

**VIRULENCE CHARACTERISTICS OF ENTEROCOCCI FROM CURED MEAT AND  
POTENTIAL FOR INTER-GENETIC TRANSFER OF ANTIBIOTIC RESISTANCE  
DETERMINANTS**

By

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## ABSTRACT

The genus *Enterococcus* has an exceptional ability to acquire and transmit antibiotic resistance genes and is considered to be a major vector in their dissemination. Enterococci are part of the normal gut microbiota of humans and animals and are frequently encountered in food products including dry fermented sausage. Since fermented sausages are not heat-treated before consumption they might be a vehicle for transmitting resistance and virulence traits of enterococci by conjugation with commensal bacteria present in the human gut and pathogenic bacteria that might be present, such as *Listeria* species. A PCR-based assay was developed to detect enterococci in dry fermented sausage meat at the generic level by targeting a 16S rRNA sequence and a total of 29 *Enterococcus* strains (15 *E. faecalis*, 13 *E. faecium*, and one *E. gallinarum*) were identified. The susceptibility of these enterococci to antibiotics was tested and it was found that 27/29 were resistant to more than one antibiotic and possessed antibiotic resistance determinants. All strains were positive for at least one virulence gene. Strong biofilm formation occurred at lower than optimum temperature in all three species of enterococci and probably contributed to their survival in the harsh conditions experienced during dry sausage fermentation and drying. *Sma*I pulsed-field gel electrophoresis (PFGE) patterns exhibited genomic heterogeneity within and between the two larger groups of isolates. In spite of this heterogeneity, the phenotypic similarities observed suggested that food could still be a vehicle for distribution of antibiotic resistant bacteria among humans. *In vitro* conjugation experiments demonstrated transfer of the tetracycline resistant determinant, *tet*(M), from *E. faecium* S27 isolated from fermented sausage to clinical isolates of both *E. faecium* and *E. faecalis*. The streptomycin resistance of *E. faecium* S27 was also transferred to a clinical strain, *E. faecalis* 82916, which was confirmed by the presence of the streptomycin resistance gene, *aadA*, in the donor and transconjugant strains. *E. faecium* S27 also transferred

*tet(M)* and streptomycin resistance to *Listeria monocytogenes* GLM-2 by *in vitro* mating. Evidence suggests that enterococci in fermented meats may contribute to the spread of resistance determinants.

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## THESIS FORMAT

The thesis is comprised of three manuscripts published in peer-reviewed scientific journals (Chapters 3, 4 and 6), and these are indicated below. The formatting of manuscripts was altered to standardize their presentation as is required for this thesis. Chapter 1 gives an overall introduction to the subjects of study in this thesis. Chapter 2 presents a comprehensive literature review of all the work described in the following chapters.

Chapter 3 entitled “Antimicrobial resistance of *Enterococcus* species from meat and fermented meat products isolated by a PCR-based rapid screening method” by M. Jahan, D. O. Krause, and R. A. Holley, 2013 was originally published in the International Journal of Food Microbiology, 163:7 89–95.

Chapter 4 entitled “Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25° C and 37° C” by M. Jahan and R. A. Holley, 2014, was originally published in the International Journal of Food Microbiology, 170: 65-69.

Chapter 5 is entitled “Allyl isothiocyanate resistance among enterococci isolated from raw and fermented meat” and has not been published elsewhere.

Chapter 6 entitled “Horizontal transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to clinical isolates of *Enterococcus faecium* and *Enterococcus faecalis*” by M. Jahan, G. G. Zhanel, R. Sparling and R. A. Holley, is accepted for publication in the International Journal of Food Microbiology.

Chapter 7 is entitled “Transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to *Listeria monocytogenes* and *Listeria innocua*” and has not been published elsewhere.

Chapter 8 provides an overall discussion and conclusion. Chapter 9 ends this work with a section on future directions for research. Lastly, references cited are documented in alphabetical order.

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**List of abbreviations**

Agg	Aggregation substance
AITC	Allyl Isothiocyanate
API 20	Analytical Profile Index 20
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion Broth
CDC	Center for Disease Control and Prevention
CFU	Colony forming unit
CHEF	Counter-clamped Homogeneous Electric Field Electrophoresis
CLSI	Clinical and Laboratory Standards Institute
CRIFS	Canadian Research Institute for Food Safety, University of Guelph
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
EFSA	European Food Safety Authority
Esp	Extracellular surface protein
Gel	Gelatinase
GRAS	Generally Regarded As Safe

HGT	Horizontal Gene Transfer
IS	Insertion Sequences
ITC	Isothiocyanates
KF-SA	KF-Streptococcus Agar
LAB	Lactic Acid Bacteria
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MRS	DeMan-Rogosa-Sharpe
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
RDP	Ribosomal Database Project
Rep	Replication initiating genes
Tra	Plasmid-encoded transfer genes
TSB	Tryptic Soy Broth
UPGMA	Unweighted pair-group method using arithmetic averages

## Chapter 1

### Introduction

Bacteria of the genus *Enterococcus* are wide spread in the environment and are common among food and clinical isolates. Depending on the strain, they are considered as indicator, spoilage, or potentially pathogenic organisms. Enterococci are a group of extremely versatile bacteria found predominantly in the gastrointestinal tract of humans and animals. They also survive in external environments and can colonize diverse niches due to their ability to tolerate extreme conditions. Enterococci are known to be opportunistic pathogens that are harmless to healthy individuals, but cause disease in those who have severe underlying disease or who are immunocompromised.

Most enterococci have naturally occurring or inherent resistance to various antibiotics which is a specific cause for concern and a contributing factor to their pathogenesis. Intrinsic resistance to antibiotics is mediated by genes located on the bacterial chromosome. However, acquired resistance is mediated by genes residing on plasmids or transposons. They are also known for their capacity for horizontal gene transfer via numerous mobile genetic elements by conjugation (Clewell, 1990). It has been reported that *Enterococcus faecalis* (RE25) isolated from raw fermented sausage was resistant to several antibiotics (Schwarz et al., 2001) and contained a 50-kbp plasmid which had the ability to transfer resistance by conjugation to other *Enterococcus faecalis* strains, *Lactococcus* spp. and *Listeria* spp. But the pathogenesis of enterococci is not only due to their resistance to a wide variety of antibiotics, but also to the fact that they harbor subtle virulence traits. There are reports of enterococci isolated from foods containing virulence factors, such as cytolysins, gelatinase, protease, aggregation substance, extracellular surface protein and other proteins involved in binding to host cells (Eaton and Gasson, 2001; Franz et al., 2001).

The source of enterococci in foods is thought to be fecal contamination. Their resistance to pasteurization temperatures and adaptation to different growth conditions such as extremes of temperatures, pH and salinity are reasons why they can be found in food products either manufactured from raw materials, such as milk and meat or in heat-treated food products. They can also contaminate finished products during food processing. However, the ability of enterococci to grow in food processing plants and many other environments long after their introduction compromises the reliability of enterococcal numbers as an indicator of fecal contamination and demonstrates that finding enterococci in foods does not equate with 'fecal' presence. Moreover, many foods, such as fermented sausage may contain significant numbers of enterococci of non-animal origin. Marchesini et al. (1992) reported the presence of  $10^3$  to  $10^5$  CFU/g enterococci at the end of sausage ripening whether sausages were manufactured with or without starter cultures.

Most meat products are cooked before consumption and hence few viable 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 microbiota that are not inhibited by the conditions that develop during fermentation, such as the lactic acid bacteria (LAB) and enterococci, may be found in the final ready-to-eat product. Unlike most food-associated LAB, there is debate whether enterococci should be considered 'Generally Regarded As Safe' (GRAS) organisms. Under certain circumstances they can act as 'protective cultures' by their production of bacteriocins and through their probiotic characteristics. Probiotic enterococci strains are also available as supplements for animals (Foulquie Moreno et al., 2006).

Unfortunately, the beneficial contributions of enterococci to human and animal health are offset by the large number of nosocomial infections attributed to these organisms. *E. faecalis* and *E.*

*faecium* are the third most common cause of hospital-acquired infections (Giraffa, 2002). Treatment options are often limited when an infection is complicated by the antibiotic resistance of the infecting enterococcal strains. Moreover, as enterococci have a high propensity for genetic exchange via conjugation, antibiotic resistance can be readily spread through a bacterial population. This is of special concern in terms of food safety. Since enterococci are common contaminants of foods from animal origin, such as fermented sausages, antibiotic resistant strains can easily come into contact with an otherwise safe starter or probiotic strain and pass on genes encoding antibiotic resistance, which would then be consumed and exposed to a second population of organisms in the host gastrointestinal tract, perpetuating the spread of antibiotic resistance genes. Thus, the potential health risk from the transfer of antibiotic resistance genes in enterococci to bacteria in the resident microbiota of the human gastrointestinal tract and possibly pathogenic bacteria is unquantified. It is certainly possible that adventitious organisms carrying antibiotic resistant determinants may interact with pathogens in the animal/human gastrointestinal tract and enhance pathogen virulence. However, these hypotheses are yet to be confirmed.

From a food safety perspective it would be of value to know the prevalence of enterococci in fermented meat products and whether they are resistant to antibiotics, whether they possess virulence traits of concern, and whether there is a possibility that these characteristics might be transmitted to human commensals or pathogens.

Therefore, this work was carried out to achieve following objectives:

- i) To assess the occurrence of enterococci in commercial fermented dry sausage and determine the risk associated with these products.

- ii) To determine the minimum inhibitory concentration (MIC) of antibiotics against enterococci isolated from fermented meats.
- iii) To determine the presence of virulence factors associated with these enterococci.
- iv) To assess the sensitivity of the enterococci toward natural antimicrobials, such as allyl isothiocyanate (AITC).
- v) To understand whether the transfer of antibiotic resistance genes from meat enterococci to human isolates of enterococci can occur and the molecular mechanism(s) underlying these transfers.
- vi) To determine whether the transfer of antibiotic resistance genes from enterococci to starter cultures and pathogens associated with meat and fermented sausages can occur.

## Chapter 2

### Review of Literature

#### 2.1 Taxonomy and habitat of enterococci

The genus *Enterococcus* belongs to the group of microorganisms known as lactic acid bacteria (LAB). They are Gram-positive, catalase-negative, oxidase-negative, facultatively anaerobic, non-spore forming bacteria occurring as single cocci or in chains. Historically, enterococci had been classified as group D streptococci. When Schleifer and Kilpper-Balz (1984) used DNA-DNA and DNA-rRNA hybridization and found that *Streptococcus (S.) faecalis* and *S. faecium* were distantly related to the genus of *Streptococcus*, they were transferred to the genus *Enterococcus*. Today on the basis of phylogenetic evidence, strengthened by 16S rRNA, DNA sequencing and/ or DNA–DNA hybridization studies, 28 species are included in this genus (Moreno et al., 2006). Among the recognized species, *E. faecium* and *E. faecalis* are the two most prominent and they have the most significant influence among this group of organisms on human disease, in fermented foods and in probiotics (Franz et al., 1999).

Enterococci are ubiquitous microorganisms, but their predominant habitat is the gastrointestinal tract of humans and warm-blooded animals. *E. faecium* and *E. faecalis* are often found to be human-associated species, varying in abundance among individuals and along the gastrointestinal tract (Ogier and Serror, 2008). But they are also found in abundance in cattle, pigs and poultry (Bager et al., 1997; Devriese et al., 1992; Manero et al., 2002), sheep, goats, dogs, horses, rabbits (Devriese et al., 1987; Wheeler et al., 2002) and wild birds (Poeta, 2005). Certain species of enterococci are also associated with plants, such as *E. mundtii*, *E. sulfureus* and *E. casseliflavus* (Müller et al., 2001). Enterococci are commonly isolated from foods, water and soils, probably as



a result of dissemination from fecal sources and their natural tolerance to adverse environmental conditions (Aguirre and Collins, 1993; Giraffa, 2002). They have the ability to ferment carbohydrates to L-lactic acid and are recognized as homo-fermentative LAB. Enterococci are often resistant to pasteurization temperatures and can adapt to different growth conditions. They can grow in the presence of 6.5% NaCl, 40% bile salt, at pH (4.5–10.0) and temperatures between 10°C and 45°C (Fisher and Phillips, 2009; Manero and Blanch 1999). This means that they can be found in food products either manufactured from raw materials, such as milk or meat and in heat-treated products. They can also contaminate finished products during food processing. Enterococci are an important part of the fermented food microbiota, especially fermented cheese and sausages. Enterococci of food origin also share a number of useful biotechnological traits, such as bacteriocin production (Herranz et al., 2001) and probiotic characteristics (Fuller, 1991). As they are commonly isolated from human and animal excreta they also serve as indicators of sanitary quality (Franz et al., 1999).

## **2.2 Enterococci in food**

Enterococci can be readily isolated from foods, including a number of fermented foods. *E. faecalis* is often the predominating *Enterococcus* species in the human bowel, and to a lesser extent, *E. faecium*. Although enterococci can act as an indicator of fecal contamination, their presence in many food products may not always be related to direct fecal contamination (Mundt, 1982). Birollo et al. (2001) showed that enterococci had little value as hygiene indicators in the industrial processing of foods because enterococci are not only associated with warm blooded-animals, but they also occur in soil, surface waters and on plants, vegetables, and insects (Jay et al. 2005; Mundt, 1982).

Enterococci are the most thermotolerant of non-sporulating bacteria and some can survive pasteurization temperatures and have the ability to survive in different substrates under extremes of temperature, pH, and salinity. Tolerance to these extremes explains their survival during processing of cooked and uncooked food, and their presence in processed foods (Hugas et al., 2003; Simpson et al., 1994). Enterococci can easily colonize raw foods of animal origin and can survive and even multiply during fermentation (Girraffa, 2002). Some authors consider enterococci as technologically unacceptable in finished products, such as in dry fermented sausages (Holley et al., 1988). Although there is agreement that enterococci belong to the LAB, there is controversy whether they should be considered GRAS (Generally Recognized As Safe) microorganisms since they may represent a risk to human health (Hugas et al., 2003; Moreno et al., 2006).

The contribution of enterococci to the organoleptic properties of fermented food products and their ability to produce bacteriocins (enterocins) are important characteristics of value in food technology. In recent years because of their antibiotic resistance and potential virulence traits, enterococci have been considered by some to be undesirable in foods (Franz et al., 1999). The main discussion has focused on whether enterococci transmitted by food can cause disease in a hospital setting. Despite this concern, research has focused on the use of *Enterococcus* strains for control of pathogens, such as *Listeria* in fermented meat and cheese products (Lauková and Turek, 2011; Vera Pingitore et al., 2012).

### 2.2.1 Presence of enterococci in raw and processed meat

Since enterococci are commonly present in the gastrointestinal tract of animals, there is significant potential for their contamination of meat at slaughter. In a study of enterococci from raw meat

products, *E. faecalis* and *E. faecium* were shown to be the predominant isolates in raw meat from beef and hog carcasses (Knudtson and Hartman, 1993; Stiles et al., 1978) and *E. faecalis* was the most frequent isolate among the Gram-positive cocci found in a study on poultry (Turtura and Lorenzelli, 1994).

Antibiotic resistant enterococci can be consistently isolated from fresh meat (Franz et al., 2003) and it is evident that meats generally become contaminated during processing and handling. Heating of processed meats during production may delay the growth of spoilage bacteria but enterococci are thermo-tolerant and may survive heat-processing (Franz et al., 2003; Moreno et al., 2006). Both *E. faecalis* and *E. faecium* which survived commercial thermal processing have been implicated in spoilage of canned hams and chub-packed luncheon meats (Magnus et al., 1988). Danyluk et al. (2013) observed that *E. faecium* had the ability to survive normal pasteurisation of canned meat. The heat resistance of enterococci in these products is influenced by salt, nitrite, and meat tissue (Franz et al., 1999; Danyluk et al., 2013). To prevent enterococci from causing problems in processed meats, it was suggested that initial contamination of product by these organisms should be avoided (Magnus et al., 1988).

### 2.2.2 Presence of enterococci in fermented cured meat

Enterococci are frequently isolated from fermented, cured meat products, especially from fermented sausages. Fermented sausages are made from a batter of pork meat, pork fat and/or beef and includes salt, sugar, nitrate and/or nitrite and some spices. This mixture is then stuffed into casings and subjected to fermentation followed by ripening/drying (Graumann and Holley, 2008). Traditional sausages are generally manufactured without the addition of starter cultures and therefore the micro-flora of these products comes from the natural contamination of raw meat

during slaughtering and manufacturing (Talon et al. 2007). Enterococci have been frequently isolated from many types of fermented sausages over the past few decades in different parts of the world (Table 2.1). *E. faecium* and *E. faecalis* are the predominant enterococci species and are found in relatively high numbers during meat fermentation. They may contribute, together with lactobacilli, to the process of pH reduction during meat fermentation. Often present as contaminants, enterococci can multiply during fermentation (Hugas, 2003). Metaxopoulos et al. (2001) isolated enterococci as contaminants in naturally fermented sausage batter where they were able to grow during the fermentation and ripening of sausages in manufacturing plants in Greece. In commercially fermented sausages manufactured with competitive starter cultures consisting of lactobacilli, pediococci and staphylococci, the enterococci are inhibited, but their presence in finished products has been observed (Marchesini et al., 1992). Enterococci have also been isolated from dry fermented sausages where they produced antilisterial bacteriocins, and there has been a suggestion that such strains may be suitable for use as adjunct starter cultures to improve food safety (Franz et al., 2011).

Table 2.1 Studies on dry fermented sausages and the presence of enterococci

Types of sausage	Enterococci species	Important features of isolated enterococci	References
Commercially fermented German salami	<i>Enterococcus</i> spp.	Enterococci were 4 log CFU/g after 90 d fermentation and ripening	Marchesini et al., 1992
Milano style salami	<i>Enterococcus</i> spp.	Enterococci were 4.7 log CFU/g after 70 d fermentation and ripening	Papa et al., 1995
Chorizo, Spanish dry fermented sausage	<i>E. faecium</i>	Produced enterocin P that inhibited <i>Listeria</i> , <i>Staphylococcus</i> , <i>Clostridium</i>	Cintas et al., 1997
Traditional Greek salami	<i>Enterococcus</i> spp.	Enterococci were 5 log CFU/g after 28 d fermentation and ripening	Samelis et al., 1998
Spanish salami and sausages with olives	<i>E. faecium</i>	Showed resistance towards erythromycin and rifampicin	Ben Omar et al., 2004
French naturally fermented sausages	<i>Enterococcus</i> spp.	Enterococci were 7.7 log CFU/g after about 65 d fermentation and ripening	Lebert et al., 2007
Iberian dry fermented sausages	<i>E. faecium</i>	Used as a probiotic	Ruiz-Moyano et al., 2008
Thailand fermented sausage ('mum')	<i>E. thailandicus</i>	Newly discovered enterococcal species from fermented sausages	Tanasupawat et al., 2008
Chorizo and Fuet	<i>E. faecalis</i> ; <i>E. faecium</i>	Showed antibiotic resistance and presence of virulence gene	Martin et al., 2005
Spanish dry-cured sausages	<i>E. faecium</i>	Showed resistance towards tetracycline, rifampicin, ciprofloxacin, erythromycin and nitrofurantoin	Landeta et al., 2013

## 2.3 Pathogenesis of enterococci

During the past several decades, enterococci have emerged as important nosocomial pathogens (Arias and Murray, 2012; Murray 2000; Nguyen et al., 2010; van Schaik et al., 2010). The importance of their pathogenesis is attributed primarily to the high degree of multi-drug resistance exhibited by most enterococci, and they usually affect patients who are debilitated by other concurrent, chronic illnesses. The species responsible for most community infections is *E. faecalis* and it has caused 90% of all enterococcal infections. Since the late 1980s a rapid increase in nosocomial *E. faecium* infections has occurred (Top et al., 2008; Treitman et al., 2005). Enterococci are associated with a variety of different clinical infections including intra-abdominal, pelvic, soft tissue infections, those of the urinary tract, bacteremia and endocarditis. Some uncommon infections, such as meningitis, hematogenous osteomyelitis, septic arthritis, and pneumonia due to *E. faecium* have also been diagnosed in clinical settings (Teixeira and Merquior, 2013).

### 2.3.1 Antibiotic resistance of enterococci

Enterococci can be resistant to a wide variety of antibiotics commonly used in human medicine as well as those that are used for animal therapy, prophylaxis or for growth promotion. The organisms are becoming the focus of attention because of their increasing resistance towards antibiotics. Enterococci are able to survive in the hospital environment where antibiotics are used and this phenomenon has enabled the dissemination of resistant organisms in the environment (Murray

1990). Moreover, being part of the gastrointestinal flora, the enterococci are commonly exposed to antibiotic resistance genes from other commensals, and are able to transfer these to other perhaps more pathogenic bacteria present in the gastrointestinal tract (Engel et al., 1980; Moubareck et al., 2003). This illustrates the potential clinical importance of enterococci as a reservoir for antimicrobial resistance determinants.

Antibiotics are defined as substances having a biological, semi-synthetic or synthetic, origin which show selective activity against bacteria, yeast or fungi and may thus be used in treatment of infections caused by these organisms. Antimicrobial susceptibility testing of a clinical isolate is usually based on establishing a Minimum Inhibitory Concentration (MIC) for effectiveness. The MIC value is the lowest concentration of an antimicrobial agent which inhibits the growth of that organism, most often bacteria. Clinical MIC breakpoints are designed to guide therapy and do not provide any information on resistance mechanisms. Thus, epidemiological cut off values (the highest MIC value of the wild-type population) are designed to describe low level resistance and monitor resistance development. The clinical MIC breakpoints generally divide bacteria into three categories of sensitivity: susceptible, intermediate, or resistant (Kahlmeter, 2003). Antibiotic resistance was described early after the introduction of the first clinically useful antibiotic – penicillin (Levy, 2002). Subsequently with other antibiotics similar observations were made. Consistently, a few years after the introduction of new antibiotics in clinical practice, resistance tends to emerge (Martinez, 2008). From a biochemical point of view, bacterial antibiotic resistance mechanisms can be divided into 4 major classes, but these can overlap, i.e. an organism can become resistant to an antimicrobial by more than one mechanism. The biochemical mechanisms of antibiotic resistance include: i) production of drug inactivating enzymes; ii) modification of an existing antimicrobial target; iii) reduced access due to altered penetration and/or efflux that

actively gets rid of the antimicrobial agent before it reaches its target site, iv) metabolic bypass by overproduction or through an alternative pathway (Sefton, 2002).

Antimicrobial resistance in enterococci has been characterized as being either intrinsic or acquired.

#### *Intrinsic resistance in enterococci*

Intrinsic resistance in bacteria is due to the lack of a target site for the antibiotic in question or results from its inability to access the target site within the cell. This type of resistance is specified by naturally occurring genes found on the host's chromosome, (Alekshun and Levy, 2007; Top et al., 2008). Examples of intrinsic resistance in enterococci include resistance towards cephalosporins,  $\beta$ -lactams, sulfonamides and low levels of resistance to clindamycin or aminoglycosides (Franz et al., 1999).

*Enterococcus* spp. are generally considered naturally resistant to cephalosporins and  $\beta$ -lactam drugs due to their low affinity for penicillin binding proteins (PBPs). It is notable that the level of susceptibility towards  $\beta$ -lactam drugs varies between enterococcal species (Top et al., 2008). Low level resistance of aminoglycosides is due to inefficient uptake of the drugs, and resistance to lincosamides is due to putative efflux mechanisms. *E. gallinarum* and *E. casseliflavus* are, in addition, intrinsically resistant to low levels of vancomycin due to the production of D-Alanine–D-Serine-ending peptidoglycan side chain precursors for which vancomycin has a lower binding affinity compared to the normal D-Alanine-D-Alanine side chains (Top et al., 2008).

