de mogelijke rol die de niet-pathogene bacteriële flora kan hebben in het behoud en de verspreiding van antibiotica-resistentie via de voedselketen.

APPENDIX

TABLES OF ISOLATES/STRAINS RECOVERED/USED IN THIS STUDY

Table A.1. Selection of Tc^r LAB isolates from fermented dry sausage end products (n = 24) (Chapter 2, 3 and 4)

Strain number [a]	Other number [b]	Source [c]	Taxon	Antibiotic resistance profile
DG 013	LMG 21677	FDS-01A	*Lb. plantarum*	Tc^r, *tet* (M)-*1* on transferable R-plasmid
DG 048	LMG 21678	FDS-08A	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*1*
DG 142	LMG 21679	FDS-07A	*Lb. curvatus*	Tc^r, chromosomal *tet* (M)-*2*
DG 143	R-12148	FDS-07A	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*1* , and Rif^r
DG 165	LMG 21680	FDS-11A	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 483	R-12482	FDS-09B	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 484	LMG 21681	FDS-09B	*Lb. curvatus*	Tc^r, chromosomal *tet* (M)-*2*
DG 485	R-12484	FDS-09B	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 488	R-12487	FDS-11B	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 489	R-12488	FDS-11B	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 493	LMG 21682	FDS-07B	*Lb. sakei* subsp. *sakei*	Tc^r, *tet* (M)-*1* on transferable R-plasmid, and Rif^r
DG 498	R-12497	FDS-12B	*Lb. alimentarius*	Tc^r, *tet* (M)-*2* on transferable R-plasmid
DG 499	R-12498	FDS-12B	*Lb. alimentarius*	Tc^r, plasmid located *tet* (M)-*2*
DG 500	LMG 21683	FDS-12B	*Lb. alimentarius*	Tc^r, *tet* (M)-*2* on transferable R-plasmid
DG 507	LMG 21684	FDS-02B	*Lb. plantarum*	Tc^r, *tet* (M)-*1* and *erm* (B) on 2 different transferable R-plasmid
DG 509	LMG 21685	FDS-08C	*Lb. plantarum*	Tc^r, plasmid located *tet* (M)-*1* , and Pen^r
DG 512	R-12511	FDS-08C	*Lb. plantarum*	Tc^r, plasmid located *tet* (M)-*1*
DG 515	LMG 21686	FDS-06	*Lb. plantarum*	Tc^r, *tet* (M)-*2* on transferable R-plasmid
DG 516	R-12515	FDS-06	*Lb. sakei* subsp. *carnosus*	Tc^r, plasmid located *tet* (M)-*2*
DG 520	R-15519	FDS-08D	*Lb. plantarum*	Tc^r, plasmid located *tet* (M)-*1*
DG 522	LMG 21687	FDS-08D	*Lb. plantarum*	Tc^r, *tet* (M)-*1* on transferable R-plasmid
DG 524	LMG 21688	FDS-14	*Lb. curvatus*	Tc^r, chromosomal *tet* (M)-*2*
DG 525	R-12886	FDS-14	*Lb. sakei* subsp. *sakei*	Tc^r, chromosomal *tet* (M)-*1* , and Rif^r
DG 533	R-12894	FDS-08E	*Lb. plantarum*	Tc^r, plasmid located *tet* (M)-*1*

a/ DG numbers are the original numbers that were assigned to the isolates; b/ All isolates were included in the research database of the Laboratory of Microbiology and received a R-number, whereas a selection was deposited in the BCCM^{TM}/LMG Bacteria Collection and received a LMG number; c/ FDS: fermented dry sausage, the numbers correspond to a type and the letter to a batch

Table A.2. Selection of Tc^r LAB isolates from FDS-08 process line batch I (n = 53) (Chapter 5)