#### *Acquired resistance in enterococci*

Acquired resistance to an antimicrobial drug is the result of an alteration of the genome of a microorganism. The alteration may occur by the mutation of a gene to become resistant or by the



incorporation of one or more resistance genes from a donor organism into an acceptor organism via conjugation, transformation or transduction (Catry et al., 2003). The later methods are known as horizontal gene transfer (HGT) and are considered to be the major mechanisms for spread of antimicrobial resistance (Rowe-Magnus and Mazel, 1999). Examples of antibiotics where development of acquired resistance has occurred include chloramphenicol, erythromycin, high levels of clindamycin and aminoglycoside resistance, tetracycline,  $\beta$ -lactam (by  $\beta$ -lactamase or penicillinase), fluoroquinolone and glycopeptide resistance (Franz et al., 1999).

Intrinsic resistance to some commonly used antibiotics afforded the enterococci an opportunity to acquire genes encoding high levels of resistance. This has happened with the development of resistance to high levels of aminoglycosides, penicillin, tetracycline, chloramphenicol and vancomycin (Mundy et al., 2000).

### 2.3.2 Virulence factors

A virulence factor is an effector, often a molecule, that enhances the ability of a microorganism to cause disease beyond that intrinsic to the species (Mundy et al., 2000). Enterococcal virulence factors are widely present among clinical strains and these have been detected in food isolates (Eaton and Gasson, 2001; Franz et al., 2001). Over the years several virulence factors have been identified in food enterococci which include: aggregation substances, gelatinase, enterococcal surface protein (Aslam et al., 2012; Foulquié Moreno et al., 2006; Franz et al., 2001), cytolysin, pheromone, (Martin et al., 2005; Ribeiro et al., 2011; Valenzuela et al., 2009) and the ability to form biofilms (Barbosa et al., 2010).

### 2.3.2.1 Aggregation substances

Aggregation substance (Agg) is a pheromone-inducible surface protein that promotes bacterial aggregation to facilitate bacterial conjugation. Agg enables efficient contact between donor and recipient and facilitates transfer of mobile genetic elements (e.g., plasmid). It is an essential component of the pheromone-responsive plasmid exchange system (Clewell, 1993).

This trait may contribute to the pathology of enterococcal infection through different mechanisms. The cells that express this trait form large aggregates *in vivo*. Agg may bind and present a common ligand at the surface of the organism, possibly resulting in superantigen activity (Mundy et al., 2000). Agg also increases the hydrophobicity of the enterococcal surface, which may induce localization of cholesterol to phagosomes, and prevent or delay fusion with lysosomal vesicles (Mundy et al., 2000). Agg is exclusively found in clinical *E. faecalis* strains; however, its incidence among food isolates seems to be high (Eaton and Gasson, 2001; Franz et al., 2001).

### 2.3.2.2 Cytolysin

Cytolysin is a novel hemolytic, post-translationally modified proteinaceous toxin that is considered to be a virulence factor mainly of *E. faecalis* strains (Mundy et al., 2000). The cytolysin genes are located on the highly transmissible pheromone-responsive conjugative plasmid or integrated into the bacterial chromosome (Ike et al., 1990). The cytolysin of *E. faecalis* possesses additional bacteriocin activity against a broad range of Gram-positive bacteria (Mundy et al., 2000). Although cytolysin is common in *E. faecalis* from clinical settings, it has frequently been found in food isolates of *E. faecalis* (Aslam et al., 2012).

### 2.3.2.3 Gelatinase

Gelatinase (Gel) is an extracellular metallo-endopeptidase involved in the hydrolysis of gelatin, collagen, haemoglobin, and other bioactive peptides (Coque et al., 1995). This protease gene, *gelE*, was first sequenced by Su et al. (1991), and the enzyme is involved in pathogenicity of *Enterococcus* species. Singh et al. (1998) demonstrated that Gel is commonly produced by nosocomial, fecal, and clinical enterococcal isolates. The frequency of Gel production among food *E. faecalis* strains is also high (Eaton and Gasson, 2001; Franz et al., 2001). Eaton and Gasson (2001) also found that *gelE* could be present in spite of the absence of any phenotypic gelatinase activity in enterococci strains isolated from food. Silent genes may occur when there are low levels of gene expression, or sometimes they are due to down-regulation or to an inactive gene product, all of which can be influenced by environmental factors (Eaton and Gasson, 2001; Finlay and Falkow, 1997).

### 2.3.2.4 Extracellular surface protein (Esp)

Extracellular surface protein (Esp) is a cell associated and chromosomally encoded protein containing 1,873 amino acids. Esp was originally discovered in *E. faecalis* (Shankar et al., 1999) and later was also found in *E. faecium* (Willems, 2001). This gene has been linked to pathogenesis, possibly through tissue colonization and biofilm formation with increased virulence (Shankar et al., 1999). Esp is thought to play a role in adhesion and evasion of the immune response of the host. There are reports of the presence of the *esp* gene in enterococci from foods (Eaton and Gasson, 2001; Valenzuela et al., 2009). The *esp* gene is considered to be an infection-associated virulence factor and thus it is undesirable when in enterococci isolated from food.

### 2.3.2.5 Biofilm formation by enterococci

Biofilms can be defined as matrix-embedded bacterial populations adhered to a surface or to each other (Poulsen, 1999). The formation of biofilms is a dynamic strategy used by all kinds of microorganisms, which allows them to survive under adverse environmental situations. Bacterial biofilms are more resistant to environmental stress than their free living counterparts and their attachment to food products or food contact surfaces accelerates spoilage, complicates cleaning and facilitates disease transmission. Enterococci have an extraordinary ability to form biofilms and in biofilms they are more resistant to antibiotic treatments and experience increased levels of genetic exchange than planktonically growing cells (Mohamed and Huang, 2007). Biofilms also provide protection for cells from environmental stress including UV light, shear forces and host immune defense. Thus biofilms have been implicated as an important etiological agent of chronic infection in clinical settings involving catheters (Kristich, 2004). Biofilm formation by an organism depends on both environmental and genetic factors. Environmental factors include nutrient content of the growth medium, such as glucose, serum, availability of iron and carbon dioxide (CO<sub>2</sub>), osmolarity, pH, and temperature. Baldassarri et al. (2001) showed that tryptic soy broth (TSB) medium with 1 % glucose supplementation enhances biofilm production in *E. faecalis* compared to TSB without glucose. Changes in the osmotic strength also affect biofilm formation in enterococci. Kristich et al. (2004) showed that biofilm production was abolished by exposure to a medium having high osmolarity (2–3 % sodium chloride) without affecting the growth of the bacteria. Temperature is another important factor influencing biofilm formation in enterococci. In a study it was revealed that temperature and oxygen played an important role in biofilm formation and early stages in infection (Van Wamel et al., 2007).

Several enterococcal virulence factors have been reported to have important roles in biofilm formation and these include Agg, Esp, Gel, biofilm enhancer or putative cell wall-anchored protein (Bee) and endocarditis as well as biofilm-associated pilus (*ebp*) which is highly conserved and part of the core genome (Nallapareddy et al., 2006; Rosa et al., 2006; Schlüter et al., 2009; Soares et al., 2014; Tendolkar et al., 2004; Toledo-Arana et al., 2001).

Biofilm formation is an issue of concern in the food industry because biofilms facilitate the survival of pathogenic bacteria which may contaminate food-processing equipment and products, particularly in the dairy industry where enterococci adhere to stainless steel and may contaminate equipment. They have been of considerable interest in food hygiene since cells in biofilms are more resistant to cleaning and disinfection processes. Most of the studies with biofilms have been conducted with clinical enterococci and only a few studies have been conducted on biofilm formation by enterococci isolated from food. Gomes et al. (2008) showed that enterococci species isolated from food in Brazil were able to form biofilms. Biofilm formation has been observed in enterococci isolated from traditional fermented meat products produced in the north of Portugal (Barbosa et al., 2010). Gundogan et al. (2013) found that *E. faecalis* and *E. faecium* isolated from meat and milk products were able to form biofilms. Thus, *Enterococcus* spp. may contaminate meat and milk products by surviving in biofilms on equipment which normally should be removed during adequate cleaning and sanitation to minimize risk.

### 2.3.3 Sensitivity of enterococci towards natural antimicrobials: allyl isothiocyanate

There is growing interest today in using plant-derived antibacterial compounds, such as extracts of spices and herbs for food preservation (Shan et al., 2007). Allyl isothiocyanate (AITC), a natural compound in plants belonging to the family Brassicaceae (Cruciferae), has been shown to have

strong antimicrobial activity in liquid media as well as in its vapor form. AITC is a major pungent flavor compound in horseradish, mustard and wasabi and is generated from its precursor allyl glucosinolate or sinigrin. Sinigrin, which is found widely in mustard and other cruciferous vegetable species, such as cabbage and cauliflower, is hydrolyzed by the action of myrosinase when the plant tissue is disrupted to release isothiocyanates (ITCs), including AITC (Cui and Eskin, 1998). AITC in the form of oil of mustard is used primarily as a flavoring agent in variety of foods. Mustard seeds and mustard powder, having the potential to form quantities of ITCs sufficient to be bactericidal to pathogenic bacteria, have been used as common spices in fermented sausages to improve flavour, texture and the safety of these products (Chacon et al., 2006; Graumann and Holley, 2008; Nadarajah et al., 2005).

Studies are going on to know about the specific mechanism by which isothiocyanates kill bacteria. AITC affects the integrity of cell membranes, in particular the plasma membrane, and causes leakage of cellular metabolites, such as ATP from *E. coli* (Lin et al, 2000; Luciano and Holley, 2009). In contrast, Ahn et al. (2001) found there was a reduction of internal ATP levels in *Listeria monocytogenes* when exposed to AITC, but ATP leakage was not detected. AITC was found by transmission electron microscopy to modify the internal structure of treated organisms when compared to non-treated cells. In another instance, inhibition of the activities of thioredoxin reductase and acetate kinase by AITC in *E. coli* has been shown and a mechanism of action that would target different metabolic pathways has been suggested (Luciano and Holley, 2009). It was observed by Kassie and Knasmüller (2000) that AITC was able to cause DNA damage in *Salmonella*, *E. coli* and also in human Hep G2 cells.

AITC derived from sinigrin successfully eliminated 2.7 log CFU/g *E. coli* O157:H7 from ground beef, roast beef and hamburger patties (Nadarajah et al., 2005). Mustard powder containing AITC

has been reported to control *E. coli* O157:H7 numbers in meat and fermented meat products (Chacon et al., 2006; Graumann and Holley, 2008). AITC has been demonstrated to also have high bactericidal activity in the vapor form against a variety of bacteria (*Bacillus*, *Staphylococcus*, *Salmonella*, *E. coli*, *Pseudomonas* and *Vibrio*) as well as yeast and molds (Isshiki et al., 1992). Very little is known about the effect of AITC on enterococci. It is of interest that plant essential oils have been shown to have activity against *E. faecalis* (Holley and Patel, 2005), and that there are reports of Gram-positive bacteria being sensitive to essential oils (Blaszyk and Holley, 1998; Palaniappan and Holley, 2010). Park et al. (2013) showed that AITC from horse radish root had antibacterial activity against *E. faecalis* and other oral microorganisms. In another study by Wilson et al. (2013) it was observed that there were measurable antibacterial effects of AITC (1.7 to 2  $\mu$ M) on *E. faecalis* and *E. faecium*.

#### **2.4 Gene transfer mechanisms and the development of antimicrobial resistance in enterococci**

Genetic transfer from a donor to a recipient bacterial cell can occur either vertically or horizontally. Vertical inheritance is the transmission of genetic material from a mother cell to its offspring during cell division, whereas horizontally acquired genes come from other bacteria or the environment. All successful antibiotics target important bacterial functions which are typically carried out by chromosomally-encoded proteins. A mutation occurring in the bacterial chromosome can change the sensitivity of a protein to which an antibiotic is directed or is inhibitory, and eventually the organism will become drug resistant. However, in an environment where exposure to lethal levels of antibiotics is not continuous, it is not likely that a single mutation leading to resistance would become an epidemic problem (Summers, 2006). Recently there has been growing concern about increased antibiotic resistance in bacteria and the main contributing

factor is their increased use. This has resulted from the wide-spread use of antibiotics both in humans and animals as therapeutics and prophylactics. Some of these antibiotics are also used continuously in animal feed to promote growth, increase feed efficacy and decrease waste production. When used for these purposes, antibiotics are commonly known as growth promoters or performance enhancers (van den Bogaard and Stobberingh, 2000). The use of an antibiotic in veterinary practice can be a contributor to the emergence of antibiotic-resistant bacteria. To become drug resistant, first the organism must come into contact with the antibiotic and then have a mechanism to transfer that resistance to daughter cells or to other members of the same species (Khachatourians, 1998). The acquisition of new DNA by gene transfer has a relatively high impact on the development of antimicrobial resistance compared to mutations.

## **2.5 Horizontal gene transfer**

Horizontal gene transfer (HGT) refers to the acquisition of foreign genes by organisms. HGT is recognized as the major and dominant force in bacterial evolution. HGT of functional units can provide the recipient organism with the tools necessary to occupy new ecological niches. This way of acquiring new genetic material has had a great impact on the evolution and genome plasticity of prokaryotic organisms, and is the major reason for the spread of resistance genes (Sørensen, 2005). Resistance genes can be transferred not only within related species but also across major bacterial divisions. Some HGT is natural and it confers a selective advantage on that organism. Natural transformation occurs under normal bacterial growth conditions where bacteria can uptake, integrate and stably express extracellular DNA. The genetic information may be transferred naturally in three major ways; transformation, transduction, and conjugation. Not all bacteria are able to use all three processes, but most free living bacteria are able to use at least two (Summers, 2006). Integration of a foreign DNA element is often limited by defense mechanisms



of the recipient, so gene transfer is only successful if the element is able to replicate in the host by itself, or after insertion in the host genome it is expressed and affords an advantage to the recipient (Thomas and Nielsen, 2005).

### 2.5.1 Transformation

Transformation is a process in which naked, exogenous DNA is taken up and recombined into the genome of a competent bacterial cell. The DNA can be taken up by cells in its vicinity to serve as a nutrient source, for DNA repair or as a source of genetic innovation. If the bacterium taking up the DNA is closely related to the one that released it, some DNA can be integrated into the bacterial genome of the recipient cell by homologous recombination. Heterologous genes would not be able to recombine and would be degraded by the recipient's nucleases (Dubnau, 1999). Enterococci are not known to be naturally competent and thus this process is not considered an important factor in the evolution of this genus (Johnsen et al., 2002).

### 2.5.2 Transduction

Transduction is a process of DNA acquisition by which non-viral DNA can be transferred from an infected host bacterium to a new host via infectious or noninfectious virus particles known as bacteriophages. The injected bacterial DNA can then be integrated into the recipient chromosome. Normally transduction is only successful in transferring alleles of homologous genes among bacteria which are closely related. Bacteriophages often transfer toxin genes or virulence factors, but rarely antibiotic resistance genes (Summers, 2006). However, bacteriophages have been demonstrated in enterococci and it is believed by others that phages may play a role in the spread of antibiotic resistance in enterococci (Yasmin et al., 2010; Werner et al., 2013).

### 2.5.3 Conjugation

Conjugation is the process whereby a DNA molecule is transferred from a donor to a physically attached recipient cell via a modified flagellum (pilus) which makes contact between the mating cells. Conjugation is initiated by mobile genetic elements called conjugative plasmids or by chromosomally-integrated conjugative elements which include transposons. This process involves movement of DNA from the donor to the recipient through a pore in the pilus and DNA is recombined into the recipient genome or circularized as a plasmid (Frost et al., 2005; Summers, 2006). Gram-positive bacteria, and especially enterococci, use conjugation as a system for genetic exchange and transfer antibiotic resistance as well as virulence factors among related species (Murray, 1998).

## 2.6 Mobile genetic elements

Mobile genetic elements (MGEs) are segments of DNA that mediate the movement of DNA within genomes or between bacterial cells (Frost et al., 2005). A large variety of mobile genetic elements have been identified and new elements are continuously being found. MGEs increase the likelihood that new strains with novel or improved selective advantages over their neighbors will arise. In addition, MGEs can make rapid changes in virulence potential that influence genome evolution in many bacterial pathogens (Heuer and Smalla, 2007).

### 2.6.1 Plasmids

Plasmids are usually circular, self-replicating DNA molecules that exist in cells as extra-chromosomal replicons. Plasmids have a dominant role in the horizontal transfer of genetic information between bacteria and can transfer DNA between genera, phyla and even major

domains. Plasmids replicating autonomously in bacteria may sometimes be integrated into the bacterial chromosome. Some plasmids are self-transmissible or may be mobilized by other plasmids, enabling them to spread to other hosts and deliver new traits, such as virulence and antibiotic resistance. In enterococci a plasmid classification system has been developed which is based on replication initiating (*rep*) genes. In addition, for successful plasmid transfer a number of plasmid-encoded transfer (*tra*) genes are also involved. Proteins involved in transfer have been studied but their functions still need to be explained. Plasmid transfer machinery may be co-opted by co-resident plasmids which are mobilizable, but lack the genes for the type IV secretion system needed to complete the action. A single strand DNA transfer occurs first followed by rolling-circle or theta-replication and synthesis of the second DNA strand in both the donor and recipient (Jensen et al., 2010). The conjugative plasmids in enterococci are often divided into two major groups; pheromone responsive plasmids and broad host range plasmids.

#### *Pheromone responsive plasmids*

Pheromone responsive plasmids are mainly found in *E. faecalis* (rarely in *E. faecium*), and they are responsible for rapid dissemination of antibiotic resistance. Communication by these plasmids involves small hydrophobic peptide sex pheromones that are secreted by potential recipient cells, and when this happens in close contact with a potential donor, the transcription of a gene on the donor plasmid is switched on. The result is production of an aggregation substance on the donor-cell surface mediating contact between donor and recipient cells. Once the plasmid is transferred from the donor to the recipient, expression of that particular sex pheromone will be turned off (Palmer et al., 2010). Vancomycin and erythromycin resistant pheromone-responsive plasmids were isolated during a Japanese outbreak of *E. faecalis* infection (Zheng et al., 2009). A few reports

were found for *E. faecium* pheromone responsive plasmids encoding antibiotic resistance (Handwerger, 1990).

### *Broad host range plasmids*

Broad host range conjugative plasmids also occur in enterococci and are able to transfer between enterococci and other Gram-positive organisms including streptococci and staphylococci. The frequency of transfer is generally much lower than with the pheromone responsive plasmids. Since staphylococci, streptococci, and enterococci share several resistance genes, the broad host range plasmids may well be a vehicle through which some of these resistance genes have spread among these different genera of bacteria (Murray, 1998). Most broad host range plasmids are identified in streptococci and enterococci and often contain resistance genes to a wide spectrum of antibiotics. The lower limits in size for these types of plasmids are in the range of 15-20 kb which is the size of the transfer region with no additional genes. The most common maintenance system on these plasmids is a post-segregational toxin–antitoxin killing system, which ensures the persistence of plasmid containing cells in a population even in the absence of direct antibiotic selection. Some of the most characterized broad host range plasmids identified in enterococci are pAM $\beta$ 1 (originating in *E. faecalis*), pIP501, pRE25 and pRUM (Palmer et al., 2010). It has been shown that pAM $\beta$ 1 and pIP501 encode resistance toward multiple antibiotics and were able to transfer resistance to other Gram-positive bacteria, including *Streptomyces lividans*, *Leuconostoc* spp., and *Listeria* spp. (Kurenbach et al., 2003; Pucci et al., 1998; Vicente et al., 1998). The plasmid pIP501 has also been shown to be transferable to the Gram-negative bacterium, *Escherichia coli* (Kurenbach et al., 2003), and pRE25 can transfer by conjugation into *Listeria innocua* and *Lactococcus lactis* (Schwarz et al., 2001), suggesting their capability for broad host range transmission of antibiotic resistance and other genes.

### 2.6.2 Transposable elements

Transposable elements are mobile DNA segments that can repeatedly insert into one or more sites of genomes (Roberts et al., 2008). They encode all the necessary functions for intracellular transposition and intercellular conjugation. They are present in a wide variety of Gram-positive and Gram-negative bacteria and are important for the spread of antibiotic resistance genes. They usually encode a transposase that catalyses the transposition event and can carry determinants for antibiotic resistance and other properties. They are flanked by inverted repeat DNA sequences and have the capability to move within or between replicons. The simplest transposable elements are “Insertion Sequences” (IS) which are small DNA elements. They encode a transposase which is an enzyme that cuts out the transposable element and inserts it into the DNA by homologous or non-homologous recombination between short repeats (Roberts and Mullany, 2006).

Transposable elements may be mobilized by other conjugative elements; in some cases this includes elements in the host chromosome. However transposable elements are not influenced by incompatibility, so conjugative transposons may interact with each other and with other replicative and non-replicative elements. Different variations and rearrangements of mobile and non-mobile elements may be formed, resulting in the generation of a mosaic of larger and more complex elements providing benefit to the host (Roberts and Mullany, 2006). After the sequencing era started, new and more complex variants of elements were frequently discovered and described. Different transposable elements found in enterococci are described in Table 2.2.

Table 2.2 Transposable elements: definitions and some examples in enterococci

Types of transposable elements	Definition	Examples in enterococci			Reference
		Designation	Resistant gene	Host range	
Composite transposons	Flanked by IS elements. Instead of each IS element being transposed separately, the whole length of DNA spanning from one IS element to the other is moved in one unit. Often carry one or more antibiotic resistance genes.	Tn924	Aminoglycosides	<i>E. faecalis</i>	Thal et al., 1994
		Tn1547	Vancomycin	<i>E. faecalis</i>	Quintiliani and Courvalin, 1996
		Tn5281	Aminoglycosides	<i>Enterococcus</i> spp.	Hegstad et al., 2010
		Tn5385	Aminoglycosides, erythromycin, tetracycline, penicillin, mercuric chloride, streptomycin	<i>E. faecalis</i>	Hegstad et al., 2010
Unit transposons	Unit transposons encode an enzyme which is engaged in the excision and integration of the element. These are often site-specific recombinases or resolvases. In addition, the elements include one or more accessory genes in their genetic unit and are often flanked by inverted repeats (IR).	Tn917	Macrolides, streptogramin B, lincosamides,	<i>E. faecalis</i>	Shaw and Clewell, 1985
		Tn1546	Glycopeptides ( <i>vanA</i> )	<i>Enterococcus</i> spp.	Arthur et al., 1993
Conjugative transposons	They are also known as integrative conjugative elements, carry genes for excision, conjugative transfer and for integration within the new host genome. They carry a wide range of accessory genes, including antibiotic resistance.	Tn916	Tetracycline	<i>Enterococcus</i> spp.	Franke and Clewell, 1981
		Tn1545	Tetracycline, macrolides, lincosamides, streptogramin B, kanamycin	<i>Enterococcus</i> spp.	Hegstad et al., 2010

### 2.6.3 Integrons

Integrons are genetic units that are able to capture small mobile elements called gene cassettes and ensure their expression by site-specific recombination. An integron may contain dozens of cassettes which are transcribed from a common strong promoter and can eventually be transferred as a whole between different replicons. They are frequently associated with transposons and conjugative plasmids that mediate the spread of integrons and integrated gene cassettes (Werner et al., 2013). Integrons possess two conserved segments separated by a variable region which includes integrated antibiotic resistance genes and one or more gene cassettes of unknown function. They are a natural expression vector that permits the insertion of antibiotic resistance gene(s) by a site-specific recombination mechanism (Lévesque et al., 1995). The most common integron classes 1 and 2, encoding antimicrobial resistance determinants, are mainly described for Enterobacteriaceae, but have recently been described in enterococci (Xu et al., 2010). They resemble genetic elements from Gram-negative bacteria and it has been assumed that they might have escaped from Gram-negative bacteria rather than being an integral entity of the enterococcal genome.

## **2.7 Molecular epidemiology and typing methods**

Molecular epidemiology is a field which quite recently emerged from the integration of molecular biology with traditional epidemiologic research. This is the area where the coordinated development of medical diagnostics and public health prevention strategies has occurred and it has proven important in the determination of the physical sources of outbreaks, biological relationships, transmission routes and presence of important virulence genes which pathogenic organisms contain. Many molecular typing techniques, such as DNA restriction fragment analysis,

total plasmid profile analysis, randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and ribotyping, have proven invaluable for epidemiological investigations of enterococcal outbreaks. These typing methods are of importance in cases where pathogens increase in prevalence or acquire characteristics that increase their capacity to cause disease (van den Braak et al., 2000).

#### *Pulsed Field Gel Electrophoresis (PFGE)*

Pulsed-field gel electrophoresis (PFGE) has been considered the gold standard typing method for several medically important bacteria including enterococci. This method compares large genomic DNA fragments created by digestion of the DNA with restriction enzymes after separation of the fragments by agarose gel electrophoresis. This method then, will give a fingerprint of the whole genome. The bacterial chromosome is typically a circular molecule and enzymatic digestion yields several linear DNA molecules of different length. By comparing two isolates that are the same strain (i.e. clonal), the sites at which the restriction enzymes act on the DNA and the length between these sites would be identical. If the DNA banding patterns between isolates is identical, the isolates are considered the same strain. If two isolates are not the same strain, then the sites at which the restriction enzymes act on the DNA and the length between these sites will be different, as will their DNA banding patterns. PFGE has been important for the identification of clonal relatedness between isolates that are epidemiologically associated in time and space (Carriço et al., 2005). PFGE restriction patterns are stable over time, they are reproducible and since standard criteria for interpretation are available, this method has been the most useful for classification and identification of strains (Domig et al., 2003). Transfer of genetic elements from a donor to a recipient will be visible as a shift of bands on the gel. A disadvantage is that several differences in



bands may lead to the conclusion that the isolates compared are distantly or not related, where they in fact may be closely related isolates with a high frequency of horizontal gene transfer.

PFGE is now a widely used method because of its ability to discriminate among enterococcal strains. Counter-clamped homogeneous electric field electrophoresis (CHEF) is currently the basis for most of the recent PFGE studies. PFGE has been extensively evaluated for epidemiological characterization of enterococcal outbreaks, showing improved strain discrimination and allowing the identification of clonal complexes that predominate among multidrug-resistant enterococci. The most frequently used restriction enzyme to digest enterococcal DNA is *SmaI*. Other enzymes used for enterococci include *ApaI* or *SfiI* (Teixeira and Merquior, 2013).

PFGE for enterococci is mostly recommended for the purpose of evaluating genetic relatedness and tracing transmission of strains that are associated in time and location. One of the important advantages of this technique is that rearrangements in the genome, such as insertions and deletions of genetic elements may be visible.

## **2.8 Research goal**

The research goal in this thesis was to evaluate the hypothesis that food might be a source for the dissemination of antibiotic resistance genes to the human gastrointestinal microbiota. To that end, the aim of this research was to find out whether enterococci isolated from raw and fermented food can transfer their antibiotic resistance to human enterococci isolates and to other related bacterial genera.

## Chapter 3

### **Antimicrobial resistance of *Enterococcus* species from meat and fermented meat products isolated by a PCR-based rapid screening method**

#### **3.1 Abstract**

Enterococci are predominantly found in the gastrointestinal tract of humans and animals, but species commonly resident on vegetation are known. Their presence in large numbers in foods may indicate a lapse in sanitation and their ability to serve as a genetic reservoir of transferable antibiotic resistance is of concern. Conventional culture methods for identification of enterococci are slow and sometimes give false results because of the biochemical diversity of the organisms in this genus. This work reports the development of a PCR-based assay to detect enterococci at the genus level by targeting a 16S rRNA sequence. Published 16S rRNA sequences were aligned and used to design genus specific primers (EntF and EntR). The primers were able to amplify a 678 bp target region from *E. faecalis* ATCC 7080 and 20 other strains of enterococci from 11 different species, but there was no amplification by 32 species from closely related genera (*Pediococcus*, *Lactobacillus*, *Streptococcus* and *Listeria*) or species of *E. coli* and *Salmonella*. The PCR positive samples were plated, screened by a colony patch technique and their identities were confirmed by API 20 Strep panels and sequencing. When dry fermented sausage and ham as well as fresh meat batter for dry cured sausage manufacture were tested for enterococci by the method, 29 *Enterococcus* strains (15 *E. faecalis*, 13 *E. faecium*, and one *E. gallinarum*) were identified. When susceptibility of these enterococci to 12 antibiotics was tested, the highest incidence of resistance was to clindamycin (89.6%), followed by tetracycline hydrochloride (65.5%), tylosin (62%), erythromycin (45%), streptomycin and neomycin (17%), chloramphenicol (10.3%), penicillin

(10.3%), ciprofloxacin (10.3%) and gentamicin (3.4%). None was resistant to the clinically important drugs vancomycin or ampicillin. Most strains (27/29) were resistant to more than one antibiotic while 17 of 29 of strains were resistant to from three to 8 antibiotics. The molecular method developed was validated for speciation of enterococci and was useful in assessing uncooked processed meat products as a reservoir for multi-drug resistant *Enterococcus* species.

### **3.2 Introduction**

Enterococci are ubiquitous microorganisms related to lactic acid bacteria (LAB) and are capable of surviving extremes of temperature (5 to 60 °C) (Murray, 1990), pH (4.6 to 9.9) and high sodium chloride concentration (6.5% w/v). They are capable of growth in the presence of 40% (w/v) bile salts (Fisher and Phillips, 2009) and they commonly occur in foods, especially those of animal origin, such as meat and milk (Giraffa, 2002). Since they have the gastrointestinal tract of animals as their primary habitat, there is significant potential for their contamination of meat at slaughter. *Enterococcus* (*E.*) *faecalis* and *E. faecium* were shown to be the predominant isolates in raw meat from beef and hog carcasses (Knudtson and Hartman, 1993; Stiles et al., 1978), but *E. faecalis* was the most frequent isolate among the Gram-positive cocci found in a study on poultry (Turtura and Lorenzelli, 1994). Enterococci are the most thermotolerant of non-sporulating bacteria and some can survive pasteurization temperatures. Tolerance to environmental extremes explains their survival during processing of cooked and uncooked cured meats and their ability to multiply during fermentations (Hugas et al., 2003; Simpson et al., 1994). In processed meat the presence of enterococci reflects the extent of initial contamination or, in the case of dry sausage a weak fermentation by desirable bacteria. Thus they are considered technologically undesirable in dry fermented sausage (Holley et al., 1988).

Although long known as opportunistic human pathogens, enterococci more recently have distinguished themselves as major nosocomial pathogens and can cause bacteremia, endocarditis and other infections (Franz et al., 1999). Enterococci can be resistant to a wide variety of antibiotics commonly used in human medicine as well as those that are used for animal therapy, prophylaxis or for growth promotion. They are also known for their capacity to exchange genetic information by conjugation (Clewell and Dunny, 2002) and may spread antibiotic resistance among non-pathogenic organisms (Cocconcelli et al., 2003; Fisher and Phillips, 2009). Thus, there is concern about their presence in uncooked fermented meats because of the contribution they may make to the baseline level of antibiotic resistance in other genera and the potential for transfer of antibiotic resistant bacteria from the indigenous animal microflora to the human gastrointestinal tract (Mathur and Singh, 2005). Therefore, the health risk concern related their presence in the food chain would seem to be warranted (Hummel et al., 2007a; Klein et al., 1998; Vignaroli et al., 2011), and it appears prudent to restrict the spread of enterococci in food.

The objective of this study was to develop a rapid molecular-based method for identification and subsequent isolation of enterococci from meat and commercial meat products. The goal was to enable their more accurate detection, avoid lengthy culture incubation intervals and eliminate ambiguous phenotypic results (Knudtson and Hartman, 1992; Moore et al., 2006). Work undertaken examined the suitability of API-20 biochemical panels to support the PCR method and identify the less frequently encountered enterococci (Devriese et al., 1995; Kirschner et al., 2001). The intent was to design and use primers to rapidly detect enterococci at the genus level to reduce the labour required for screening isolates from large groups of samples. Following their identification, *Enterococcus* isolates from commercial fermented and dry sausages prepared in this

laboratory were examined for both antibiotic resistance profiles and for genes conferring those resistances.

### 3.3 Materials and Methods

#### 3.3.1 Bacterial strains

Eighteen different enterococcal reference strains used in this study were obtained from Dr. C. M. A. P. Franz, Max-Rubner Institute, Karlsruhe, Germany, and three were from the American Type Culture Collection (ATCC, Manassas, VA, USA). These plus 32 non-enterococcal isolates from the Department of Food Science, University of Manitoba culture collection, were used to verify the specificity of the PCR-based assay (Table 3.1). The reference enterococci strains were grown from frozen stocks kept at -80 °C in Brain Heart Infusion Broth (BHI, Difco, Fisher Scientific, Edmonton, AB, Canada) containing 50% (v/v) glycerol (Sigma, St. Louis, MO, USA) and were cultured on KF-Streptococcus Agar (KF-SA, Difco). *Lactobacillus*, *Listeria*, *Pediococcus* and *Streptococcus* species were grown from frozen (-80 °C) stocks in Lactobacilli MRS broth (Difco) plus 50% (v/v) glycerol and were cultured on Lactobacilli MRS agar. *Staphylococcus*, *Escherichia* and *Salmonella* species were grown from frozen (-80 °C) stocks in Tryptic Soy Broth (Difco) plus 50% (v/v) glycerol and were cultured on Tryptic Soy Agar. Strains were grown for 24 h at 37 °C before use.

#### 3.3.2 PCR primers

Enterococcal genus specific primers were designed after downloading 16S ribosomal RNA gene sequences from the Ribosomal Database Project (RDP: Center for Microbial Ecology, Michigan State University). The sequences were aligned using the ClustalW multiple sequence alignment

program (Thompson et al., 1994) and database search tools developed by the National Center for Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov/>). The primer EntF (5'-AGCGCAGGCGGTTTCTTAA-3') and EntR (5'-CTCGTTGTACTTCCCATTTGT-3') binding to positions 638-656 and 1339-1358, respectively and complementary to conserved enterococcal sequences were designed and used to amplify enterococcal DNA. These primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada).

### 3.3.3 Meat sampling

Fifty samples of fermented dry sausage and two samples of dry cured ham purchased at retail (Winnipeg, MB, Canada) plus 8 samples of dry sausage batter prepared in this laboratory after starter culture (*Pediococcus* and *Staphylococcus*) inoculation and before stuffing in casings were used in this study. The sausage types included Genoa, Hungarian dry salami, peppered dry salami, Gypsy dry salami, parmesan dry salami and pepperoni. These sausages were produced commercially and purchased from Canadian retail chains.

### 3.3.4 Meat sample processing

Twenty-five gram samples of meat were aseptically weighed and separately placed in a stomacher bag (Filtro-Bag, VWR, Edmonton, AB, Canada). Then 225 ml of BHI broth was added and samples were homogenized by stomaching (BagMixer 400, Intersciences Inc., Markham, ON, Canada) for 1 min. The bags were statically incubated 16 h for enrichment at 37 °C.

### 3.3.5 Screening for enterococci in meat samples

After incubation, DNA was extracted from the enriched cultures using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA). Polymerase chain

reaction (PCR) with the specific 16S rRNA primers EntF and EntR was used to screen positive samples. The DNA was amplified in a final volume of 25  $\mu$ L, where each PCR reaction contained 2  $\mu$ L of sample DNA, 5  $\mu$ L of 1X PCR buffer, 0.6  $\mu$ L of MgCl<sub>2</sub> (50 mM), 0.5  $\mu$ L dNTPs (10mM), 0.5  $\mu$ L of each primer (25 pmol), 1 unit of Taq polymerase (Lucigen Corporation, Middleton, USA), and water. Thermal cycling conditions were: 1 cycle of denaturation (94 °C, 2 min); 36 cycles of denaturation (94 °C, 1 min), annealing (56 °C, 1 min), and extension (72 °C, 1 min), followed by a final extension (72 °C, 5 min).

### 3.3.6 Isolation of enterococci

Broth cultures that yielded positive PCR results for enterococci were streaked onto two selective isolation media: KF-SA and Bile Esculin Azide Agar (BEAA, EMD Chemicals Inc., Darmstadt, Germany) to promote isolation. Plates were then incubated at 37 °C for 16-18 h and enterococci were screened by a colony patch technique (Islam et al., 2007). This involved selection of single colonies (including different morphological types) from both KF-SA and BEAA which were spot-plated on KF-SA to create a (6X6) grid pattern of 36 colonies and incubated at 37 °C for 16-18 h. From these plates, colonies in each row or column were pooled and DNA from each group of 6 colonies was extracted as previously described. Isolates from groups with a positive PCR result (with Ent primers) were confirmed individually by PCR before concluding they belonged to the genus *Enterococcus*. The isolates were also subjected to Gram staining, the catalase test, and tested for growth at 6.5% NaCl or with 40% bile and at 10 °C or 45 °C as well as at pH 9.6. All isolates were then identified to species using the API 20 Strep (BioMérieux, Marcy l'Etoile, France) biochemical test kit. Species identities were confirmed by 16S rRNA sequencing using universal primers 27f (5' AGAGTTTGATCMTGGCTCAG 3') and 1492r (5' TACGGYTACCTTGTTACGACTT 3') (Wang et al., 2003). The PCR sequencing reaction

contained 4  $\mu\text{L}$  of chromosomal DNA, 5  $\mu\text{L}$  of 1X PCR buffer, 1.2  $\mu\text{L}$   $\text{MgCl}_2$  (50 mM), 1  $\mu\text{L}$  dNTPs (10mM), 1  $\mu\text{L}$  of each primer (25 pmol), 2 units of Taq polymerase (Lucigen Corporation), and water to 50  $\mu\text{L}$ . Thermal cycling conditions were: 1 cycle of denaturation (94 °C, 1 min); 36 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 1.5 min), followed by a final extension (72 °C, 8 min). These sequencing reactions were done by Sanger Sequencing Services (McGill University and Génome Québec Innovation Centre, Montréal, QC, Canada).

### 3.3.7 Antibiotic preparation

Ten antibiotics currently registered in Canada for use in food animals plus vancomycin and ciprofloxacin were used in this study. Antibiotic powders of known potencies were obtained from Sigma-Aldrich, Canada Ltd. (Oakville, ON). The ranges of antibiotic concentration used were 0.125 to 64  $\mu\text{g}/\text{ml}$  for erythromycin, clindamycin and tylosin; 0.250 to 128  $\mu\text{g}/\text{ml}$  for ampicillin, penicillin G, chloramphenicol, tetracycline and vancomycin; 16 to 8192  $\mu\text{g}/\text{ml}$  for gentamicin; 32 to 16384  $\mu\text{g}/\text{ml}$  for streptomycin and neomycin; and 0.0625 to 32  $\mu\text{g}/\text{ml}$  for ciprofloxacin. Antimicrobials were dissolved in distilled water and filter sterilized through 0.20  $\mu\text{m}$  pore-sized syringe filter units (Fisher Scientific) except for tetracycline which was dissolved in ethanol (25%, v/v) as a solubility mediator. The effect of ethanol on the growth of enterococci was examined in susceptibility tests and found not to be measurable.

### 3.3.8 Antibiotic susceptibility and minimum inhibitory concentrations (MICs)

Fifty  $\mu\text{L}$  of double-strength sterile MH broth was placed into each well of 96-well microtiter plates (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA). Lysed horse blood was not used. To the first wells 50  $\mu\text{L}$  of antibiotic solutions were added and serial two-fold dilutions



were made to the desired concentrations. Wells were then inoculated with 50  $\mu$ L of bacterial suspension to obtain a final concentration in each well of approximately  $5 \times 10^5$  CFU/ml and then plates were covered and incubated overnight at 35 °C (CLSI, 2002). Reference strain *E. faecalis* ATCC 7080 and *E. faecalis* ATCC 29212 (CLSI, 2002) were used as controls to monitor the accuracy of this study.

Following incubation, 40  $\mu$ L of p-iodonitrotetrazolium violet was added to each well and plates were further incubated for 2 h (Eloff, 1998). The trials were conducted in triplicate. The MICs for enterococci were determined according the Clinical and Laboratory Standards Institute (CLSI, 2002), the European Food Safety Authority (EFSA, 2008) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, DANMAP (Bager and Emborg, 2001). The MIC was considered to be the lowest antimicrobial concentration at which no red color (signifying no metabolic activity) appeared. When no microbiological breakpoint was found using these reference guides, breakpoint values from structurally or functionally-related antibiotics were used. Isolates with MICs above these breakpoints were considered resistant.

### 3.3.9 Determination of resistance genes

DNA was extracted from overnight cultures grown in BHI broth, using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA). PCR assays were used to determine the genes associated with resistance to macrolides (*ermA*, *ermB*, *ermC* and *mefA/E*), tetracycline [genes for ribosomal protection proteins *tet(M)*, *tet(O)*, *tet(S)* or genes for efflux proteins *tet(K)*, and *tet(L)*], chloramphenicol (*cat*) and ciprofloxacin (*gyrA* and *perC*). The oligonucleotide primers used included those reported previously for *ermA*, *ermB*, *ermC* and *mefA/E* (Gevers et al., 2003; Sutcliffe et al., 1996), for *tet(M)*, *tet(O)*, *tet(S)*, *tet(K)*, and *tet(L)*

(Gevers et al., 2003) and for *cat*, *gyrA* and *perC* (Hummel et al., 2007a), and PCR was performed as described before (Gevers et al., 2003; Hummel et al., 2007a; Sutcliffe et al., 1996).

### 3.4 Result

#### 3.4.1 Development of *Enterococcus* genus specific primers

A pair of *Enterococcus* genus specific primers (EntF, EntR) was developed from two highly conserved enterococcal 16S ribosomal DNA sequences. The primers were tested with 21 known strains of enterococci plus bacteria from other related genera including *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Staphylococcus* and *Listeria* as well as with three non-related strains from the Gram-negative genera, *Escherichia* and *Salmonella*.

The enterococcal primers gave 678 base pair (bp) fragments when tested with enterococcal DNA from species including *E. avium*, *E. casseliflavus*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. flavescens*, *E. gallinarum*, *E. hirae*, *E. mundtii*, and *E. pseudoavium* (Fig 3.1). Under PCR conditions used each of these species gave a single predominant band. No amplification products were obtained when DNA from lactobacilli, pediococci, streptococci, staphylococci, *Listeria*, *Escherichia coli* or *Salmonella* was used (Table 3.1).

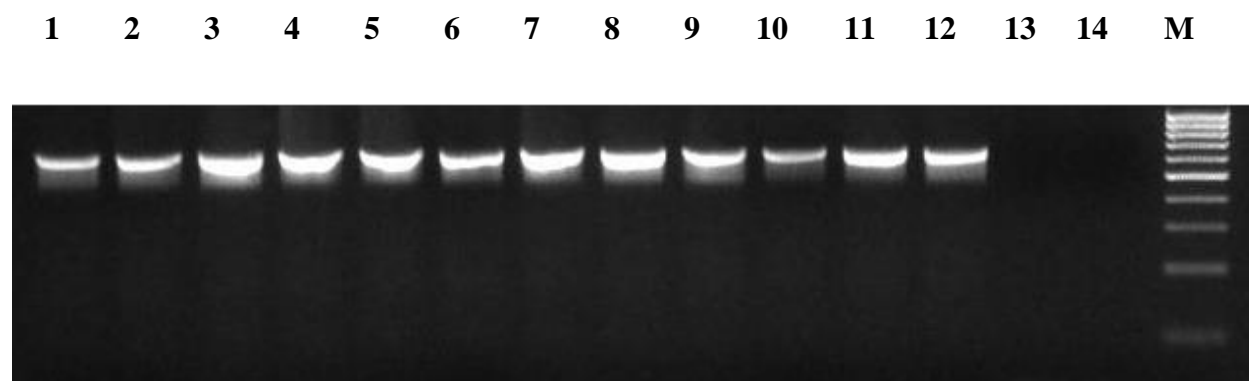
Table 3.1 Specificity of genus specific *Ent* primers in PCR assays with DNA from a variety of Gram-positive and Gram-negative bacterial species

Bacterial strain (Antibiotic resistance) <sup>a</sup>	PCR	Bacterial strain (Antibiotic resistance)	PCR
<i>Enterococcus avium</i> DSM 20679	+ <sup>b</sup>	<i>Lactobacillus paracasei</i> ATCC 24302	-
<i>Enterococcus casseliflavus</i> LMG 10745	+	<i>Lactobacillus sharpeae</i> ATCC 49974	-
<i>Enterococcus dispar</i> DSM 6630	+	<i>Listeria monocytogenes</i> GLM-1	-
<i>Enterococcus durans</i> LMG 10746	+	<i>Listeria monocytogenes</i> 2-138	-
<i>Enterococcus faecalis</i> FAIR-E-309	+	<i>Pediococcus pentosaceus</i> 2UM/114	-
<i>Enterococcus faecalis</i> JH2-2	+	<i>Pediococcus pentosaceus</i> UM/116P	-
<i>Enterococcus faecalis</i> LMG 7937	+	<i>Pediococcus pentosaceus</i> 127UM	-
<i>Enterococcus faecium</i> FAIR-E32	+	<i>Pediococcus pentosaceus</i> 2UM 121	-
<i>Enterococcus faecium</i> DSM 207477	+	<i>Pediococcus pentosaceus</i> 1168	-
<i>Enterococcus faecium</i> 472	+	<i>Pediococcus pentosaceus</i> MPL-E	-
<i>Enterococcus flavescens</i> DSM 7370	+	<i>Pediococcus pentosaceus</i> 2UM/102	-
<i>Enterococcus gallinarum</i> DSM 20628	+	<i>Pediococcus pentosaceus</i> 2UM/105	-
<i>Enterococcus hirae</i> LMG 6399	+	<i>Pediococcus pentosaceus</i> 2UM/108	-
<i>Enterococcus mundtii</i> DSM 4838	+	<i>Pediococcus pentosaceus</i> 2UM/113	-
<i>Enterococcus pseudoavium</i> FAIR-E 105	+	<i>Pediococcus pentosaceus</i> 116P	-
<i>Enterococcus faecalis</i> ATCC 19433	+	<i>Pediococcus acidilactic</i> ATCC 8081	-
<i>Enterococcus faecalis</i> ATCC 7080	+	<i>Pediococcus acidilactic</i> UM/104P	-
<i>Enterococcus gallinarum</i> ATCC 49573	+	<i>Pediococcus acidilactic</i> UM/119P	-
<i>Enterococcus faecium</i> FAIR-E 84 (Em, Te) <sup>a</sup>	+	<i>Pediococcus acidilactic</i> 122P	-
<i>Enterococcus faecalis</i> FAIR-E 85 (Ci, Cm, Em, Te) <sup>a</sup>	+	<i>Pediococcus acidilactic</i> UM/129P	-
<i>Enterococcus faecalis</i> FAIR-E 324 (Cm, Te)	+	<i>Pediococcus acidilactic</i> 1288	-
<i>Lactobacillus plantarum</i> UM/131L	-	<i>Streptococcus pyogenes</i> ErmB	-
<i>Lactobacillus plantarum</i> UM/134L	-	<i>Staphylococcus carnosus</i> 109M	-
<i>Lactobacillus plantarum</i> UM/135L	-	<i>Escherichia coli</i> O157:H7 02:1840	-
<i>Lactobacillus plantarum</i> ATCC 8014	-	<i>Escherichia coli</i> O157:H7 NOO-666	-
<i>Lactobacillus bulgaricus</i> ATCC 11842	-	<i>Salmonella</i> Typhimurium SGI1(Tet R)	-
<i>Lactobacillus rhamnosus</i> ATCC 7469	-		

<sup>a</sup>Ciprofloxacin (Ci), Chloramphenicol (Cm), erythromycin (Em) and tetracycline (Te).