Strain number	Source [a]	Taxon	*tet* genes
DG 830	3A	*Lb. plantarum*	Plasmid located *tet* (M)-*1*
DG 842	3A	*Lb. plantarum*	*tet* (M)
DG 850	3A	*Lb. plantarum*	Plasmid located *tet* (M)-*1*
DG 862	1C	*Lb. curvatus*	*tet* (M)-*1*
DG 864	1C	*Lb. reuteri*	RPP
DG 866	1C	*St. parauberis*	*tet* (S)
DG 867	1C	*Lc. garvieae*	*tet* (S)
DG 869	1C	*Lc. garvieae*	*tet* (S)
DG 870	1C	*Lc. garvieae*	*tet* (S)
DG 872	1C	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 873	1C	*Lc. garvieae*	*tet* (S)
DG 876	1C	*Lc. garvieae*	*tet* (S)
DG 878	1C	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 881	1C	*Lc. garvieae*	*tet* (S)
DG 883	1C	*Lc. garvieae*	*tet* (S)
DG 884	1C	*Lb. plantarum*	*tet* (M)
DG 887	1C	*Lb. brevis* -like	Plasmid located *tet* (M)-*1*
DG 888	1C	*Lb. brevis* -like	*tet* (M)
DG 893	1A	*Lc. lactis* subsp. *lactis*	*tet* (M)-*1* & *tet* (S)
DG 906	1A	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 909	1A	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 910	1A	*Lc. lactis* subsp. *lactis*	*tet* (M) & *tet* (S)
DG 914	1A	*Lc. lactis* subsp. *cremoris*	*tet* (M)
DG 915	1A	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 916	1A	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 919	1A	*Lb. curvatus*	*tet* (M)
DG 923	1B	*Lc. garvieae*	*tet* (S)
DG 926	1B	*Lb. sakei* subsp. *sakei*	Plasmid located *tet* (M)-*1*
DG 928	1B	*Lc. lactis* subsp. *cremoris*	*tet* (S)
DG 929	1B	*Lc. lactis* subsp. *cremoris*	*tet* (S)
DG 931	1B	*Lc. garvieae*	*tet* (S)
DG 933	1B	*Lc. garvieae*	*tet* (S)
DG 935	1B	*Lc. garvieae*	*tet* (S)
DG 937	1B	*Enterococcus sp.*	*tet* (S)
DG 938	1B	*Lc. garvieae*	*tet* (S)
DG 939	1B	*Lc. garvieae*	*tet* (S)
DG 941	1B	*Lc. garvieae*	*tet* (S)
DG 942	1B	*Lc. garvieae*	*tet* (S)
DG 944	1B	*Lb. curvatus*	Plasmid located *tet* (M)-*2*
DG 945	1B	*Lb. curvatus*	Chromosomal located *tet* (M)-*2*
DG 947	1B	*Lb. curvatus*	*tet* (M)
DG 948	1B	*Lb. curvatus*	*tet* (M)
DG 954	2B	*P. pentosaceus*	No RPP, *tet* (K) or *tet* (L)
DG 957	2B	*Lc. lactis* subsp. *lactis*	*tet* (S)

Table A.2. (continued)

Strain number	Source [a]	Taxon	*tet* genes
DG 973	4	*Lb. plantarum*	Plasmid located *tet* (M)-*1*
DG 974	4	*Lb. plantarum*	Plasmid located *tet* (M)-*1*
DG 976	4	*Lb. sakei* subsp. *sakei*	Chromosomal located *tet* (M)
DG 978	4	*Lb. curvatus*	*tet* (M)-*2*
DG 985	4	*Lb. plantarum*	Plasmid located *tet* (M)-*1*
DG 986	4	*Lb. paracasei*	*tet* (M)-*2*
DG 989	4	*Lb. sakei* subsp. *carnosus*	Plasmid located *tet* (M)-*2*
DG 990	4	*Lb. curvatus*	*tet* (M)
DG 997	4	*Lb. plantarum*	*tet* (M)

a/ 1A: frozen lard; 1B: frozen raw pork; 1C: fresh raw pork; 2B: meat batter after addition of the starter culture and spices; 3A: fermented sausage; 4: sliced and packed end product

Table A.3. Selection of Tc^r LAB isolates from FDS-08 process line batch II (n = 32) (Chapter 5)