<sup>b</sup>PCR amplification with Ent primers; positive (+), no amplification (-).

Figure 3.1 Example of PCR amplifications by the *Enterococcus* genus specific PCR primers



Lanes: 1, *Enterococcus faecalis* ATCC 7080; 2, *Enterococcus faecalis* ATCC 19433; 3, *Enterococcus avium* DSM 20679; 4, *Enterococcus casseliflavus* LMG 10745; 5, *Enterococcus dispar* DSM 6630; 6, *Enterococcus durans* LMG 10746; 7, *Enterococcus flavescens* DSM 7370; 8, *Enterococcus gallinarum* DSM 20628; 9, *Enterococcus hirae* LMG 6399; 10, *Enterococcus pseudoavium* FAIR-E 105; 11, *Enterococcus mundtii* DSM 4838; 12, *Enterococcus faecium* FAIR-E32; 13, *Escherichia coli* O157:H7 02:1840; 14, Blank; M, standard 100-bp molecular size ladder.

### 3.4.2 Screening of *Enterococcus* from meat products

When the 60 meat samples were analyzed for the presence of enterococci using the enterococcal genus specific primers developed, 56.7% (34/60) of the samples were found positive for enterococci.

### 3.4.3 Isolation of *Enterococcus* from meat products

After plating the 34 PCR positive samples on KF-SA and BEAA, 29 isolates were identified as having biochemical properties characteristic of *Enterococcus*, i.e. they were Gram-positive, catalase negative and showed visible growth at 10 °C and 45 °C. They also grew in broth containing 6.5% NaCl or 40% bile and at pH 9.6. Bacteria in the remaining 5 PCR positive samples did not grow when plated on KF-SA and BEAA, and were not tested further. Thus 8.3% (5/60) of meat samples gave an initially false positive result when tested with *Enterococcus* specific PCR primers.

The selected 29 isolates were identified to the species level using API 20 Strep and 25 strains were randomly selected and confirmed by 16S rRNA sequencing. The species found in the meat products were *E. faecalis* (51.7%), *E. faecium* (44.8%) and *E. gallinarum* (3.4%) (Table 3.2).

Table 3.2 *Enterococcus* species isolated from fermented dry sausage, dry cured ham and unfermented dry sausage batter confirmed at the species level by API 20 STREP and sequencing

<i>Enterococcus</i> isolates	API 20 Strep	Sequencing <sup>a</sup>
<i>E. faecalis</i> <sup>b</sup> S3	+	
<i>E. faecalis</i> S5	+	+
<i>E. faecium</i> S6	+	+
<i>E. faecium</i> S9	+	+
<i>E. faecalis</i> S10	+	+
<i>E. faecalis</i> S11	+	+
<i>E. faecalis</i> S13	+	+
<i>E. faecalis</i> S14	+	
<i>E. faecium</i> S15	+	+
<i>E. faecalis</i> S18	+	+
<i>E. gallinarum</i> S19	+	+
<i>E. faecium</i> S22	+	+
<i>E. faecalis</i> S25	+	+
<i>E. faecium</i> S27	+	+
<i>E. faecium</i> S28	+	+
<i>E. faecium</i> S29	+	+
<i>E. faecium</i> S30	+	+
<i>E. faecium</i> S31	+	+
<i>E. faecium</i> S34	+	+
<i>E. faecalis</i> S36	+	+
<i>E. faecalis</i> S38	+	+
<i>E. faecalis</i> S39	+	+
<i>E. faecalis</i> S40	+	+
<i>E. faecalis</i> S41	+	
<i>E. faecalis</i> S48	+	
<i>E. faecium</i> S50	+	+
<i>E. faecalis</i> <sup>c</sup> H1	+	+
<i>E. faecium</i> <sup>d</sup> M1	+	+
<i>E. faecium</i> M2	+	+

<sup>a</sup> Sequencing reaction with universal primers 27f and 1492r (Wang et al., 2003; Appendix I);

confirmed by API 20 Strep or sequencing using universal primers (+).

<sup>b</sup>(S) enterococci isolated from fermented dry sausage;

<sup>c</sup>(H) enterococci isolated from dry cured ham;

<sup>d</sup>(M) enterococci isolated from unfermented dry sausage batter.

#### 3.4.4 Antibiotic resistant phenotypes

It was found that 28 of the 29 enterococci isolates exhibited resistance to at least two antibiotics and 17/29 isolates were resistant to from three to 8 antibiotics, but none was resistant to ampicillin or vancomycin (Table 3.3). Of *E. faecium* strains, 23% were resistant to either ciprofloxacin or penicillin. Ten of 13 *E. faecium* strains were resistant to clindamycin or erythromycin, and 8 were resistant to tylosin. Almost half (46%) were resistant to tetracycline with at least three strains resistant to each of the remaining antibiotics. In contrast, all 15 *E. faecalis* strains were resistant to clindamycin, 11 were resistant to tetracycline and 9 were resistant to tylosin. *E. faecalis* strains were not resistant to penicillin, but 4/13 isolates were resistant to erythromycin and  $\leq 3$  strains were resistant to each of the remaining antibiotics. The *E. gallinarum* strain was also resistant to tetracycline and clindamycin.

#### 3.4.5 PCR detection of antibiotic resistant genes

When meat isolates were tested for the presence of genes that confer resistance to tetracycline, macrolides, chloramphenicol and fluoroquinolones (Table 3.3), of 19 isolates showing phenotypic resistance to tetracycline 15 were positive for the gene encoding the ribosomal protection protein *tet(M)*, but *tet(O)* and *tet(S)* were absent. Genes encoding the tetracycline efflux pumps *tet(K)* and *tet(L)* were amplified in 11 isolates. When three erythromycin ribosomal methylase genes (*erm*) were targeted in 13 erythromycin resistant isolates, only *ermB* genes were detected, and they were present in 7 isolates. While no isolates were able to amplify *ermA* and *ermC* genes, the gene encoding macrolide efflux (*mefA/E*) was detected in one strain each of *E. faecalis* and *E. faecium*. Only one isolate, *E. faecium* S15, was positive for the chloramphenicol resistance (*cat*) gene. Although three isolates showed phenotypic resistance to 4 $\mu$ g/mL ciprofloxacin, neither *gyrA* nor *perC* genes were amplified.

Table 3.3 Antibiotic susceptibility and the occurrence of resistance genes in 29 enterococci isolates from meat and fermented meat products

Enterococci meat Isolates	MIC (resistance breakpoint [ $\mu\text{g/ml}$ ]) <sup>a</sup>										Antibiotic resistance gene(s)	
	Cm (16)	Em (4)	Te (8)	Sm (1024)	Ne (1024)	Ge (512)	Ty (4)	Cl (4)	Ci (2)	PG (8)		
<i>E. faecalis</i>												
<sup>b</sup> S3								<sup>c</sup> 32 <sup>R</sup>				
S5			32 <sup>R</sup>					16 <sup>R</sup>				<i>tet</i> (M, K)
S10							8 <sup>R</sup>	16 <sup>R</sup>				
S11							8 <sup>R</sup>	16 <sup>R</sup>				
S13	32 <sup>R</sup>	64 <sup>R</sup>	64 <sup>R</sup>	>4096 <sup>R</sup>	>4096 <sup>R</sup>		64 <sup>R</sup>	64 <sup>R</sup>				<i>tet</i> (M, L), <i>ermB</i>
S14			32 <sup>R</sup>					16 <sup>R</sup>				<i>tet</i> (M)
S18			64 <sup>R</sup>					32 <sup>R</sup>				<i>tet</i> (M, K, L)
S25			64 <sup>R</sup>				8 <sup>R</sup>	16 <sup>R</sup>				<i>tet</i> (M, L)
S36		64 <sup>R</sup>	64 <sup>R</sup>	>4096 <sup>R</sup>	>4096 <sup>R</sup>		64 <sup>R</sup>	64 <sup>R</sup>				
S38							8 <sup>R</sup>	8 <sup>R</sup>				
S39			128 <sup>R</sup>					16 <sup>R</sup>				<i>tet</i> (M)
S40			64 <sup>R</sup>					16 <sup>R</sup>				<i>tet</i> (M, K)
S41		64 <sup>R</sup>	64 <sup>R</sup>	>4096 <sup>R</sup>		>4096 <sup>R</sup>	64 <sup>R</sup>	64 <sup>R</sup>				<i>tet</i> (M, K), <i>ermB</i> , <i>mefA/E</i>
S48			64 <sup>R</sup>				8 <sup>R</sup>	32 <sup>R</sup>				<i>tet</i> (M, K)
<sup>d</sup> H1		16 <sup>R</sup>	64 <sup>R</sup>					16 <sup>R</sup>				<i>ermB</i>
<i>E. faecium</i>												
S6							8 <sup>R</sup>	16 <sup>R</sup>	4 <sup>R</sup>			
S9		8 <sup>R</sup>	32 <sup>R</sup>	>4096 <sup>R</sup>				8 <sup>R</sup>		16 <sup>R</sup>		<i>tet</i> (M, L)
S15	32 <sup>R</sup>	16	64 <sup>R</sup>	>4096 <sup>R</sup>	>4096 <sup>R</sup>			8 <sup>R</sup>	4 <sup>R</sup>	16 <sup>R</sup>		<i>tet</i> (M, L), <i>ermB</i> , <i>cat</i>
S22		16 <sup>R</sup>					8 <sup>R</sup>	32 <sup>R</sup>				
S27		16 <sup>R</sup>	128 <sup>R</sup>	>4096 <sup>R</sup>	>4096 <sup>R</sup>			8 <sup>R</sup>	4 <sup>R</sup>			<i>tet</i> (M), <i>ermB</i>
S28		8 <sup>R</sup>										
S29		8 <sup>R</sup>					8 <sup>R</sup>	8 <sup>R</sup>				
S30	32 <sup>R</sup>		16 <sup>R</sup>				8 <sup>R</sup>	8 <sup>R</sup>				<i>tet</i> (M)
S31		16 <sup>R</sup>					8 <sup>R</sup>			16 <sup>R</sup>		<i>tet</i> (M)
S34			64 <sup>R</sup>					16 <sup>R</sup>				
S50		64 <sup>R</sup>	128 <sup>R</sup>				64 <sup>R</sup>	64 <sup>R</sup>				<i>tet</i> (M), <i>ermB</i>
<sup>e</sup> M1		16 <sup>R</sup>	16 <sup>R</sup>				8 <sup>R</sup>					<i>mefA/E</i>
M2							8 <sup>R</sup>	16 <sup>R</sup>				
<i>E. gallinarum</i> S19			64 <sup>R</sup>					16 <sup>R</sup>				<i>tet</i> (M, K, L)



<sup>a</sup>Ciprofloxacin (Ci), chloramphenicol (Cm), clindamycin (Cl), erythromycin (Em), gentamicin (Gm), neomycin (Ne), penicillin G (PG), streptomycin (Sm), tetracycline (Te) and tylosin (Ty). Breakpoint values are from CLSI (2002), EFSA (2008) and DANMAP Bager and Emborg, 2001).

<sup>b</sup>(S) enterococci isolated from fermented dry sausage;

<sup>c</sup>R, antibiotic resistant;

<sup>d</sup>(H) enterococci isolated from dry cured ham;

<sup>e</sup>(M) enterococci isolated from unfermented dry sausage batter.

### 3.5 Discussion

Enterococci are widely distributed in nature and are associated with the spoilage of meat, especially fermented meat products (Hugas et al., 2003). The major concerns with enterococci today are that they can cause serious infection in humans and are becoming an increasing problem in nosocomial settings. Enterococci in foods may act as reservoirs for antibiotic resistance genes (Valenzuela et al., 2009) and, thus, there is need for methods to screen for their presence and reduce the opportunity for their spread. Conventional identification by phenotypic characteristics is difficult and requires several days to complete. While molecular genetic techniques have been developed, these are used mainly for detection and identification of enterococci in clinical samples (Ke et al., 1999; Palladino et al., 2003; Roger et al., 1999). Since interfering strains and substances likely differ among samples of different origin, it would be desirable to have a rapid assay for the detection of enterococci in food. Deasy et al. (2000) developed 16S rRNA gene primers which were used in PCR to distinguish between enterococci and lactococci, largely of dairy origin, but food samples were not analysed. While the method had significant discriminatory power, enterococcal and lactococcal gene sequences used to construct primers could not be confirmed in the present work to be consistent with database descriptions of DNA for these organisms. Nonetheless, since it is accepted that 16S rRNA gene sequences are highly conserved and can be used to measure the evolutionary distance and relatedness of organisms (Clarridge, 2004), enterococcal 16S ribosomal gene sequences were used to design primers. By analyzing 16S rRNA sequences obtained from the Ribosomal Database Project, which were aligned using the ClustalW multiple sequence alignment program, as well as the database search tools developed by the National Center for Biotechnology Information, a set of *Enterococcus* specific primers EntF and EntR were developed for testing with 53 bacterial species including 21 enterococci and 32 non-

enterococci. With these primers it was possible to detect all reference enterococcal species, but there was no DNA amplification by 8 strains of *Lactobacillus*, 17 strains of *Pediococcus* and one strain of *Staphylococcus carnosus* which can occur in processed meats. Most of these organisms are used as starter cultures for the production of fermented sausage (Hugas and Monfort, 1997; Smith and Palumbo, 1983). Further, there was no amplification (by the enterococci genus specific primers designed) of DNA from two *Listeria monocytogenes* strains and one *Streptococcus pyogenes* strain or by that from two strains of *Escherichia coli* and one strain of *Salmonella* Typhimurium.

The PCR assay found enterococci in 34 of 60 meat samples. When the analysis was repeated on groups of 6 culture mixtures by colony patch screening, it was found that only 29 isolates required biochemical confirmation by API 20 Strep tests. The API kit confirmed all PCR positive results at the species level, perhaps because meat product isolates are among commonly encountered strains. However, Deasy et al. (2000) did not find the same extent of agreement between kit and PCR results. The accuracy of results was further confirmed in the present work by sequence analysis of the 16s rRNA gene from isolates. These sequences have been deposited with GenBank and accession numbers have been assigned (Appendix I).

In this study, the most common enterococcal species found in meat and fermented dry sausage and ham were *E. faecalis* (51.7%) and *E. faecium* (44.8%). *E. gallinarum* (3.4%) was also isolated from fermented sausage. In foods of animal origin, such as minced beef and pork, fermented sausage, cheese and poultry meat, *E. faecalis* is most often isolated, followed by *E. faecium*. Other species such as *E. gallinarum*, *E. durans*, *E. hirae* and *E. avium* are less frequently identified (Klein, 2003). Peters et al. (2003) also found a higher percentage of *E. faecalis* than *E. faecium* in foods of animal origin. In contrast, Martin et al. (2005) found *E. faecium* to be the predominant

species in slightly fermented sausage. It is significant that *E. faecalis* and *E. faecium* are the most prominent species among enterococci causing human disease (Franz et al., 1999). These two species have also developed resistance to a wide variety of clinically important antibiotics (Huycke et al., 1998) and together they are a leading cause of healthcare-associated infections reported to the Center for Disease Control and Prevention, CDC (Hidron et al., 2008). Use of enterococci as an indicator of the sanitary quality of food has been popular, particularly since *E. faecalis* is often of fecal origin (Franz et al., 1999; Jay et al. 2005; Knudtson and Hartman, 1992). However, their sanitary significance in food is uncertain because some strains present may be naturally resident on plants and insects (Jay et al. 2005; Mundt, 1982). Although enterococci naturally present on plants are considered non-pathogenic to humans, antibiotic resistance can also be found in these types of enterococci (Müller et al., 2001).

It is possible that the initial false positive PCR results observed here with meat samples were caused by *Carnobacterium* species which could have been present. These organisms appear to have some homology in 16S rRNA gene sequences with enterococci (Deasy et al., 2000). It is planned that *Lactococcus* species together with *Carnobacterium* species and *Brochothrix thermosphacta* will be examined for reactivity with the primers designed in the present work.

Results from the present study revealed high incidences of resistance to clindamycin, tetracycline, erythromycin and tylosin, with all isolates being resistant to at least one antibiotic tested. One strain of *E. faecium* S15 was resistant to 8 antibiotics and *E. faecalis* S13 was resistant to 7 antibiotics. Among ribosomal protection protein genes tested, most of the tetracycline resistant ( $Tet^R$ ) strains carried either *tet(M)*, *tet(K)* or *tet(L)* or a combination of two or three of these determinants with *tet(M)* being the most common. This was also observed by Huys et al. (2004) in enterococci isolated from food. Genes for *tet(O)* and *tet(S)* were not detected as reported by

Hummel et al. (2007b). Wilcks et al. (2005) showed a very low incidence of *tet(O)* and *tet(S)* in Tet<sup>R</sup> enterococci isolated from raw food. However, the two genes *tet(K)* and *tet(L)* occurred in 36% and 32% of enterococci, respectively, in the present study. In contrast, Huys et al. (2004) did not detect the *tet(K)* gene among their isolates, whereas Hummel et al. (2007b) found *tet(K)* in 56% of Tet<sup>R</sup> food enterococci, which is consistent with the present results. In *E. gallinarum tet(M)*, *tet(K)* and *tet(L)* were detected (Table 3.3).

Thirteen (44.8%) of the 29 isolated enterococci strains were resistant to erythromycin (Em<sup>R</sup>). When tested for the presence of *ermA*, *ermB*, *ermC* or *mefA/E*, none of the Em<sup>R</sup> strains was able to amplify genes for *ermA* or *ermC*. The *ermB* gene was detected in 7 (53.8%) of the Em<sup>R</sup> strains. This is consistent with the observation by Hummel et al. (2007b), where *ermB* was the dominant Em<sup>R</sup> gene and *ermA* or *ermC* were not detected. The gene for the *mefA/E* type efflux pump was present in one strain of *E. faecalis* S41 along with *ermB*, but *mefA/E* was solely responsible for erythromycin resistance in *E. faecium* M1. For 6 antibiotic resistant enterococci strains, no PCR product was obtained with any of the primers, indicating that other factors influence resistance in these strains.

Of the 3/29 isolates that were resistant to chloramphenicol only one contained the *cat* gene. The occurrence of chloramphenicol resistance among these fermented sausage enterococci is consistent with an earlier observation by Huys et al. (2004) where 2 of the 33 enterococci from foods were resistant. In another investigation Hummel et al. (2007b) showed that of 38 enterococci from foods 25 were resistant to chloramphenicol, which is much higher than the low incidence reported in the present study.

Enterococci possess a natural low level of resistance to aminoglycosides (Murray, 1990). Results revealed that 5 (17%) of the 29 strains tested had a high level of resistance to both streptomycin and neomycin. A high level of aminoglycoside resistance among clinically relevant enterococci from food animals, especially among *E. faecalis* and *E. faecium*, has been reported (Donabedian, 2003). One *E. faecalis* isolate from the present work showed high gentamicin resistance, which is similar to an earlier observation by Peters et al. (2003).

Enterococci are intrinsically resistant to  $\beta$ -lactam antibiotics (Kak and Chow, 2002). Results here are not in agreement with this generalization, since all isolates were sensitive to ampicillin and only three strains were resistant to penicillin. That only *E. faecium* strains were resistant to penicillin is consistent with the historical observation that *E. faecium* strains were more frequently resistant to penicillin than those of *E. faecalis* (Murray, 1990).

Although the enterococci in the present study showed resistance to a number of antibiotics, they did not show resistance to vancomycin, and had a low frequency of resistance towards ciprofloxacin. These food enterococcal strains were largely still susceptible to these clinically relevant antibiotics. This result is similar to an earlier study where all 182 *Enterococcus* species isolated from traditional fermented meat products were susceptible to vancomycin and one showed resistance towards ciprofloxacin (Barbosa et al., 2009). However several earlier investigations showed the occurrence of vancomycin resistant enterococci in foods of animal origin, which is in contrast to this study (Klein et al., 1998; Van den Braak et al., 1998).

In conclusion, results presented here suggest that the enterococci isolated from meat and fermented meats may act as a reservoir for antimicrobial resistance genes. The method developed can rapidly and accurately screen enterococci from large numbers of meat samples within 24 h. In contrast, 2

to 3 days are necessary for traditional methods and these often generate inconclusive results. Thus, this method has value in both experimental as well as in industrial settings for the rapid screening of enterococci in food to understand the contribution they make to the burden of illness and in developing effective policy to reduce the frequency of antibiotic resistance in medically important bacteria.

## Chapter 4

### **Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25 °C and 37 °C**

#### **4.1 Abstract**

Twenty-nine *Enterococcus* strains from raw and fermented meat products were screened for the presence of virulence genes, including those for aggregation substances (*asa1* and *asa373*), cytolysin activator (*cylA*), collagen binding protein (*ace*), endocarditis antigen (*efaA*), enterococcal surface protein (*esp*) and gelatinase (*gelE*). Virulence gene occurrence, expression of gelatinase and pheromone aggregation was greater in *Enterococcus faecalis* than in *Enterococcus faecium* strains. All *E. faecalis* and 54% of *E. faecium* were positive for at least one or more virulence gene. The only strain of *Enterococcus gallinarum* tested also contained virulence genes. The effect of different growth temperatures (25 and 37 °C) on biofilm formation using polystyrene plates was also assessed. Strong biofilm formation occurred at lower than optimum temperature in all three species of enterococci. Neither *esp* nor *gelE* were necessary for biofilm formation and this relationship was species rather than strain specific. This study emphasizes the importance of enterococci as a reservoir of virulence genes and the potential for their genetic transfer to human strains following consumption of uncooked or undercooked contaminated meat.

#### **4.2 Introduction**

Enterococci inhabiting the gastrointestinal tract of humans and other animals have emerged as a major cause of nosocomial infections in recent decades (Franz et al., 1999). They can be found in other environments including soil, plants and water, especially when contaminated with fecal



materials (Aarestrup et al., 2002). Enterococci have been isolated from various foods of animal origin and they can be resistant to a wide variety of antibiotics commonly used in human medicine as well as those that are used for animal therapy, prophylaxis or for growth promotion (Chapter 3). They are also known for their capacity to exchange genetic information by conjugation (Clewell and Dunny, 2002) and may spread antibiotic resistance among non-pathogenic organisms (Cocconcelli et al., 2003; Fisher and Phillips, 2009).

Although enterococcal virulence factors are found more frequently among clinical strains, they are also detected in food isolates (Eaton and Gasson, 2001; Franz et al., 2001). Over the years several virulence factors have been identified in food enterococci which include: aggregation substances, cytolyisin, pheromone, gelatinase, enterococcal surface protein and biofilm formation (Aslam et al., 2012; Barbosa et al., 2010; Foulquié Moreno et al., 2006; Franz et al., 2001; Martin et al., 2005; Ribeiro et al., 2011; Valenzuela et al., 2009). Occurrence of several virulence traits together with a high level of resistance to a wide variety of antibiotics suggest that enterococci may represent a reservoir of virulence and antibiotic resistant genes in the food chain which is a matter of concern (Ogier and Serror, 2008).

Biofilms can be defined as matrix-embedded bacterial populations adhered to a surface or to each other (Poulsen, 1999). Bacterial biofilms are more resistant to environmental stress than their free living counterparts and their attachment to food products or food contact surfaces accelerate spoilage, complicate cleaning and facilitate disease transmission. In the food industry biofilms produced by *Listeria monocytogenes*, *Salmonella* species, *Escherichia coli*, *Pseudomonas* species, *Brochothrix thermosphacta* and *Lactobacillus* species have been the subjects of greatest study (Di Bonaventura et al., 2008; Giaouris et al., 2013; and Rode et al., 2007). Although biofilms have been suggested to be important factors in the pathogenesis of enterococcal infection (Sandoe et al.

2003), few studies of biofilms have been done with food enterococci. Moreover, most of the studies on enterococcal biofilms have been conducted using optimum environmental conditions, allowing the cells to grow and divide normally. However, there are several studies on biofilms demonstrating that survival of enterococci involves stress-response mechanisms (George et al., 2005; Lleo et al., 2007). The present study examined the influence of suboptimal temperature exposure (25 °C), similar to conditions used for dry sausage fermentation, upon the ability of enterococci from meat to form biofilms. Very little information is currently available on the distribution of virulence genes in and biofilm formation by enterococci from fermented meat, especially in commercially fermented dry sausages and ham. Therefore, the current work also investigated the presence of virulence genes in antibiotic resistant enterococci isolated from raw sausage batter, commercially fermented sausage and dry cured ham.

### **4.3 Materials and Methods**

#### **4.3.1 Bacterial strains**

Twenty-nine enterococcal strains showing some antibiotic resistant traits isolated from raw and fermented meat, identified as *Enterococcus faecalis* (n=14), *Enterococcus faecium* (n=11) and *Enterococcus gallinarum* (n=1) were examined. Reference strains of *E. faecalis* FAIR-E 324 (*asaI*<sup>+</sup>, *cylA*<sup>+</sup>, *efaA*<sup>+</sup>, *gelE*<sup>+</sup> and *esp*<sup>+</sup>), *E. faecalis* JH2-2 (*ace*<sup>+</sup>; sex pheromone producer), *E. faecalis* OG1X:pCF10 (*asa373*<sup>+</sup>) and *E. faecalis* ATCC 29212 (a strong biofilm producer) were used (Chapter 3). Enterococci strains were grown from frozen stocks kept at -80 °C in Brain Heart Infusion Broth, BHIB (Difco, Fisher Scientific, Edmonton, AB, Canada) containing 50% (v/v) glycerol (Sigma, St. Louis, MO, USA) and were cultured on KF-Streptococcus Agar (Difco) at 37 °C.

#### 4.3.2 Detection of virulence genes by PCR

DNA was extracted from overnight cultures grown at 37 °C in BHIB (Difco), using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA, USA). The sequences of oligonucleotide primers used to amplify virulence genes are listed in Table 4.1. Primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada) and PCR was performed as described before (Creti et al., 2004). Briefly, 2 µL of chromosomal DNA was used as the template in a final volume of 25 µL of PCR mixture, which contained 12.5 µL of PCR Master Mix 2X (Promega, Madison, WI, USA), 0.5 µL of each primer (25 pmol) and water to 25 µL. Thermal cycling conditions were: 1 cycle of denaturation (94 °C, 3 min); 36 cycles of denaturation (94 °C, 1 min), annealing (temperatures indicated in Table 4.1, 1 min), and extension (72 °C, 1 min), followed by a final extension (72 °C, 5 min).

Table 4.1 PCR primers and product sizes for detection of *Enterococcus* virulence determinants

Gene	Primer sequence (5'→3')	Product size (bp)	Annealing temperature (°C)	Reference
<i>esp</i>	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	932	61	Shankar et al., 1999
<i>gelE</i>	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	405	52	Eaton and Gasson, 2001
<i>asa1</i>	CCAGCCAACACTATGGCGGAATC CCTGTCGCAAGATCGACTGTA	529	51	Creti et al., 2004
<i>asa373</i>	GGACGCACGTACACAAAGCTAC CTGGGTGTGATTCCGCTGTTA	619	59	Creti et al., 2004
<i>cylA</i>	GACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTTAC	688	54	Creti et al., 2004
<i>ace</i>	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	56	Creti et al., 2004
<i>efaA</i>	GCCAATTGGGACAGACCCTC CGCCTTCTGTTTCCTTCTTTGGC	688	56	Creti et al., 2004

#### 4.3.3 Gelatinase assay

Production of gelatinase was determined using two methods. First, Todd-Hewitt Agar (IBI Scientific, Peosta, IA, USA) containing 30g/L gelatin (Difco) was used. After inoculation of pure test cultures previously grown at 37 °C for 24h, the plates were incubated overnight at 37 °C and then cooled at 4 °C for 5 h. The appearance of a turbid halo around the colonies was considered positive for gelatinase activity. In the second method 0.8% Nutrient Broth (Oxoid Ltd., Basingstoke, England) with 12% gelatin (Difco) was used (Marra et al., 2007). Pure cultures were individually stabbed into tubes, incubated for 24 to 72 h at 37 °C and then held at 4 °C for 30 min. In tubes where an organism produced sufficient gelatinase, the gelatin remained liquefied upon cooling.

#### 4.3.4 Pheromone clumping assay

The production of aggregation substances was determined in enterococci isolates using the clumping assay described by Dunny et al. (1979) in the presence of the sex pheromone-producing *E. faecalis* strain JH2-2. Briefly, *E. faecalis* JH2-2 was grown in Todd-Hewitt Broth, THB (IBI Scientific) for 18 h at 37 °C. The pheromone-containing supernatant was obtained by centrifuging at 10,823 x g (Franz et al., 2001) and sterilized by autoclaving for 15 min. Each well of a 96 well polystyrene microtiter plate (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA) containing 200 µL of serially diluted pheromone in THB was inoculated with 20 µL of each test culture (grown at 37 °C for 24 h) and incubated at 37 °C with shaking. Cell clumping (positive response) was examined at 2, 4, 6 and 18 h when samples were mounted on glass slides and observed by phase-contrast microscopy. Strains of *E. faecalis* OG1X:pAM373 and *E. faecalis*

JH2-2 (which does not react with its pheromone) were used as positive and negative controls, respectively.

#### 4.3.5 Biofilm assay

Detection of biofilm formation by enterococci *in vitro* was studied using the method described by Schlüter et al. (2009). Briefly, the strains were grown in Tryptic Soy Broth, TSB (Difco) with 1% glucose (Sigma, St. Louis, MO, USA) and incubated overnight at 37 °C and at 25 °C. The cells were pelleted, re-suspended in fresh medium and normalized to an absorbance of 1.00 at 595 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). The cultures were diluted 1:40 and 200 µL of cells were dispensed into wells of a flat-bottom polystyrene microtiter plate (Becton Dickinson and Co.). The plates were incubated for 24 h at 37 °C and 25 °C without agitation to allow bacterial growth and formation of biofilm. After incubation, wells were washed with sterile phosphate buffered saline (PBS) and air-dried in a 60 °C incubator (Isotemp, Fisher Scientific) for 1 h. Biofilm formation was quantified using crystal violet staining and absorbance measured at 595 nm with a Synergy H4 Hybrid Micro-plate Reader (Biotek Instruments, Winooski, VT, USA). Biofilms were scored as:  $OD_{595} < 0.120$ , non-producer;  $OD_{595}$  between 0.120 and 0.240, medium producer;  $OD_{595} > 0.240$  to 0.70, strong producer (Schlüter et al., 2009);  $OD_{595} > 0.71$ , very strong producer (Macovei et al., 2009).

#### 4.3.6 Statistical analysis

Biofilm assays were performed for each strain in groups of 12 replicates and repeated twice. The statistical significance of mean differences ( $p < 0.05$ ) was assessed by analysis of variance using JMP® 8.02 (SAS Institute Inc.) and differences in results between two temperatures were compared using Student's *t*-test.

#### 4.4 Result and Discussion

Results from examining enterococcal isolates for virulence genes that encode aggregation substance (*asa1* and *asa373*), surface protein (*esp*), gelatinase (*gelE*), cytolysin activator (*cylA*), collagen binding protein (*ace*) and endocarditis antigen (*efaA*) by PCR as well as for gelatinase and the clumping response to the presence pheromone by phenotypic assay are shown in Table 4.2.

The aggregation substance gene, *asa1*, was present in 48.3% (14/29) of isolates. It was associated more frequently with *E. faecalis* (10/15) than with *E. faecium* (3/13). Franz et al. (2001) also reported finding *asa1* in only *E. faecalis* food isolates. Similarly, Aslam et al. (2012) found *E. faecalis* carrying the *asa1* gene more frequently in meat isolates which is in agreement with results from the present study. Only 3/29 strains (*E. faecalis* S13 and S41 and *E. faecium* S27) were positive for the *asa373* gene and these were also positive for the *asa1* gene. The clumping reaction occurred in all strains carrying either or both the *asa1* and *asa373* genes in the presence of sex pheromone produced by *E. faecalis* JH2-2, except *E. faecalis* H1, which was *asa1* positive. In this study it was not surprising that aggregation substance genes were more frequently associated with *E. faecalis* than with *E. faecium* since plasmids responsible for pheromone production are more commonly associated with *E. faecalis* (Dunny, 1990). The only strain of *E. gallinarum* tested also carried the *asa1* gene and showed the clumping phenotype.

The *cylA* gene which is associated with cytolysin production was found in two *E. faecalis* (S10 and S11) strains. This result is consistent with that of Aslam et al. (2012) where cytolysin producing genes were found in *E. faecalis* isolated from poultry meat.

Table 4.2 Occurrence of virulence factors among enterococci isolated from raw and fermented meat

Enterococci meat isolates	Virulence gene							Gelatinase activity		Clumping phenotype
	<i>esp</i>	<i>gel</i> <i>E</i>	<i>asa-</i> <i>I</i>	<i>asa-</i> <i>373</i>	<i>cyl-</i> <i>A</i>	<i>ace</i>	<i>efa-</i> <i>A</i>	Gelatinase (3% gelatin)	Gelatinase (12% gelatin)	
<i>E. faecalis</i> <sup>b</sup> S3						+	+			
<i>E. faecalis</i> S5		+	+			+	+	+	+	+
<i>E. faecalis</i> S10		+			+	+	+			
<i>E. faecalis</i> S11		+			+	+	+			
<i>E. faecalis</i> S13	+	+	+	+			+	+	+	+
<i>E. faecalis</i> S14		+	+			+	+	+	+	+
<i>E. faecalis</i> S18			+				+			+
<i>E. faecalis</i> S25		+	+							+
<i>E. faecalis</i> S36		+	+			+	+	+		+
<i>E. faecalis</i> S38		+				+	+			
<i>E. faecalis</i> S39		+				+	+	+	+	
<i>E. faecalis</i> S40		+	+			+	+	+	+	+
<i>E. faecalis</i> S41	+	+	+	+		+	+	+	+	+
<i>E. faecalis</i> S48			+			+	+			+
<i>E. faecalis</i> <sup>c</sup> H1		+	+			+	+			
<i>E. faecium</i> S6										
<i>E. faecium</i> S9										
<i>E. faecium</i> S15		+	+				+	+	+	+
<i>E. faecium</i> S22			+							+
<i>E. faecium</i> S27	+			+			+			+
<i>E. faecium</i> S28										
<i>E. faecium</i> S29										
<i>E. faecium</i> S30		+	+							+
<i>E. faecium</i> S31		+								
<i>E. faecium</i> S34		+						+	+	
<i>E. faecium</i> S50										
<i>E. faecium</i> <sup>d</sup> M1										
<i>E. faecium</i> M2		+								
<i>E. gallinarum</i> S19			+			+	+			+

<sup>a</sup>(+) presence of virulence factor;

<sup>b</sup>(S) enterococci isolated from fermented dry sausage;

<sup>c</sup>(H) enterococci isolated from dry cured ham;

<sup>d</sup>(M) enterococci isolated from unfermented dry sausage batter



The gene encoding collagen binding protein, *ace*, was found in 12/15 (80%) of *E. faecalis* strains, but none of the strains of *E. faecium* was positive for *ace*. Similarly, Creti et al. (2004) reported that the *ace* gene was present in all clinical *E. faecalis* strains and Aslam et al. (2012) also found *ace* in a majority of *E. faecalis* strains isolated retail meat.

The *efaA* gene encoding an endocarditis antigen was found in 14/15 (93%) of *E. faecalis* strains, whereas only 2/13 *E. faecium* strains were positive. *E. gallinarum* S19 was positive for both the *ace* and *efaA* genes. Valenzuela et al. (2009) reported a high incidence of *efaA* in *E. faecalis* strains (67%) isolated from foods of animal origin, which is in agreement with results from this study. Although the incidence of this gene in *E. faecium* was also high (63%) in that study, in the current study its frequency was low (15%).

The *esp* gene that encodes an enterococcal surface protein (Shankar et al., 1999) was present in 3/29 (10.3%) of enterococci. It was found in two *E. faecalis* (S13 and S41) and one *E. faecium* (S27) strain isolated from fermented dry sausage. This result is in accord with those of Klibi et al. (2012) where 3/119 of enterococci isolated from beef were positive for the *esp* gene. Other authors have reported this gene in enterococci from foods and humans, but in a higher proportion than that found in the present study (Eaton and Gasson, 2001; Valenzuela et al., 2009). The *esp* gene is considered to be an infection-associated virulence factor and thus it is undesirable when in enterococci isolated from food.

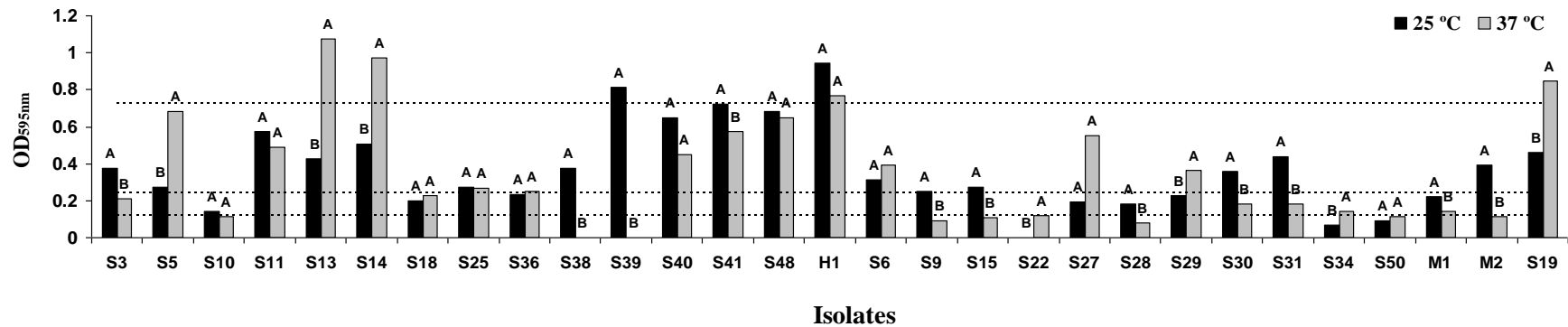
The *gelE* gene was detected in 59% (17/29) of all isolates. The gene was more frequent in *E. faecalis* (12/15) than *E. faecium* (5/13) strains. Aslam et al. (2012) also found the *gelE* gene in a majority of *E. faecalis* strains isolated from retail meat. In the present work silent (expressed at undetected levels or non-expressed) *gelE* genes occurred in both *E. faecalis* (S10, S11, S25, S38

and H1) and *E. faecium* (S30, S31 and M2) strains since gelatinase activity was not detected in these organisms, although the *gelE* gene was present. This result is in agreement with those of Eaton and Gasson (2001) where it was found that although the *gelE* gene was present, no phenotypic gelatinase activity was expressed by enterococci strains isolated from food. Silent genes may occur when there are low levels of gene expression, or sometimes they are due to down-regulation or to an inactive gene product, all of which can be influenced by environmental factors (Eaton and Gasson, 2001; Finlay and Falkow, 1997).

The extent of biofilm production by 62% (18/29) of strains was significantly different ( $p \leq 0.05$ ) at 25 °C and 37 °C. Seven strains (three *E. faecalis*, three *E. faecium*, and the *E. gallinarum* strain) produced more extensive biofilms at 37 °C than 25 °C, but with 11 strains (4 *E. faecalis* and 7 *E. faecium*), biofilm production at 25 °C was greater than at 37 °C (Fig. 4.1). It was found that at 37 °C, 20% (3/15) of *E. faecalis* (S13, S14 and H1) strains were very strong biofilm ( $OD_{595} > 0.71$ ) producers; however, the former two strains were only strong biofilm ( $OD_{595} > 0.240-0.70$ ) producers ( $p \leq 0.05$ ) at 25 °C. In contrast, *E. faecalis* S41 produced very strong biofilms at 25 °C, but when grown at 37 °C they were only strong producers of biofilms ( $p \leq 0.05$ ). None of the *E. faecium* strains produced biofilms that could be characterized as being very strong. As with some of the very strong *E. faecalis* biofilm producers, *E. gallinarum* S19 produced very strong biofilms at 37 °C but less extensive ( $p = 0.0004$ ), strong biofilms at 25 °C. *E. faecalis* strain S3 was a moderate biofilm producer at 37 °C, but at 25 °C it produced a strong biofilm which was significantly different. Interestingly, *E. faecalis* S10, S38 and S39 produced no biofilms at 37 °C, but when tested at 25 °C *E. faecalis* S38 and S39 produced strong biofilms. Similarly, three strains of *E. faecium* (S9, S15 and M2) produced no biofilms at 37 °C, but were able to produce strong biofilms when grown at 25 °C. Although the organisms were different, this result is consistent with

results from the study by Herald and Zottola (1988), where it was shown that *Yersinia enterocolitica* had higher adherence capacity at 21 °C than at 30 °C. *Listeria monocytogenes* showed higher adherence properties and biofilm formation at suboptimal temperatures (Chavant et al., 2002). In a similar study by Rode et al. (2007), higher biofilm forming capacity of *Staphylococcus aureus* on polystyrene was found at sub-optimal temperatures (20, 25 and 30 °C), which is in agreement with the present work. It is of interest that the greatest biofilm production by *Salmonella* spp. was found at 30 °C after 24 h and after 48 h at 22 °C (Stepanović et al., 2003).

Figure 4.1 Biofilm formation by enterococci strains at 25 and 37 °C



The dashed lines indicate biofilm formation capacity (OD<sub>595</sub> < 0.120 = nonproducer; OD<sub>595</sub> 0.120 to 0.240 = medium producer; OD<sub>595</sub> > 0.240 to 0.70 = strong producer; OD<sub>595</sub> > 0.710 = very strong producer). Ef, *E. faecalis*; Em, *E. faecium*; Eg, *E. gallinarum*. A/B, different letters indicate a significant difference ( $p \leq 0.05$ ) in biofilm formation at 25 and 37 °C.

When the presence of the *esp* and *gelE* genes plus gelatinase activity were examined in the 29 strains of enterococci, no correlation was found between the occurrence of these genes, the activity of gelatinase and the formation of biofilms at either temperature. The observation that isolates with or without *esp* and *gelE* were able to form biofilms is consistent with results reported by Marra et al. (2007) and Rosa et al. (2006). Only two strains of *E. faecalis* (S13 and S41) that were positive for both *esp* and *gelE* were strong or very strong biofilm producers at both 37 and 25 °C; however, one strain of *E. faecium* (S27) which was positive for *esp* but negative for *gelE*, was a strong biofilm producer at 37 °C and a medium biofilm producer at 25 °C. The *E. gallinarum* strain was a strong biofilm producer despite the absence of *esp* and *gelE* genes. It should be noted that there are some studies concluding that *esp* gene expression might lead to a significant increase in biofilm formation which is contradictory to the present findings (Tendolkar et al., 2004 and Toledo-Arana et al., 2001). Data from the current study showed that formation of biofilms by *E. faecalis*, *E. faecium* and *E. gallinarum* isolated from food did not appear dependent upon the presence of either *esp* or *gelE* genes. It is possible in the absence of *esp* or *gelE* or at low levels of their expression that the expression of a combination of other virulence factors, such as *ebp* (for endocarditis and biofilm associated pili) which is highly conserved and part of the core genome (Nallapareddy et al., 2006) and the *bee* locus (for biofilm enhancement in *Enterococcus*) (Schlüter et al., 2009) may contribute to biofilm formation in enterococci.

#### **4.5 Conclusion**

This study revealed that *E. faecalis* strains more frequently carried virulence genes than *E. faecium* strains and that the temperature optimum for biofilm formation was strain rather than species dependent. As potential virulence factors were identified in the three species of enterococci

examined, the possibility should not be excluded that these organisms, when present in meat and commercially fermented meat, may contribute to enterococcal infections, particularly in susceptible consumers.

## Chapter 5

### **Allyl isothiocyanate resistance among enterococci isolated from raw and fermented meat**

#### **5.1 Abstract**

This study investigated susceptibilities of enterococci from raw and fermented meat to allyl isothiocyanate (AITC), a natural antimicrobial isolated from Oriental or brown mustard seeds. Susceptibility tests and the minimum inhibitory concentrations (MICs) of AITC were determined using broth macro-dilution in screw-capped tubes at 25 °C. A total of 29 multi-drug resistant enterococci were included in this study. The highest MIC of AITC was 2.5 mM and the lowest was 1.0 mM. *E. faecium* showed higher MIC values of AITC than *E. faecalis*. Resistance of the enterococci to AITC was similar to that of other Gram-positive bacteria.

#### **5.2 Introduction**

Enterococci are Gram-positive, facultatively aerobic bacteria ubiquitous in the gastrointestinal tract of humans and animals. They are not generally considered pathogenic, but for immunocompromised individuals they may cause severe disease including endocarditis, wound and urinary tract infections (Hummel et al., 2007b; Lanthier et al., 2011). Enterococci are resistant to a wide variety of antibiotics commonly administered in human medicine as well as some that are used for animal growth promotion or for treatment and control of animal diseases. Their resistance to antimicrobials may be intrinsic or acquired, and the enterococci are known for their capacity to exchange genetic information by conjugation (Clewell and Dunny, 2002). They may spread antibiotic resistance among other non-pathogenic enterococci or enhance the virulence of other pathogens (Cocconcelli et al., 2003; Fisher and Phillips, 2009). Thus, there are potential food

safety concerns because if they are present in foods that are not cooked before consumption (like dry-fermented sausages), they may serve as vehicles for the transfer of antimicrobial resistance from the indigenous animal microbiota to the human gastrointestinal tract (Mathur and Singh, 2005). Bacterial pathogens from the animal gastrointestinal tract can contaminate raw and fermented meats during production or processing. One approach that has gained considerable interest recently has been the use of plant-derived antibacterial compounds, such as extracts of spices and herbs for food preservation and control of bacterial pathogens contaminating different stages in food processing and preservation (Shan et al., 2007). AITC is a component in the essential oil of mustard that contributes to its hot spiciness. In both its vapor and liquid forms it has high bactericidal activity, and thus has been tested for its ability to eliminate pathogenic bacteria from meat and fermented meat products (Chacon et al., 2006; Graumann and Holley, 2008).

In the present work, broth macro-dilution of AITC was used to assess the level of resistance of 29 enterococci strains isolated from raw and fermented meat. Very little information is currently available about fermented meat enterococci and their resistance towards AITC. Thus, the aim of this study was to generate, by means of phenotypic susceptibility tests, a representation of AITC resistance patterns of enterococci present in commercially prepared dry fermented sausages, dry cured ham and unripened sausage batter.

## **5.3 Materials and Methods**

### **5.3.1 Bacterial strains and culture conditions**

Enterococci were isolated from fermented dry sausage and dry cured ham samples purchased at retail (Winnipeg, MB, Canada) and dry sausage batter before addition of starter cultures (prepared in the Department of Food Science, University of Manitoba, Winnipeg, MB, Canada). The strains



were confirmed to be enterococci at the species level by API 20 Strep strips (BioMérieux, Marcy l'Etoile, France) and sequencing using universal primers. The isolates were identified as *Enterococcus faecalis* (n=15), *Enterococcus faecium* (n=13) and *Enterococcus gallinarum* (n=1) (Chapter 3). All these enterococci were multi-drug resistant and possessed virulence traits (Chapter 3, 4). From KF- Streptococcus agar plates (Difco, Fisher Scientific, Edmonton, AB, Canada), isolated colonies were inoculated into Mueller-Hinton broth (MH, Fisher Scientific) and incubated overnight at 25°C. The bacterial density was adjusted using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England) at 600 nm to achieve a concentration near 7.4 log CFU/ml.