Strain number	Source [a]	Taxon	*tet* gene(s)
DG 678	3A	*Lb. plantarum*	*tet* (M)
DG 681	3A	*Lb. plantarum*	*tet* (M)
DG 693	3A	*Lb. plantarum*	*tet* (M)
DG 696	3A	*Lb. plantarum*	*tet* (M)
DG 697	3A	*Lb. plantarum*	*tet* (M)
DG 700	3A	*Lb. plantarum*	*tet* (M)
DG 704	3A	*Lb. plantarum*	*tet* (M)
DG 706	3A	*Lb. brevis*-like	*tet* (M)
DG 708	1C	*Lc. garvieae*	*tet* (S)
DG 709	1C	*Lc. garvieae*	*tet* (S)
DG 710	1C	*Lc. lactis* subsp. *lactis*	*tet* (S)
DG 718	1C	*Lc. garvieae*	*tet* (S)
DG 719	1C	*Lc. garvieae*	*tet* (S)
DG 721	1C	*Lb. plantarum*	*tet* (M)
DG 730	1C	*Lc. garvieae*	*tet* (S)
DG 731	1C	*Lc. garvieae*	*tet* (S)
DG 734	1C	*Lc. garvieae*	*tet* (S)
DG 737	1C	*Lb. brevis*-like	*tet* (M)
DG 759	1B	*Leuc. citreum*	*tet* (S)
DG 775	1B	*Lb. sakei* subsp. *sakei*	*tet* (M)
DG 761	1B	*Lb. sakei* subsp. *sakei*	*tet* (M)
DG 786	1B	*P. pentosaceus*	*tet* (M)
DG 787	1B	*P. pentosaceus*	*tet* (S)
DG 788	1B	*Lc. garvieae*	*tet* (S)
DG 790	1B	*Lc. garvieae*	*tet* (M) & *tet* (S)
DG 794	1A	*St. parauberis*	*tet* (M)
DG 796	1A	*Lc. lactis* subsp. *cremoris*	*tet* (M)
DG 798	1A	*Lc. garvieae*	*tet* (S)
DG 799	1A	*Lc. garvieae*	*tet* (S)
DG 800	4	*Lb. sakei* subsp. *carnosus*	*tet* (M)
DG 806	4	*Lb. brevis*-like	*tet* (M)
DG 807	4	*Lb. brevis*-like	*tet* (M)

a/ 1A: frozen lard; 1B: frozen raw pork; 1C: fresh raw pork; 2B: meat batter

Table A.4. Transconjugants obtained in this study (chapter 4)

Recipient X Donor	Strain No	Transferred resistance gene(s)
E. faecalis JH2-2 X		
DG 013	TC 013-1	*tet* (M)
DG 493	TC 493-1	*tet* (M)
	TC 493-4	*tet* (M)
DG 498	TC 498-1	*tet* (M)
	TC 498-2	*tet* (M)
DG 500	TC 500-1	*tet* (M)
	TC 500-3	*tet* (M)
	TC 500-5	*tet* (M)
DG 507	TC 507-1	*tet* (M) and *erm* (B)
	TC 507-2	*tet* (M)
	TC 507-4	*tet* (M) and *erm* (B)
DG 515	TC 515-1	*tet* (M)
DG 522	TC 522	*tet* (M)
Lc. lactis subsp. *lactis* Bu2-60 X		
DG 493	TC 493-21	*tet* (M)
DG 515	TC 515-21	*tet* (M)

Table A.5. Other strains and plasmids used in this study

Recipient strain for conjugation experiments		
E. faecalis JH2-2 (LMG 19456)	No plasmids, Rif^r, Fus^r	Jacob and Hobbs (1974)
Lc. lactis subsp. *lactis* Bu2-60 (LMG 19460)	No plasmids, Rif^r, Fus^r, Str^r	Neve *et al.* (1984)
Other strains		
Lc. lactis subsp. *cremoris* AC1	Plasmid size marker	Neve *et al.* (1984)
Lb. plantarum 5057	Plasmid located *tet* (M)-*1*	Danielsen (2002)
Plasmids/transposons		
Tn*1545*	*Reference construct for erm* (B) and *int*	Courvalin P.
pJI3	*Reference construct for tet* (M)	Morse *et al.* (1986)
pAT121	*Reference construct for tet* (O)	Collard J.-M.
pVP2	*Reference construct for tet* (S)	Perreten *et al.* (1997)
pAT102	*Reference construct for tet* (K)	Courvalin P.
pAT103	*Reference construct for tet* (L)	Courvalin P.