### 5.3.2 Antimicrobial susceptibility testing and MIC determination

Concentrations of AITC (Aldrich Chemical Co., Milwaukee, WI, USA) used were from 0.5 mM/L to 5 mM/L. In each screw-capped tube 0.1 mL culture was added to 9.9 mL of MH broth. Then AITC was added to each tube to give the concentrations noted above. The tubes were incubated at 25 °C with a shaker speed of 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 24 h. Absence of growth (no increase in measured OD) was considered to be the MIC.

## 5.4 Results and Discussion

MIC results from tests of AITC against the 29 enterococcal strains are presented in Table 5.1. It was found that three *E. faecium* strains were only sensitive to 2.5 mM/L AITC, which was high compared to *E. faecalis* isolates, but 11 of 14 *E. faecalis* strains had MICs of 2.0 mM/L. The lowest MIC of 1.0 mM/L was found with one *E. faecium* strain. In earlier studies, the Gram-positive *Listeria monocytogenes* with an MIC of 1.42 mM/L AITC showed greater resistance than

*Escherichia coli* O157:H7 and *Salmonella* Montevideo (Lin et al., 2000). In another study (Palaniappan and Holley, 2010), MIC values for the Gram-positives *Streptococcus pyogenes* (0.63 mM/L) and *Staphylococcus aureus* (0.15 mM/L) were much lower than the lowest MIC observed in the present study for the enterococci.

Table 5.1 MIC values of AITC against enterococcal strains isolated from commercial raw and fermented meat

AITC (mM/L)	<i>E. faecalis</i> strains (n=14)	<i>E. faecium</i> strains (n=11)	<i>E. gallinarum</i> strains (n=1)
1.0		S50, <sup>c</sup> M1	
1.5	<sup>a</sup> S3, S5, S13, <sup>b</sup> H1	S6, S9, M2 S31, S34	
2.0	S10, S11, S14, S18, S25, S36, S38, S39. S40, S41, S48	S15, S22, S30	S19
2.5		S27, S28, S29	

<sup>a</sup>(S) Enterococci isolated from fermented dry sausage; <sup>b</sup>(H) enterococci isolated from dry cured ham; <sup>c</sup>(M) enterococci isolated from unfermented dry sausage batter

There is a growing interest today in using plant-derived antibacterial compounds, such as extracts of spices and herbs for food preservation (Shan et al., 2007). AITC, a natural compound in plants belonging to the family Brassicaceae (Cruciferae), has been shown to have strong antimicrobial activity in liquid media as well as in its vapor form. AITC is a major pungent flavor compound in horseradish, mustard and wasabi and is generated from its precursor, allyl glucosinolate or sinigrin. Sinigrin, which is found widely in mustard and other vegetables belonging the family Brassicaceae, such as cabbage and cauliflower, is hydrolyzed by the action of endogenous myrosinase when the plant tissue is damaged to yield isothiocyanates (ITCs), including AITC (Cui and Eskin, 1998). It can cause an increase in cell membrane permeability and result in leakage of cellular metabolites (Lin et al, 2000). AITC has been found effective for reducing *E. coli* O157:H7 viability in fermented meat products (Chacon et al., 2006; Graumann and Holley, 2008). Plant essential oils have been shown to have inhibitory activity against *E. faecalis* (Holley and Patel, 2005). Park et al. (2013) showed that AITC from horse radish root had antibacterial activity against *E. faecalis* and other oral microorganisms which is consistent with the present results. In another study by Wilson et al. (2013) it was observed that there were measurable antibacterial effects of AITC against *E. faecalis* or *E. faecium*. Since AITC has a pungent taste and odor, it is likely that these characteristics will limit its use in fermented sausages. Chacon et al. (2006) showed that fermented sausages containing 5 mM/L AITC were the most acceptable of AITC-treated sausages as assessed by a sensory panel, and thus these sausages could be marketed as a safe, hot specialty salami product. Clark (1992) noted that AITC up to 5.0 mM/L has been acceptable as a flavor fortifier when a sharp spice effect is needed to simulate the mustard or horseradish taste. These values are much higher than the highest MIC (2.5 mM/L) of enterococci tested. Thus, this compound can be used to effectively control enterococci in fermented sausages.

## 5.5 Conclusion

The frequent occurrence of antibiotic resistant enterococci in commercially produced fermented dry sausage may be of concern because these products may be vehicles for dissemination of bacteria with multiple, transferable antibiotic resistance. This study showed that three strains of *E. faecium* had the greatest AITC tolerance, but a larger number of *E. faecalis* strains showed slightly greater tolerance than *S. faecium* strains. It was evident that the enterococci strains examined could be controlled in dry fermented sausage environments by AITC at levels that were unlikely to cause sensory rejection of the treated products.

## Chapter 6

### **Horizontal transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to clinical isolates of *Enterococcus faecium* and *Enterococcus faecalis***

#### **6.1 Abstract**

*Enterococcus* species are part of the normal intestinal flora of a large number of mammals including humans, and consequently they can be used as indicators of fecal contamination in food and water for human consumption. Their presence in large numbers in foods may indicate a lapse in sanitation and their ability to serve as a genetic reservoir of transferable antibiotic resistance is of concern. In the present study, *Enterococcus* spp. isolated from commercially fermented meat and human clinical specimens were studied to determine genetic relationships. *Sma*I pulsed-field gel electrophoresis (PFGE) patterns exhibited genomic heterogeneity within and between both groups of isolates. However, in spite of this heterogeneity there were still substantial phenotypic similarities which suggested that food might be a potential vehicle for distribution of resistant bacteria among humans. *In vitro* conjugation experiments demonstrated transfer of the tetracycline resistant determinant, *tet*(M), from *Enterococcus faecium* S27 isolated from fermented sausage to clinical isolates of both *E. faecium* and *Enterococcus faecalis*. The streptomycin resistance of *E. faecium* S27 was also transferred to a clinical strain, *E. faecalis* 82916, which was confirmed by the presence of the streptomycin resistance gene, *aadA*, in the donor and transconjugant strains. Since the *aadA* gene is associated with a class 1 integron, results also suggested that resistance transfer might have occurred via an integron. It appears this is the first identification of a class 1 integron in *E. faecium* isolated from food. The importance of food enterococci as a reservoir of

antibiotic resistance genes and the potential for their genetic transfer to human strains following consumption of uncooked or undercooked contaminated meat is underlined by this work.

## **6.2 Introduction**

Enterococci are ubiquitous microorganisms related to the lactic acid bacteria. They are predominantly inhabitants of the gastrointestinal tract of humans and animals and also commonly occur in vegetables, other plant materials, soil and water (Aguirre and Collins, 1993). Enterococci are considered to be an important part of the natural microflora responsible for ripening and aroma development of certain traditional cheeses and sausages, especially those produced in the Mediterranean area. As enterococci are able to survive and compete in the human gastrointestinal tract, they have been used as active strains in commercial probiotic preparations (Foulquié-Moreno et al., 2006; Franz et al., 1999; Holzapfel et al., 1998; Lund et al., 2002). Enterococci have been used in the food industry as starter cultures (Foulquié-Moreno et al., 2006), and some strains which produce the bacteriocin, enterocin, have been used as bio-preservatives (Stiles and Holzapfel, 1997).

Enterococci have emerged as important nosocomial pathogens over the past decade (Vankerckhoven et al., 2004). Being normal human commensals, enterococci are commonly associated with human infection and have intrinsic resistance to many classes of antibiotics most commonly administered in human medicine (Murray, 2000). Further, it has been suggested that antibiotic use for animal growth promotion or for treatment or control of animal disease expedite the appearance of antibiotic resistant bacteria in humans (Smith et al., 2002). Enterococci have the capacity to easily acquire and express new resistance genes and can thus tolerate selective antibiotic pressure and become resistant to new classes of antibiotics (Rice, 2001). These

antibiotic resistant enterococci can be found in large numbers in foods of animal origin (Aslam et al., 2012; Giraffa, 2002; Jamet et al., 2012) and can be transmitted to humans through consumption and handling of contaminated animal-derived food (Sørensen et al., 2001). Enterococci are the most thermotolerant of non-sporulating bacteria and some can survive pasteurization temperatures. Tolerance to environmental extremes explains their survival during processing of cooked and uncooked cured meats and their ability to multiply during fermentations (Hugas et al., 2003; Simpson et al., 1994). This high survival capacity in changing environments, such as food might be explained in part by their capacity to form biofilms (Creti et al., 2004; Chapter 4; Tendolkar et al., 2006).

Gazzola et al. (2012) found that tetracycline and erythromycin resistance genes resident on plasmids in a clinical isolate of *E. faecalis* were transferred with high frequency to other enterococci and bacterial genera present during raw fermented dry sausage ripening. Therefore, it is certainly possible that enterococci from foods of animal origin may play a significant role in the dissemination of antibiotic resistance genes, particularly since these genes are located on transferable genetic elements (Clewell et al., 1995; Vignaroli et al., 2011).

The evolution of antibiotic resistance in microbial communities may occur by horizontal transfer of resistance genes across species and genus borders not only by conjugative plasmids and transposons, but also by the possession of integrons and insertion elements, as well as by lytic and temperate bacteriophages (Teuber et al., 1999). Conjugation in *Enterococcus* species normally occurs via the sex pheromone system, which mediates conjugative plasmid transfer (Clewell and Weaver, 1989). Transposons also are recognised to be as important for the dissemination of antibiotic resistance genes as plasmids and they contribute to both long-term bacterial evolution and short-term adaptation, enabling rapid responses to environmental change (Scott, 2002).



Integrations are novel DNA elements characterized by their ability to incorporate gene cassettes present as part of a transposon (which can change position in the genome) or they can occur independently on several groups of broad host range plasmids. Integrations possess two conserved segments separated by a variable region which includes integrated antibiotic resistance genes and one or more gene cassettes of unknown function. They are a natural expression vector that permits the insertion of antibiotic resistance gene(s) by a site-specific recombination mechanism (Lévesque et al., 1995).

The food chain is potentially one of the main routes for transmission of antibiotic resistant bacteria between animal and human populations (Witte, 1997). In particular, fermented meats that are not heat-treated before consumption are a likely vehicle for antibiotic resistant bacteria with a direct link between the indigenous animal microflora and the human gastrointestinal tract microflora. Few studies have been conducted to evaluate the transfer of antibiotic resistance genes mediated by conjugative plasmids or transposons among enterococci normally found in food or human clinical isolates (Gazzola et al., 2012; Hummel et al., 2007b; Vignaroli et al., 2011), and of these a single study was unsuccessful in finding antibiotic resistance gene transfer from food to human isolates (Vignaroli et al., 2012). Thus, the aim of this study was to evaluate whether organisms isolated from commercially fermented meats are a potential source for the transfer of antibiotic resistance to human-associated *Enterococcus* strains. The possible mechanism responsible for resistance transfer between the enterococci isolated from food and clinical isolates was also examined.

## 6.3 Materials and Methods

### 6.3.1 Bacterial strains

Seventeen enterococcal strains showing antibiotic resistant traits isolated from commercially fermented dry sausage (S) and dry cured ham (H) were selected. They included 11 *Enterococcus faecalis* (S13, S14, S18, S25, S36, S38, S39, S40, S41, S48, H1) and 6 *Enterococcus faecium* (S9, S15, S27, S30, S34, S50) strains (Chapter 3). Nine multi-drug resistant clinical isolates sensitive to streptomycin and tetracycline, consisting of 3 *E. faecalis* and 6 *E. faecium* strains from the Department of Clinical Microbiology, Health Sciences Centre, Winnipeg, MB, Canada were used (Table 6.1). Enterococci strains were grown from frozen stocks kept at -80 °C in Brain Heart Infusion Broth, BHIB (Difco, Fisher Scientific, Edmonton, AB, Canada) containing 50% (v/v) glycerol (Sigma, St. Louis, MO, USA) and were cultured on KF-Streptococcus Agar (Difco) at 37 °C.

Table 6.1 Enterococci isolates used, their antibiotic<sup>a</sup> resistance phenotype, genotype and source of isolation

<i>Enterococcus</i> strain	MIC ( $\mu\text{g/mL}$ ) <sup>a</sup>									Antibiotic resistance and integrase genes	Source
	Am	Ci	Cm	Em	Gm	PG	Sm	Te	Vn		
<i>E. faecium</i> S27 <sup>b</sup>	S	4 <sup>R</sup>	S	16 <sup>R</sup>	S	S	4096 <sup>R</sup>	128 <sup>R</sup>	S	<i>tet(M)</i> , <i>aadA</i>	Dry fermented sausage
<i>E. faecium</i> 82082	128 <sup>R</sup>	32 <sup>R</sup>	256 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>	<i>int</i>	Blood
<i>E. faecium</i> 83056 <sup>c</sup>	128 <sup>R</sup>	32 <sup>R</sup>	S	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>		Blood
<i>E. faecium</i> 83286	128 <sup>R</sup>	32 <sup>R</sup>	S	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	128 <sup>R</sup>	<i>int</i>	Blood
<i>E. faecium</i> 92857	128 <sup>R</sup>	32 <sup>R</sup>	32 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>		Blood
<i>E. faecium</i> 92892	128 <sup>R</sup>	32 <sup>R</sup>	128 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>		Blood
<i>E. faecium</i> 97547	128 <sup>R</sup>	32 <sup>R</sup>	128 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>		Urine
<i>E. faecalis</i> 76167	128 <sup>R</sup>	32 <sup>R</sup>	256 <sup>R</sup>	64 <sup>R</sup>	S	128 <sup>R</sup>	S	S	S	<i>int</i>	Blood
<i>E. faecalis</i> 82916 <sup>c</sup>	128 <sup>R</sup>	32 <sup>R</sup>	128 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	256 <sup>R</sup>		Blood
<i>E. faecalis</i> 94585	128 <sup>R</sup>	32 <sup>R</sup>	S	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	S	S		Blood
T1	128 <sup>R</sup>	32 <sup>R</sup>	S	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	S	128 <sup>R</sup>	256 <sup>R</sup>	<i>tet(M)</i>	Transconjugant
T2	128 <sup>R</sup>	32 <sup>R</sup>	32 <sup>R</sup>	64 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	8192 <sup>R</sup>	128 <sup>R</sup>	256 <sup>R</sup>	<i>tet(M)</i> , <i>aadA</i>	Transconjugant

<sup>a</sup> Ampicillin (Am), ciprofloxacin (Ci), chloramphenicol (Cm), erythromycin (Em), gentamycin (Gm), penicillin G (PG), streptomycin (Sm), tetracycline (Te) and vancomycin (Vn); MIC represents minimum inhibitory concentration. R, designates resistance concentration in  $\mu\text{g/mL}$ ; S, designates susceptibility toward antibiotic (CLSI 2002, DANMAP 2000, EFSA 2008);

<sup>b</sup> donor during conjugation;

<sup>c</sup> recipient during conjugation

### 6.3.2 Antibiotic susceptibility and minimum inhibitory concentrations (MICs)

The antibiotic susceptibility of food and clinical enterococci strains was determined as described in Chapter 3, using broth micro dilution. Seven antibiotics currently registered in Canada for use in food animals plus vancomycin and ciprofloxacin were used in this study. The minimum inhibitory concentrations (MICs) of enterococci were determined according the Clinical and Laboratory Standards Institute (CLSI, 2002), the European Food Safety Authority (EFSA, 2008), and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, DANMAP (Bager and Emborg, 2001). The MIC was considered to be the lowest antimicrobial concentration at which no red color from reduction of *p*-iodonitrotetrazolium violet (signifying no metabolic activity) appeared (Chapter 3). When no microbiological breakpoint was found using the reference guides, breakpoint values of structurally or functionally-related antibiotics were used. Isolates with MICs above these breakpoints were considered resistant.

### 6.3.3 Conjugation experiments

Potential donor strains were mated with various recipient strains to determine whether antimicrobial resistance could be transferred. In conjugation experiments a 1:1 ratio of donor/recipient cells was used. Tetracycline resistant, ampicillin sensitive strains from the fermented meat were used as donors. Recipients were tetracycline sensitive clinical strains that were resistant to ampicillin. The donor and recipient strains were grown overnight at 35 °C in BHIB media containing selective antibiotics. The antibiotics (Sigma) were used at the following concentrations: tetracycline 32 µg/mL and ampicillin 32 µg/mL. After incubation at 25 °C overnight, 1 mL culture was centrifuged at 8000 x g (Microcentrifuge 22R, Beckman Coulter) for 15 min and the pellets were washed twice with sterile saline solution. Donor and recipient pellets

were combined in 100  $\mu$ L of sterile saline and plated on Brain Heart Infusion Agar (BHIA, Difco) without any selective agents. After overnight incubation at 25  $^{\circ}$ C, confluent growth on plates was removed with a platinum loop and suspended in 500  $\mu$ L of sterile saline. Aliquots (100  $\mu$ L) of this suspension were spread on selective plates containing 32  $\mu$ g/mL tetracycline plus 32 $\mu$ g/mL ampicillin and incubated at 30  $^{\circ}$ C for 48 h. Transconjugants growing on both tetracycline and ampicillin were selected and their MICs were determined. Conjugation frequency was calculated as the number of transconjugant colonies (expressed as CFU/mL) divided by the number of donor cells (Sparo et al., 2011), where transconjugants were considered to be those growing on BHI agar supplemented with tetracycline and ampicillin. Each experiment was performed in triplicate; frequency values are reported as the mean ( $\pm$  standard deviation) of all three experiments.

#### 6.3.4 DNA preparation for PFGE analysis

Enterococci from fermented sausages, resistant to at least two antibiotics and containing corresponding antibiotic resistance genes (Chapter 3) plus clinical enterococci strains were selected. DNA preparation for PFGE fingerprinting of meat and clinical isolates was performed as described by Huch et al. (2008) with some modification. Briefly, cells from overnight culture in BHI broth were harvested by centrifugation at 8000  $\times$  g and 4 $^{\circ}$ C for 15 min. Then cell pellets were washed with 1 mL of TE buffer [10 mM Tris (Sigma), 10 mM EDTA (Sigma), pH 8.0]. After centrifugation pellets were suspended in 1 ml of TE buffer to obtain a suspension with an optical density ( $A_{610}$ ) of 1.85-2.3. Then 180  $\mu$ L of cell suspension was mixed with 60  $\mu$ L of lysozyme solution (100 mg/mL in distilled water, Sigma), 9  $\mu$ L of mutanolysin solution (1000 U/mL, in distilled water, Sigma) and 51  $\mu$ L TE buffer. The suspension was incubated on a shaking incubator (Symphony Incubating Orbital Shaker, VWR, Mississauga, ON, Canada) at 37  $^{\circ}$ C and 300 rpm for 45 min. After incubation, 300  $\mu$ L of cell suspension was mixed with an equal volume of agarose

solution [1.5% low melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA), 10% sodium dodecyl sulfate (Sigma) and proteinase K (20 mg/mL in distilled water, Sigma)]. This suspension was then transferred to disposable plug molds (Bio-Rad) and stored at 4°C for 15 min for complete polymerization. Plugs were then transferred to 2 mL lysis buffer (50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1% sodium lauryl sarcosine [Sigma] and 40 µl proteinase K solution [20 mg/mL, Sigma]) and incubated overnight at 54 °C and 40 rpm in a shaker/ incubator (Precision Scientific, Thermo Electron Corp., Winchester, VA, USA). The next day, lysis solution was removed and gel plugs were washed twice with pre-heated (54 °C) sterile distilled water for 10 min, followed by two washes with 54 °C TE buffer. After washing, plugs were stored at 4°C in 2 mL TE buffer until enzyme digestion. For restriction enzyme treatment, the plugs were digested with *Sma*I (New England Biolabs, Whitby, ON, Canada) using an enzyme concentration of 40 U per agarose plug for 3 h at 25 °C. The agarose plugs were loaded onto a 1 % agarose gel, which was subjected to electrophoresis in 0.5X TBE buffer at 14°C using a PFGE CHEF-DR III System (Bio-Rad). The switch times were 1–20 s at 6.0 V/cm and run for 22 h. PFGE-generated DNA profiles were analyzed using BioNumerics software, version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UPGMA), and DNA relatedness was calculated based on the Dice coefficient (Mulvey et al., 2001).

### 6.3.5 Plasmid isolation

Plasmid DNA from bacterial strains was isolated by alkaline lysis (Birnboim and Doly 1979) with minor modification (van Belkum and Stiles, 1995). Briefly, cells were lysed at 37°C in 25 mM Tris-HCl (pH 8), 10 mM EDTA containing 20 mg/mL of lysozyme and 2 µL of mutanolysin (1000

U/ml, Sigma) for 1 h. The remainder of the procedure was as described previously (Bimboim and Doly, 1979).

### 6.3.6 PCR for the identification of *tet(M)*, *int* and *aadA* genes and sequencing of these genes in donor and transconjugants

Total genomic DNA was extracted from overnight cultures grown at 37°C in BHIB (Difco), using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA, USA). The sequences of oligonucleotide primers used are listed in Table 6.2. Primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). PCR for *tet(M)* was performed as described in Chapter 3. The occurrence of conjugative transposons of the Tn916-1545 family was determined with Int-F and Int-R primers targeting the transposon integrase (*int*) gene (Gevers et al., 2003). The oligonucleotide primers chosen for amplification of streptomycin/spectinomycin adenylyltransferase (*aadA*) genes were *aadA*-F and *aadA*-R as described by Clark et al. (1999). The PCR sequencing of *tet(M)* and *aadA* of donor and transconjugants reaction contained 25 µL of PCR Master Mix 2X (Promega, Madison, WI, USA), 1 µL of each primer (25 pmol) and water to 50 µL. Thermal cycling conditions were: 1 cycle of denaturation (94 °C, 1 min); 36 cycles of denaturation (94 °C, 1 min), annealing (Table 6.2), and extension (72 °C, 1.5 min), followed by a final extension (72 °C, 8 min). These sequencing reactions were done by Sanger Sequencing Services (McGill University and Génome Québec Innovation Centre, Montréal, QC, Canada).

Table 6.2 Primers for PCR detection of *tet(M)*, *int*, and *aadA* genes

Gene	Primer sequence (5' → 3')	Product size (bp)	Annealing temperature (°C)	Reference
<i>tet(M)</i>	GAYACNCCNGGNCA YRTNGAYTT  CACCGAGCAGGGATTTCTCCAC	1513	53	Gevers et al., 2003
<i>int</i>	GCGTGATTGTATCTCACT  GACGCTCCTGTTGCTTCT	1028	60	Gevers et al., 2003
<i>aadA</i>	TGA TTT GCT GGT TAC GGT GAC  CGC TAT GTT CTC TTG CTT TTG	284	58.4	Clark et al., 1999



## 6.4 Results

### 6.4.1 Conjugative transfer of tetracycline resistance

To investigate the transferability of antibiotic resistance, plate mating trials were conducted using 13 enterococci strains containing the tetracycline resistant gene (Chapter 3) as donors and 9 clinical enterococci as recipients. Three colonies/plate (10%) were selected from the resistant transconjugants which grew on BHIA containing tetracycline and ampicillin. Of all strains studied in the mating experiments only one dry sausage isolate, *E. faecium* S27, was able to transfer its tetracycline resistance. The transfer was to two clinical isolates, *E. faecium* 83056 and *E. faecalis* 82916, and it occurred with a frequency of  $1.1 \times 10^{-6}$  and  $2.1 \times 10^{-5}$  transconjugants/donor, respectively (Table 6.3).