Danielsen, M. 2002. Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid **48**:98-103.
Jacob, A. E. and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes* . Journal of Bacteriology **117**:360-372.
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Neve, H., A. Geis, and M. Teuber. 1984. Conjugal transfer and characterization of bacteriocin plasmids in group N (lactic acid) streptococci. Journal of Bacteriology **157** :833-838.
Perreten, V., B. Kolloffel, and M. Teuber. 1997. Conjugal transfer of the Tn*916* -like transposon Tn*FO1* from *Enterococcus faecalis* isolated from cheese to other Gram-positive bacteria. Systematic and Applied Microbiology **20**:27-38.

CURRICULUM VITAE

Full name:	Dirk Maria Ludovicus Gevers
Date of Birth:	June 8th, 1976
Place of Birth:	Turnhout, Belgium
Present place of residence:	Gent
Nationality:	Belgian

EDUCATIONAL BACKGROUND

1998-2002: Ghent University, Gent, Belgium

Ph. D. student, Laboratory of Microbiology, Department of Biochemistry, Physiology and Microbiology, Faculty of Sciences, Ghent University

Specialization grant IWT (Flemish government institution)

Dissertation: Tetracycline resistance in lactic acid bacteria isolated from fermented dry sausages

1996-1998: Ghent University, Gent, Belgium

Licentiate Biochemistry

Dissertation: Isolation, characterization and identification of oxytetracycline resistant bacteria from hospital sewage

1994-1996: Limburg University, Diepenbeek, Belgium

Candidate Chemistry

1982-1994: Sint-Jozefscollege, Turnhout

Wetenschappelijke A

SCIENTIFIC ACTIVITIES

· Supervision of students

· Assistance in practical courses of microbiology

· Stay in foreign lab

December 2000: Applied Biotechnology, Chr. Hansen A/S, Denmark.

· Oral presentation at international conferences

Gevers, D., Huys, G., Debevere, J., Swings, J. (1999). Antibiotic resistance in lactic acid bacteria isolated on sliced prepacked meat products. Food microbiology and food safety into the next millennium, 17th International Conference of the International Committee on Food Microbiology and Hygiene (ICFMH), Veldhoven, Nl, 13-17 September 1999.

Gevers, D., Huys, G., Rasschaert, G., Masco, L., Baert, L., Debevere, J., and Swings,J. (2002). Tetracycline resistance in lactic acid bacteria from fermented dry sausages. Necessary and unwanted bacteria in food-microbial adaptation to changing environments, 18th International Conference of the International Committee on Food Microbiology and Hygiene (ICFMH), Lillehammer, Norway, 18-23 August 2002.

· Honours / awards

2001: The Organon Teknika Prize for best poster presentation, 2nd prize for the poster presented on the SfAM summer conference, Swansea, UK

2001: Scholarship for EuroLAB conference, Cork, Ireland

2002: Scholarship for 18th international ICFMH symposium, Food Micro 2002, Lillehammer, Norway

LIST OF PUBLICATIONS (PEER-REVIEWED)

2000

- **Gevers, D., Huys, G., Devlieghere, F., Uyttendaele, M., Debevere J., Swings, J.** 2000. Isolation and identification of antibiotic resistant lactic acid bacteria from pre-packed sliced meat products. Systematic and Applied Microbiology **23**: 279-284.

2001

- **Gevers, D., Huys, G., Swings, J.** 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters **205**: 31-36

- **Huys, G., Gevers, D., Temmerman, R., Cnockaert, M., Denys, R., Rhodes, G., Pickup, R., McGann, P., Hiney, M., Smith, P., Swings, J.** 2001. Comparison of the antimicrobial tolerance of oxytetracycline-resistant heterotrophic bacteria isolated from hospital sewage and freshwater fishfarm water in Belgium. Systematic and Applied Microbiology 24 (1), 122-130.

2002

- **Gevers, D., Danielsen, M., Huys, G., Swings, J.** 2002. Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Applied and Environmental Microbiology (revised version submitted).

- **Neysens, P., Messens, W., Gevers, D., Swings, J., De Vuyst, L.** 2002. *Lactobacillus* amylovorus DCE471, a potential strain for use in type II sourdough fermentations, displays biphasic growth patterns and a reduced amylovorin L production in the presence of sodium chloride. Microbiology (submitted).

- **Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J., Swings, J.** 2002. Prevalence and diversity of tetracycline resistant lactic acid bacteria and their *tet* genes along the process line of fermented dry sausages. Systematic and Applied Microbiology (submitted).