Table 6.3 Summary of positive conjugation experiments with enterococcal strains

Donor	Recipient	Transconjugant	Conjugation frequency <sup>a</sup> (cfu/mL)
<i>E. faecium</i> S27	<i>E. faecium</i> 83056	T1	1.1X10 <sup>-6</sup>
<i>E. faecium</i> S27	<i>E. faecalis</i> 82916	T2	2.1X10 <sup>-5</sup>

<sup>a</sup> Conjugation frequency was calculated as the number of transconjugants (cfu/ml) divided by the number of donor cells (cfu/mL) (Sparo et al., 2011)

#### 6.4.2 Phenotypic profile of antibiotic resistance

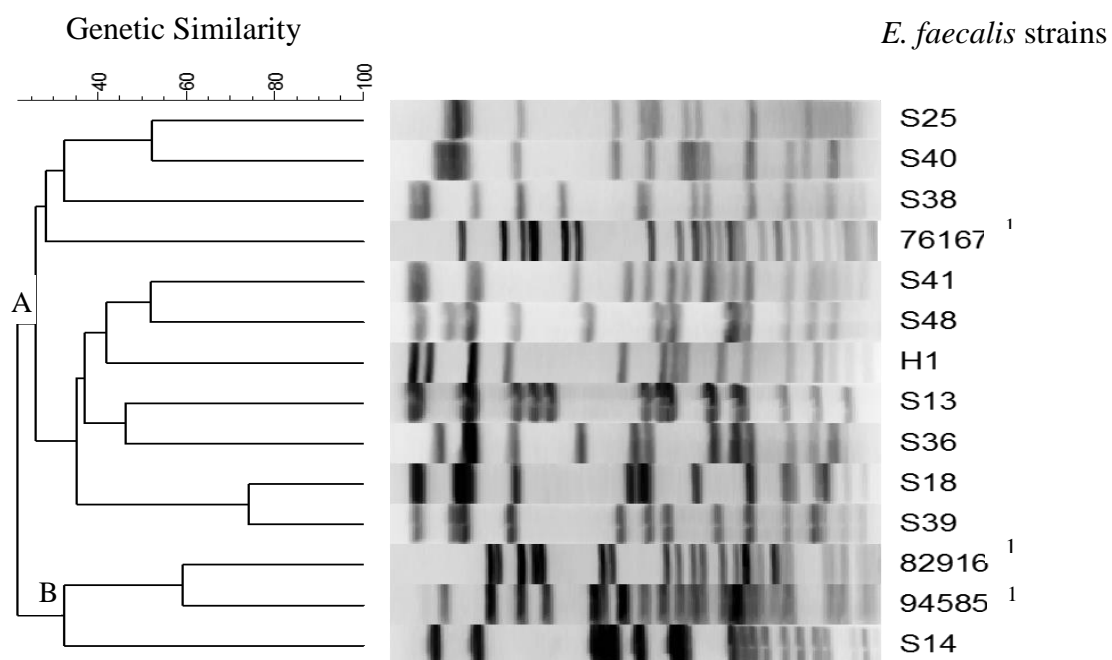
It was found that 5/9 clinical strains were resistant to 7 antibiotics tested and none was resistant to tetracycline or streptomycin (Table 6.1). Of *E. faecium* strains, two were susceptible to chloramphenicol and of *E. faecalis* strains, one (76167) was susceptible to vancomycin and gentamicin, and *E. faecalis* 94585 was susceptible to vancomycin and chloramphenicol. As anticipated, the transconjugants demonstrated an antibiotic resistant phenotype identical to their parent recipient strain except they were resistant to tetracycline. One transconjugant, T2, also demonstrated phenotypic resistance towards streptomycin (Table 6.1).

#### 6.4.3 PFGE typing of food and clinical enterococci

Enterococci isolates belonging to the two enterococcal species were typed by PFGE, and wide genotypic variability was found among the food and clinical isolates. The dendrogram generated showed no significant clustering by strain origin. The strains of *E. faecalis* examined were separable into two major clusters A and B (Fig. 6.1). The closest similarity found between meat and clinical strains was within sub-cluster B1 which included clinical strains 82916 and 84585 as well as strain S14 isolated from dry fermented sausage. The remaining isolates were included in major cluster A, with 25% similarity and could be sub-divided into 6 subgroups. Based on the similarity of *E. faecalis* isolates from fermented meat, 11 unique PFGE types were found. In addition, the three clinical *E. faecalis* isolates represented three different PFGE types.

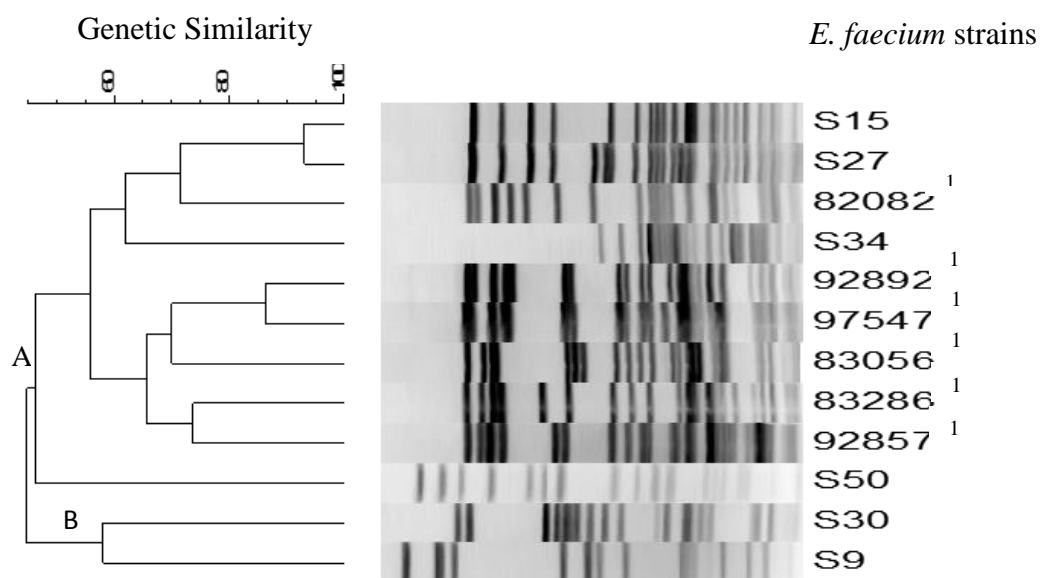
When the 12 *E. faecium* strains, represented by equal numbers of meat and clinical isolates, were subjected to PFGE, two major clusters (A and B) with 55% similarity were apparent (Fig. 6.2). The closest similarity between meat and clinical isolates (71.5%) was seen in sub-cluster A1 which included meat isolates *E. faecium* S15 and S27 with clinical isolate 82082.

Figure 6.1 UPGMA dendrogram derived from PFGE profiles (*Sma*I macrorestriction) showing restriction pattern similarity among 14 *E. faecalis* from fermented meat and human clinical isolates.



<sup>1</sup>Clinical isolates.

Figure 6.2 UPGMA dendrogram derived from PFGE profiles (*Sma*I macrorestriction) showing restriction pattern similarity among 12 *E. faecium* strains from fermented meat and human clinical isolates.



<sup>1</sup>Clinical isolates.

#### 6.4.4 PFGE typing of transconjugants

The PFGE profile from *Sma*I digestion of whole DNA from the donor (*E. faecium* S27), recipient (*E. faecium* 83056 and *E. faecalis* 82916) and transconjugant (T1 and T2) strains are shown in Fig. 6.3. *E. faecium* 83056 and the transconjugant T1 occurred in the same cluster (A) and were related to each other with 80.01% similarity. The other cluster (B) included *E. faecalis* 82916 and the transconjugant T2 which were related to each other with 87.81% similarity. Both of these clusters were distantly related to *E. faecium* S27 (44.69%).

Figure 6.3 UPGMA dendrogram derived from PFGE profiles (*Sma*I macrorestriction) showing restriction pattern similarity among enterococci strains from fermented meat, human clinical isolates and transconjugants. The donor was *E. faecium* S27. Recipients were *E. faecium* 83056 and *E. faecalis* 82916.



<sup>1</sup>Clinical isolates.

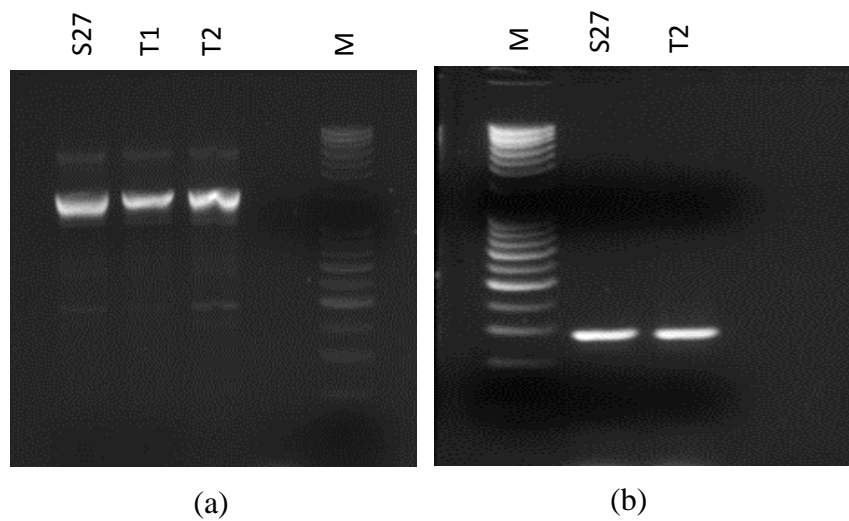
#### 6.4.5 Antibiotic resistant phenotype; resistant gene sequencing and plasmid profile analysis of transconjugants

Antibiotic resistant phenotypes of the donor, recipients and transconjugants are shown in Table 6.1. The donor *E. faecium* S27 was found resistant to tetracycline, streptomycin, erythromycin and ciprofloxacin, whereas clinical enterococci were susceptible to tetracycline and streptomycin, but resistant to penicillin G and ampicillin (Table 6.1). Phenotypic tests showed both transconjugants were resistant to tetracycline with an MIC greater than 128 µg/mL, which was the same level of resistance shown by the donor. The T2 transconjugant was also resistant to streptomycin (> 8192 µg/mL), which was 2-fold greater than the resistance to streptomycin shown by the donor, *E. faecium* S27. PCR screening to detect tetracycline resistance gene transfer using previously described primers and conditions (Chapter 3) revealed the presence of the tetracycline resistance gene *tet*(M) in transconjugants T1 and T2 (Fig. 6.4). PCR analysis for the transposon Tn916-1545 family carrying the gene *int* was positive in clinical isolates of *E. faecium* (82082, and 83286) and *E. faecalis* 76167 (although they were sensitive to tetracycline), but the tetracycline resistant *E. faecium* S27 and transconjugant strains did not contain *int*. The class 1 integron sequence containing the *aadA* gene was detected in *E. faecium* S27, the other streptomycin resistant *Enterococcus* from fermented meat (data not shown) and the transconjugant T2. When sequencing of the *tet*(M) and *aadA* gene of donor and transconjugant was done, it was revealed that they had 95 and 99% nucleotide homology, respectively (Appendix II). This result confirmed the transferred antibiotic resistance genes in the transconjugant were derived from the donor *E. faecium* S27. Plasmid profile analysis of food, clinical and transconjugant strains was done to see if antibiotic resistance was integrated into a plasmid and transferred to recipients. It was found that the plasmid profiles of the recipient strains *E. faecalis* 82916 and *E. faecium* 83056 were similar



to T1 and T2, respectively, and insertion of DNA into plasmids of these transconjugants was not observed (data not shown). PCR analysis also yielded negative results for the presence of the *tet(M)* gene on plasmids in the transconjugants.

Figure 6.4 PCR analysis of *tet(M)* and *aadA* genes in *E. faecium* S27 and transconjugants T1 (*E. faecium* S27::*E. faecium* 83056) and T2 (*E. faecium* S27::*E. faecalis* 82916). (a) PCR *tet(M)* and (b) PCR *aadA*. M, 1kb standard molecular size ladder



## 6.5 Discussion

Enterococci are known to cause disease in humans which is exacerbated when these organisms are resistant to antibiotics. Acquired antibiotic resistance combined with natural resistance to several major classes of antibiotics and the natural resistance of these organisms to low pH, high salt concentrations, and high temperatures, contribute to their survival in harsh environmental conditions. While selected strains of *Enterococcus* are used in starter cultures for the fermentation of meat products, other strains can be found in various foods as contaminants. Therefore, the food chain is considered to be one of the main routes for transmission of antibiotic resistance among the human microbiota (Rizzotti et al., 2009). Antibiotic resistant enterococci are common in foods of animal origin (Aslam et al., 2012; Chapter 3; Sánchez Valenzuela et al., 2013). These contaminating microorganisms are able to grow and colonize during sausage fermentation because they are tolerant of ripening conditions and are selected for in the process. Since fermented sausages are ready-to-eat foods, consumers are directly exposed to the bacteria present. The bacterial community in dry sausages might contain as many as  $10^8$  CFU/gm of viable enterococci or  $10^{10}$  CFU/gm lactic acid bacteria and coagulase negative staphylococci per serving (Gazzola et al., 2012). Several studies have focused on the role of commensal lactic acid bacteria as reservoirs of antibiotic resistance genes and studies on their potential transferability (Eaton and Gasson, 2001; Toomey et al., 2010; Vignaroli et al., 2011). However, little data are available on the occurrence of gene transfer between food and clinical isolates present *in vivo*.

Pulsed-field gel electrophoresis (PFGE) has emerged as one of the most widely applicable and reproducible methods to examine the origin and diversity of enterococci (Patterson and Kelly, 1998). During the present study to investigate relationships between enterococci from fermented meat and clinical samples, the chromosomal DNA of *Enterococcus* isolates was cut with *Sma*I

restriction endonuclease and restricted fragments were compared using PFGE. When the data were analyzed, it was observed that there were high levels of heterogeneity among isolates from each source belonging to the same species. Cluster analysis of macro-restricted patterns generated by *Sma*I digestion of chromosomal DNA revealed one major and a minor cluster for the 14 *E. faecalis* strains as shown in Fig. 6.1. The dry sausage isolate *E. faecalis* S14 had 62% similarity with two clinical strains (*E. faecalis* 82916 and 94585) indicating genetic proximity and suggesting that they could represent a genetic pool of *E. faecalis* strains from fermented meat with the potential to cause infection in humans. When PFGE analysis was performed with *E. faecium* it was found that only one clinical strain (82082) had high (71.5%) similarity with two meat strains (*E. faecium* S15 and S27). Although there were some close similarities in the PFGE patterns obtained, it was not possible to identify any significant association between enterococci isolated from fermented meat and human clinical samples. These results are consistent with previous work by Descheemaekker et al. (1999) and Kim et al. (2010) where a high level of genetic heterogeneity was observed between *E. faecium* strains from humans and animals. Similarly Castillo-Rojas et al. (2013) also showed that enterococci isolated from humans and those isolated from the environment had high genetic diversity according to PFGE analysis. Thus, it can be concluded that in spite of their heterogeneity there are some close relationships, and food might be a potential route for antibiotic resistance transfer to humans.

Seventeen enterococci from fermented meat carrying the tetracycline resistance gene and 9 clinical strains, sensitive to tetracycline, were subjected to mating studies in different combinations. Only one strain, *E. faecium* S27, was able to transfer its tetracycline resistance gene *tet*(M), to two of the clinical isolates (*E. faecium* 83056 and *E. faecalis* 82916) which generated two transconjugants, T1 and T2, respectively. Transconjugation was confirmed by *Sma*I restriction and

PFGE analysis (Fig. 6.3), where the PFGE profile of transconjugant T1 was 80.01% similar to that of *E. faecium* 83056. It was also concluded that transconjugant T2 was generated from *E. faecalis* 82916 because they had 87.81% similarity, respectively, by PFGE. When the antibiotic MICs for the donor, recipients and transconjugants were done, transconjugants showed profiles similar to the clinical isolates. Moreover, they also possessed the tetracycline resistance gene *tet(M)* which was present in the donor, *E. faecium* S27. These phenotypic and genotypic tests established that antibiotic resistance had been transferred from *E. faecium* S27 to clinical strains of enterococci.

To better understand the mechanism of gene transfer involved, plasmid profile analysis of the donor, recipients and transconjugants were done. Interestingly in the clinical strain transconjugants where *tet(M)* was transferred, no detectable plasmids were acquired. PCR tests for the *tet(M)* gene on transconjugant plasmids were also negative. This result was similar to that obtained by Huys et al. (2004), where no detectable plasmids were present in recipient strains, although the *tet(M)* gene was transferred. Since *tet(M)* was not on plasmids in T1 and T2, it was postulated that the antibiotic resistance gene might have been transferred on a transposon or integron. The distribution of *tet(M)* in several genera, including *Enterococcus*, is associated with the Tn916-1545 conjugative transposon family (Rizzotti et al., 2009), and this is determined by the presence of the *int* (integrase) gene. Mating experiments done in a previous study demonstrated that some strains of *E. faecalis* and *E. faecium* were able to transfer tetracycline resistance determinants by means of this transposon family (Huys et al., 2004; Rizzotti et al., 2009). Although in the present study the donor *E. faecium* S27 lacked the *int* gene it was still able to transfer the *tet(M)* gene. This finding is consistent with that from a previous study by Wilcks et al. (2005) where it was demonstrated that the presence of *int* was not necessary for transfer of the tetracycline resistant phenotype. Hummel et al. (2007) also showed that the donor in a filter-mating study transferred its tetracycline

resistant determinants *tet(M)* and *tet(L)* but were negative for the *int* gene. Thus, it can be concluded that the mobility of the *tet(M)* gene was not dependent upon the presence or absence of transposon like-elements.

Integrations are mobile genetic elements which serve as vehicles for antibiotic resistance transfer most commonly in Gram-negative bacteria, especially in Enterobacteriaceae, but are also found in Gram-positive bacteria. The streptomycin/spectinomycin adenylyltransferase gene (*aadA*) is related to class 1 integrations (Clark et al., 1999). In the present study, the class 1 integron-related gene, *aadA*, was detected in the donor strain, *E. faecium* S27, which also showed high resistance to streptomycin (Chapter 3). Aminoglycosides, including streptomycin, in combination with cell wall inhibiting antibiotics (eg. penicillins) are the antibiotics of choice for treating enterococcal infections (Davies and Wright, 1997). Since streptomycin has been used in combination with penicillin in drinking water to treat hogs in Canadian pork production facilities (Tremblay et al., 2012), there is a possibility that antibiotic resistant enterococci may develop and be transmitted by food. Pork is one of the main ingredients in dry fermented sausage formulations and thus it might serve as a vehicle for dissemination of streptomycin resistant enterococci to humans. Although the clinical isolates used here were sensitive to streptomycin, one of the transconjugants (T2) was resistant to 8192 µg/mL streptomycin, and this was twice as high as the MIC of *E. faecium* S27. In addition, the transconjugant T2 was also positive for the *aadA* gene. Since class 1 integrations have been found to contain antibiotic resistance genes and are suspected to serve as reservoirs and platforms for exchange of resistance genes within microbial populations, it can be postulated that antibiotic resistance from *E. faecium* S27 might be transferred to clinical enterococci by integrations containing two or more inserted antibiotic resistance genes. In a similar study, Schmidt et al. (2001) showed integron-mediated tetracycline resistance determinant transfer [*tet(A)*] in aeromonad spp.

even though the donors were plasmid-less. In another study it was shown that the presence of tetracycline and streptomycin resistance genes in Enterobacteriaceae, together with class 1 integrons, could play a role in the dissemination of these antibiotic resistance genes to other commensal and indigenous microorganisms (Srinivasan et al., 2008).

In conclusion, the PFGE patterns of the *Enterococcus* isolates from fermented meat/food and clinical samples showed a high level of intra-species heterogeneity. Although a small number of food and clinical enterococci isolates were used in this study, data obtained established that the transfer of antibiotic resistance from enterococci in food to enterococci isolated from humans is feasible. Moreover, the transfer of resistance determinants from *E. faecium* to both clinical *E. faecalis* and *E. faecium* strains via a natural conjugation mechanism was demonstrated and the possible mechanism was suggested to involve an integron. To our knowledge this is the first evidence of a class 1 integron being present in any enterococcal food isolate, especially from an *E. faecium* strain obtained from dry sausage. Previously, class 1 integrons have only been detected in clinical isolates of *E. faecium* (Xu et al., 2010). These results reinforce the concern expressed elsewhere about the safety of enterococcal strains present in foods, particularly in commercially fermented meat where viable antibiotic resistant enterococci may be present.

## Chapter 7

### **Transfer of antibiotic resistance from *Enterococcus faecium* of fermented meat origin to *Listeria monocytogenes* and *Listeria innocua*.**

#### **7.1 Abstract**

*Listeria monocytogenes* is an important foodborne pathogen that can cause listeriosis in children, pregnant women, the immunocompromised and the elderly. Antibiotic resistance in this species, particularly multi-drug resistance, represents a significant public health problem since the organism has a high fatality/case ratio and resistance may contribute to failure of therapeutic treatment. This study was designed to explore whether the *in vitro* transferability of antibiotic resistance from enterococci to *Listeria* spp. could occur. It was found that two of 8 *Listeria* stains were able to acquire tetracycline resistance from *E. faecium*. *Listeria monocytogenes* GLM-2 acquired the resistance determinant *tet(M)* and additional streptomycin resistance through *in vitro* mating with *Enterococcus faecium* S27 isolated from commercial fermented dry sausage. Similarly, *L. innocua* became more resistant to tetracycline, but the genetic basis for this change was not confirmed. It has been suggested that enterococci may transfer antibiotic resistance genes via transposons to *Listeria* spp., and this may explain the origin of multi-drug resistance. Thus, the presence of enterococci in food should not be ignored since they may actively contribute to enhanced antibiotic resistance of *L. monocytogenes* and other pathogens.

#### **7.2 Introduction**

*Listeria* species are ubiquitously distributed in the agri-food environment. They are found on animals and vegetables that are contaminated with animal manure, on decaying vegetation, and in



effluents from sewage treatment plants (Farber and Peterkin, 1991). The genus *Listeria* is comprised of 15 species and, except for *L. monocytogenes* and occasionally *L. ivanovii*, they are not usually recognised to cause disease in humans (Bae et al., 2014). *L. monocytogenes* is a foodborne pathogen that causes listeriosis in humans and animals (Pesavento et al., 2010). Listeriosis is a serious invasive illness mainly affecting children, pregnant women, those who are immunocompromised and the elderly. The symptoms can include meningitis, encephalitis, septicaemia, or in healthy persons, non-invasive febrile gastroenteritis and/or influenza-like symptoms (Lorber, 1990). Although all humans are routinely exposed to these pathogens, listeriosis is a relatively rare disease (Swaminathan and Gerner-Smidt, 2007).

*L. monocytogenes* is often transmitted by food. Raw meat, dairy foods, non-pasteurised milk, soft or semi-soft cheeses, and ice cream have been causes of listeriosis outbreaks. Vegetables and fish, such as smoked salmon may also act as vehicles of transmission of this pathogen (Farber and Peterkin, 1991). In foods these organisms can survive at high salt concentrations; they can tolerate acidic environments; they can survive at low temperature down to the freezing point, which means they may often grow in refrigerated foods. Additionally, *Listeria* spp., especially *L. monocytogenes* readily produce biofilms that enable the organism to survive for prolonged periods in food processing plants (Swaminathan and Gerner-Smidt, 2007).

*Listeria* spp. are naturally susceptible to penicillin, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin. They show reduced susceptibility or resistance to sulfamethoxazole, cephalosporins and the older quinolones, but are generally susceptible to fluoroquinolones. Listeriosis can be effectively treated with penicillin or ampicillin in combination with an aminoglycoside (gentamicin) or with tetracycline, erythromycin or chloramphenicol alone or in combination (Pesavento et al., 2010). *L. monocytogenes* can either acquire or transfer antibiotic

resistance genes from plasmids and transposons of other bacterial species including *Enterococcus* spp., either *in vitro* or in the intestinal tract (Poyart-Salmeron et al., 1992; Pourshaban, et al., 2002). This is a very important phenomenon as bacteria that newly acquire resistance, are not affected by antibiotics during therapy. *L. monocytogenes* is now slowly becoming resistant because of the acquisition of antibiotic resistance genes from other resistant Gram-positives including enterococci, staphylococci or other resistant *Listeria* spp. (Pesavento et al., 2010).

Enterococci inhabiting the gastrointestinal tract of humans and other animals have emerged as a major cause of nosocomial infections in recent decades (Franz et al., 1999). They can be found in a variety of environments including soil, plants and water, especially when contaminated with fecal materials (Aarestrup et al., 2002). Enterococci have been isolated from various foods of animal origin and they can be resistant to a wide variety of antibiotics commonly used in human medicine as well as those that are used for animal therapy, prophylaxis or for growth promotion. They are the most thermotolerant of non-sporulating bacteria and some can survive pasteurization temperatures. Tolerance to environmental extremes explains their survival during processing of cooked and uncooked cured meats and their ability to multiply during fermentations (Simpson et al., 1994). Their high survival capacity in changing environments, such as food might be explained in part by their capacity to form biofilms (Creti et al., 2004; Tendolkar et al., 2004). They are also known for their capacity to exchange genetic information by conjugation (Clewell and Dunny, 2002) and may contribute to the spread of antibiotic resistance among pathogenic and non-pathogenic organisms (Cocconcelli et al., 2003; Fisher and Phillips, 2009).

The food chain is potentially one of the main routes for transmission of antibiotic resistant bacteria between animal and human populations (Witte, 1997). *Listeria* commonly contaminate raw produce, dairy, meat and fish as well as other food items by direct and indirect contact. These

exposures provide opportunity for their acquisition of antibiotic resistance from other bacteria, such as enterococci that inhabit these environments. The aim of this study was to determine whether enterococci isolated from commercially fermented meats could serve as a source for the transfer of antibiotic resistance to *Listeria* spp.

## 7.3 Materials and Methods

### 7.3.1 Bacterial strains

Eight food isolates of *L. monocytogenes* (GLM-1, GLM-2, GLM-3, GLM-4, GLM-5, 2-138, 2-242 and 2-243) were provided by Dr. M.W. Griffiths, University of Guelph, Guelph, ON (Gill et al., 2002) and *L. innocua* HPB586 was obtained from the culture collection of the Food Science Department, University of Manitoba. One strain of *Enterococcus faecium* (S27) originating from dry-fermented sausage was also included in this study as a potential donor of antibiotic resistance (Chapter 3). This strain was resistant to several antibiotics including erythromycin (16 µg/mL), streptomycin (4096 µg/mL) and tetracycline (128 µg/mL), and possessed the respective resistance determinants (Chapter 3). All isolates were grown from frozen -80 °C stocks in Brain Heart Infusion Broth, BHIB (Difco, Fisher Scientific, Edmonton, AB, Canada) containing 50% (v/v) glycerol (Sigma, St. Louis, MO, USA), by incubation on *Listeria* Selective agar base (Oxoid, Basingstoke, England) with *Listeria*-selective supplement (Oxoid) at 25 °C. The *Enterococcus* strain used was also cultured from -80 °C stock in BHIB (Difco) containing 50% (v/v) glycerol (Sigma) and grown on KF-Streptococcus Agar (Difco) at 37 °C.

### 7.3.2 Antibiotic preparation

Antibiotics currently registered in Canada for use in food animals and indicated for the treatment of listeriosis were used in this study. Antibiotic powders obtained from Sigma-Aldrich, Canada Ltd. (Oakville, ON) were used at concentrations of 0.0625 to 32 µg/mL (penicillin G, ampicillin, tetracycline, erythromycin, and chloramphenicol); from 0.0312 to 16 µg/mL for gentamicin and from 0.5 to 256 µg/mL for streptomycin. Antimicrobials were dissolved in distilled water and filter sterilized through 0.20 µm pore-sized syringe filter units (Fisher Scientific), except for tetracycline which was first dissolved in 25% (v/v) ethanol as a solubility mediator. The effect of ethanol on the growth of enterococci was examined in susceptibility tests and found not to be measurable.

### 7.3.3 Antibiotic susceptibility and minimum inhibitory concentrations (MICs)

Fifty µL of double-strength sterile Mueller Hinton broth, MHB (Difco) was placed into each well of 96-well microtiter plates (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA). To the first wells 50 µL of antibiotic solutions were added and serial two-fold dilutions were made to the desired concentrations. Wells were then inoculated with 50 µL of bacterial suspension to obtain a final concentration in each well of approximately  $5 \log_{10}$  CFU/mL and incubated overnight at 35 °C. The trials were conducted in triplicate. Following incubation, 40 µL of *p*-iodonitrotetrazolium violet was added to each well and plates were further incubated for 2 h (Eloff, 1998). The MIC breakpoints for *Listeria* spp. were determined according the Clinical and Laboratory Standards Institute (CLSI, 2002), Wiggins et al. (1978) and Safdar and Armstrong (2003). The MIC was considered to be the lowest antimicrobial concentration at which no red color (signifying no metabolic activity) appeared. When no microbiological breakpoint was found

using these reference guides, breakpoint values from structurally or functionally-related antibiotics were used (CLSI, 2002). Isolates with MICs above these breakpoints were considered resistant.

#### 7.3.4 *In vitro* conjugation experiments

Potential donor strain *E. faecium* S27 was mated with various recipient *Listeria* strains to determine whether antimicrobial resistance could be transferred (Table 7.1). In conjugation experiments a 1:1 ratio of donor/recipient cells was used. The donor strain was resistant to tetracycline and susceptible to ampicillin. On the other hand, all recipient *Listeria* strains were susceptible to tetracycline and some showed resistance towards ampicillin. The donor and recipient strains were grown overnight at 35 °C in BHIB media containing selective antibiotics. The antibiotics (Sigma) were used at the following concentrations: tetracycline 32 µg/mL and ampicillin 32 µg/mL. After incubation at 25 °C overnight, 1 mL culture was centrifuged at 8000 x g (Microcentrifuge 22R, Beckman Coulter, Brea, CA, USA) for 15 min and pellets were washed twice with sterile saline solution. Donor and recipient pellets were combined in 100 µL of sterile saline and plated on Brain Heart Infusion Agar (BHIA, Difco) without any selective agents. After overnight incubation at 25 °C, confluent growth on plates was removed with a platinum loop and suspended in 500 µL of sterile saline. Aliquots (100 µL) of this suspension were spread on selective plates containing 4 µg/mL tetracycline plus 0.5 µg/mL ampicillin and incubated at 30 °C for 48 h. Transconjugants growing on both tetracycline and ampicillin were selected and their MICs were determined. Conjugation frequency was calculated as the number of transconjugant colonies (expressed as CFU/mL) divided by the number of donor cells (Sparo et al., 2011), where transconjugants were considered to be those growing on *Listeria* selective agar supplemented with tetracycline and ampicillin. Each experiment was performed in triplicate; frequency values are reported as the mean ( $\pm$  standard deviation) of all three experiments.

### 7.3.5 PCR for the identification of the *tet(M)* gene

Total genomic DNA was extracted from overnight cultures grown at 37°C in BHIB (Difco), using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA, USA). The sequences of oligonucleotide primers were for forward *tet(M)*-F: 5'-GAYACNCCNGGNCAVRTNGAYTT-3' and for reverse *tet(M)*-R: 5'-CACCGAGCAGGGATTTCTCCAC -3'. Primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). PCR for the *tet(M)* gene was performed as described in Chapter 3.

## 7.4 Result and Discussion

*Listeria* species can cause severe infections in man, including septicaemia, meningitis, meningoencephalitis and abortion. Food is the main vehicle for transmission of these organisms to humans. Members of the *Listeria* genus are considered to be susceptible to most antimicrobials used to treat human listeriosis and those used against Gram-positive bacteria in veterinary medicine. Thus, *Listeria* are normally sensitive towards ampicillin or penicillin, chloramphenicol, erythromycin, gentamicin or tetracycline (Hof, 2004). Phenotypic resistance of the *Listeria* strains examined in this study toward antibiotics is shown in Table 7.1. It was found that 3 of 9 strains were resistant to penicillin G and 6 of 9 were resistant to ampicillin. Srinivasan et al. (2005) and Yücel et al. (2005) found a high frequency of resistance to ampicillin and penicillin G in their isolates from food sources which is similar to results from the present work. One of 8 (12.5%) *L. monocytogenes* strains (strain 138) showed resistance towards streptomycin (MIC  $\geq 32$ ) which was in contrast with results found by Aase et al. (2000) where none of 160 *L. monocytogenes* isolates was resistant to streptomycin. Charpentier et al. (1995) found only 3 streptomycin resistant strains among 1100

*Listeria* spp. which was a lower frequency than observed here. *Listeria* isolates here were also resistant to gentamycin which is in contrast with those examined by Srinivasan et al. (2005) which were susceptible. Although all strains tested here were resistant to chloramphenicol, none was resistant toward tetracycline. It was notable that Arslan and Özdemir (2008) found only 2% of the strains they examined were resistant to tetracycline. *Listeria* spp. from humans, animals and food sources resistant to more than one antibiotic have been reported (Charpentier and Courvalin, 1999; Srinivasan et al., 2005). Shi et al. (2015) found 6 *Listeria* strains resistant to 10 antibiotics tested from retail ready-to-eat foods in China. Bae et al. (2014) found *L. monocytogenes* strains isolated from domestic and imported food products including seafood, vegetables, and dairy foods were resistant to more than 3 different antibiotic classes. These results are consistent with the results obtained in the present study where *Listeria* isolates resistant to at least two antibiotics were common.

Table 7.1 Antibiotic resistance phenotype of *Listeria* isolates and transconjugants

<i>Listeria</i> strain	MIC (resistance breakpoint [ $\mu\text{g/ml}$ ]) <sup>a</sup>							Source
	Am	Cm	Em	Gm	PG	Sm	Te	
	(0.5)	(8)	(0.25)	(1)	(0.5)	(32)	(2)	
<i>L. monocytogenes</i> GLM-1	S	8 <sup>R</sup>	S	8 <sup>R</sup>	S	S	S	Meat plant; <sup>c</sup> CRIFS #716
<i>L. monocytogenes</i> GLM-2 <sup>b</sup>	1 <sup>R</sup>	16 <sup>R</sup>	S	16 <sup>R</sup>	0.50 <sup>R</sup>	S	S	Meat plant; CRIFS#717
<i>L. monocytogenes</i> GLM-3	S	8 <sup>R</sup>	S	16 <sup>R</sup>	2 <sup>R</sup>	S	S	Meat plant; CRIFS#718
<i>L. monocytogenes</i> GLM-4	0.50 <sup>R</sup>	8 <sup>R</sup>	S	8 <sup>R</sup>	0.50 <sup>R</sup>	S	S	Meat plant; CRIFS#719
<i>L. monocytogenes</i> GLM-5	S	8 <sup>R</sup>	S	8 <sup>R</sup>	S	S	S	Meat plant; CRIFS#720
<i>L. monocytogenes</i> 138	0.50 <sup>R</sup>	8 <sup>R</sup>	S	16 <sup>R</sup>	S	32 <sup>R</sup>	S	Dill; University of Guelph
<i>L. monocytogenes</i> 2-242	0.50 <sup>R</sup>	16 <sup>R</sup>	S	8 <sup>R</sup>	S	S	S	Apple juice; University of Guelph
<i>L. monocytogenes</i> 2-243	0.50 <sup>R</sup>	16 <sup>R</sup>	0.25 <sup>R</sup>	8 <sup>R</sup>	S	S	S	Cucumber; University of Guelph
<i>L. innocua</i> HPB586 <sup>b</sup>	0.50 <sup>R</sup>	8 <sup>R</sup>	S	16 <sup>R</sup>	S	S	S	University of Manitoba
T3 ( <i>E. faecium</i> S27 x <i>L. innocua</i> HPB586)	0.50 <sup>R</sup>	8 <sup>R</sup>	S	8 <sup>R</sup>	S	S	4 <sup>R</sup>	
T4 ( <i>E. faecium</i> S27 x <i>L. monocytogenes</i> GLM-2)	1 <sup>R</sup>	16 <sup>R</sup>	S	32 <sup>R</sup>	4 <sup>R</sup>	$\geq 256^{\text{R}}$	$\geq 3$ 2 <sup>R</sup>	

<sup>a</sup>Ampicillin (Am), chloramphenicol (Cm), erythromycin (Em), gentamycin (Gm), penicillin G (PG), streptomycin (Sm) and tetracycline (Te); MIC represents minimum inhibitory concentration. R, designates resistance concentration in  $\mu\text{g/ml}$ ; S, designates susceptibility toward antibiotic (CLSI 2002, Wiggins et al., 1978 and Safdar and Armstrong, 2003);

<sup>b</sup> recipient during conjugation.

<sup>c</sup> CRIFS, Canadian Research Institute for Food Safety, University of Guelph



When the transferability of antibiotic resistance through plate mating trials was examined using *E. faecium* S27 containing the tetracycline resistance gene (Chapter 3) as a donor and 9 *Listeria* spp. as recipients, *E. faecium* S27 was only able to transfer its tetracycline resistance to two *Listeria* strains. Resistance transfer was to *L. innocua* HPB586 and *L. monocytogenes* GLM-2 and it occurred with a frequency of  $6.3 \times 10^{-8}$  and  $3.8 \times 10^{-8}$  transconjugants/donor. These later were represented by two transconjugants, T3 and T4, respectively (Table 7.2). When the antibiotic MICs for the donor, recipients and transconjugants were determined, transconjugants showed profiles similar to the *Listeria* isolates. Both transconjugants T3 and T4 had acquired resistance toward tetracycline, but only T4 possessed the resistance gene *tet(M)* (Fig. 7.1) which was present in the donor, *E. faecium* S27. Moreover, T4 showed higher resistance toward streptomycin which was similar to the donor. These phenotypic and genotypic observations established that antibiotic resistance had been transferred from *E. faecium* S27 to the *Listeria* isolates. Tetracycline resistance in enterococci can be conferred by various genes and mechanisms including efflux systems encoded by *tet(K)* and *tet(L)*, ribosomal protection encoded by *tet(M)*, *tet(O)*, and *tet(S)*, as well as an unknown mechanism encoded by *tet(U)* (Wilcks et al., 2005). Of these genes, *E. faecium* S27 tested positive for the presence of *tet(M)* but was not tested for the presence of *tet(U)* (Chapter 3). Thus, the tetracycline resistance of T3 might be have been due to the transfer of *tet(U)* from the donor.

Figure 7.1 PCR analysis of *tet(M)* genes in *E. faecium* S27 and transconjugants T3 (*E. faecium* S27:: *L. innocua* HPB586) and T4 (*E. faecium* S27:: *L. monocytogenes* GLM-2). GLM-2, *L. monocytogenes* GLM-2 as negative control. M, 1kb standard molecular size ladder.

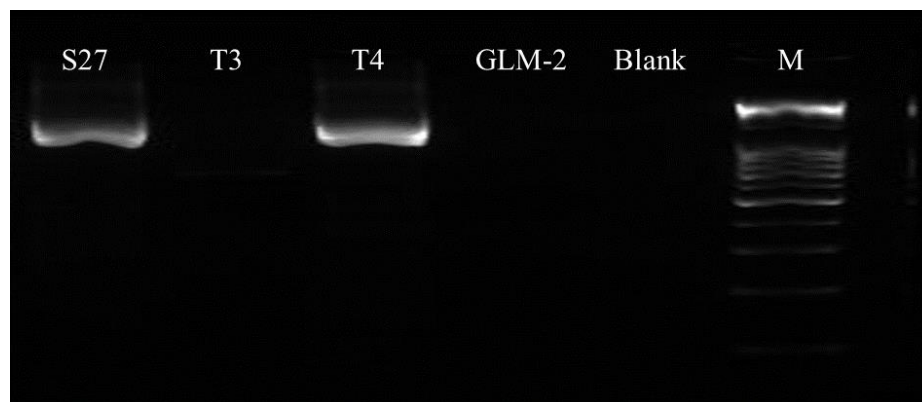


Table 7.2 Summary of positive conjugation experiments of enterococci with *Listeria* strains

Donor	Recipient	Transconjugant	Conjugation frequency <sup>a</sup> (cfu/ml)
<i>E. faecium</i> S27	<i>L. innocua</i> HPB586	T3	6.3X10 <sup>-8</sup>
<i>E. faecium</i> S27	<i>L. monocytogenes</i> GLM-2	T4	3.8X10 <sup>-8</sup>

<sup>a</sup> Conjugation frequency was calculated as the number of transconjugants (cfu/ml) divided by the number of donor cells (cfu/ml) (Sparo et al., 2011)

The evolution of antibiotic resistance in microbial communities may occur by horizontal transfer of resistance genes across species and genus borders by conjugative plasmids and transposons, but also by the action of integrons and insertion elements, and by lytic and temperate bacteriophages (Teuber et al., 1999). It has been suggested that the emergence of multiple-antibiotic resistance in *Listeria* spp. can result from the acquisition of a conjugative resistant plasmid originating in enterococci (Poyart-Salmeron et al., 1990). The donor in this study however, did not possess any detectable plasmid (data not shown). The *tet(M)* gene in many bacterial genera is often linked to the presence of conjugative transposons of the Tn916-1545 family (Rizzotti et al., 2009). Doucet-Populaire (1991) showed the transfer of the tetracycline resistant transposon Tn1545 from *E. faecalis* to *L. monocytogenes* which is likely similar to the transfer that occurred during the present work. Mating experiments done in two previous studies also demonstrated that some strains of *E. faecalis* and *E. faecium* were able to transfer tetracycline resistance determinants by means of this transposon family (Huys et al., 2004; Rizzotti et al., 2009). Biavasco et al. (1996) showed plasmid and transposon-mediated transfer of vancomycin resistance *in vitro* by conjugation or transformation from enterococci to *L. monocytogenes*.

## 7.5 Conclusion

Conjugative transposons are common in enterococci, and their involvement in the evolution of acquired antibiotic resistance in *Listeria* spp. can be confidently predicted based on the results obtained during this work. Thus, it cannot be excluded that natural transfer of antibiotic resistance to *Listeria* strains may occur in the future. These results reinforce the concern expressed elsewhere about the safety of enterococcal strains present in foods, where they may serve as reservoirs of transferrable factors which can confer antibiotic resistance to other genera/species.

## Chapter 8

### Overall Discussion and Conclusion

The increasing prevalence of antibiotic resistant bacteria may represent a serious risk for public health worldwide. Antibiotic resistance genes are easily spread via horizontal gene transfer (HGT), leading to complications in the treatment of infections caused by multi-drug resistant bacteria (Sørensen, 2005). However, antibiotic resistance is not restricted to pathogens, and the role of commensal bacteria in the dissemination of antibiotic resistance genes has gained more attention in the last decade (Wang et al., 2006). Members of the genus *Enterococcus* frequently harbor transferable antibiotic resistance genes and are assumed to play an important role in the horizontal spread of these genes (Hegstad et al., 2010). Antibiotic resistant enterococci ingested via food can transiently colonize the human gut and might transmit resistance genes to commensal or ingested bacteria in the colon. In this dissertation, it was hypothesized that foodborne bacteria, especially enterococci from dry fermented sausages, may carry antibiotic resistance genes that can be transferred to human commensal enterococci and even to pathogens, such as *L. monocytogenes*.

First, the main focus of the work was to isolate enterococci from commercially fermented dry sausages. The development of a genus specific primer and its use in the detection of enterococci in fermented sausage samples facilitated the isolation of enterococci. Real-time PCR (RT-PCR) was used as an alternative method for the detection and quantitation of enterococci from sausage samples (Domingo et al., 2003). Although RT-PCR techniques are more sensitive for detection of enterococci, the method is expensive and takes longer for large batches of samples in an industrial setting. Therefore, the method developed in Chapter 3 has value in both experimental as well as in

industrial settings for the rapid screening of enterococci in food in order to understand the contribution they may make to the burden of illness.

When the antibiotic resistance of these enterococci isolates was examined, it was revealed that most were multi-drug resistant. Antibiotic resistance genes were also present in some of these isolates (Chapter 3). In the present work it was found that some strains were resistant toward tetracycline, erythromycin, chloramphenicol and ciprofloxacin in the absence of detectable genetic determinants. Thus, it seemed probable that the enterococci isolated from meat and fermented meats may act as reservoirs for antimicrobial resistance genes.

Virulence factors and genetically encoded antibiotic resistance have been found not only in enterococci isolated from hospital patients with clinical disease, but they have also been detected in food isolates (Eaton and Gasson, 2001; Franz et al., 2001). The occurrence of several virulence traits together with a high level of resistance to a wide variety of antibiotics suggest that enterococci may represent a reservoir of virulence and antibiotic resistance genes in the food chain which are matters of concern (Ogier and Serror, 2008). In the present work it was found that the incidence of virulence factors was higher among isolated *E. faecalis* than *E. faecium* (Chapter 4). As *E. faecalis* are more often associated with human disease than *E. faecium* (Franz et al., 2001) the high frequency of virulence factors among food *E. faecalis* strains was of concern.

Biofilms have been suggested to be important factors in the pathogenesis of enterococcal infections (Sandoe et al., 2003), although few studies of biofilms have been done with food enterococci. Biofilm formation is suggested to be a stress-response mechanism for enterococci. Biofilms may greatly enhance the virulence of this organism, specifically as it relates to colonizing ability. The present study revealed that at suboptimal temperature exposure (25 °C), similar to conditions used

for dry sausage fermentation, enterococci were able to form strong biofilms. This was true for two strains of *E. faecalis* (S38 and S39) and three strains of *E. faecium* (S9, S15 and M2) which were unable to form biofilms at 37 °C, but formed strong biofilms at 25 °C. This did not appear to be dependent upon the presence of either *esp* or *gelE* genes (Chapter 4). Minimal inhibitory antibiotic concentrations were also tested at 25 °C for these 6 strains and there was no change in antibiotic resistance pattern except for one strain of *E. faecium* S9. Although this strain showed resistance towards erythromycin (8 µg/mL), penicillin (16 µg/mL), streptomycin (4096 µg/mL) and tetracycline (32 µg/mL) at 37 °C, the organism became sensitive to these antibiotics at 25 °C. The bacterial response to the stress of suboptimal temperature can cause significant changes in antibiotic resistance and associated virulence gene expression (Depardieu et al., 2007; Lenz et al., 2010). It was shown in a study that the significant differences in virulence gene expression in *E. faecalis* were influenced by growth phase and environmental conditions (Hew et al., 2007). More study is needed to understand factors influencing antibiotic resistance gene expression of food enterococci isolates.

One of the objectives of the current study was to determine if enterococci carrying antibiotic resistance and virulence factors isolated from raw and fermented meat can be controlled with natural antimicrobials present in Oriental mustard (Chapter 5). Mustard powder derived from grinding whole mustard seed is used primarily in the meat industry as an emulsifier and water binding agent or filler in cooked processed meats or other foods. The glucosinolate sinigrin (allyl glucosinolate) predominating in Oriental and brown mustard yields on hydrolysis the volatile compound allyl isothiocyanate (AITC) that has antimicrobial activity (Nadarajah et al., 2005). AITC has been found effective for reducing *E. coli* O157:H7 viability in fermented meat products (Chacon et al., 2006; Graumann and Holley, 2008). It has also been shown in some studies that

mustard flour can serve as a source of AITC and be effective in controlling *E. coli* O157:H7 in ground beef (Nadarajah et al., 2005) and also inhibit the growth of *L. monocytogenes* and *Salmonella* (Olaimat and Holley, 2014). Regarding these findings, the MIC of AITC was determined for the enterococci isolates and it was found that they varied from 1.0 mM/L to 2.5 mM/L. Since AITC up to 5.0 mM/L has been acceptable as a flavor fortifier in meat (Chacon et al., 2006), and since this level is much higher than the highest MIC value for enterococci, it is likely that AITC can be used to control these organism in dry fermented sausage environments.

Pulsed-field gel electrophoresis (PFGE) has been widely used for typing of enterococci because of its ability to discriminate between strains and its reproducibility (Patterson and Kelly, 1998). Use of epidemiological strain typing of enterococci has been well documented for human and food enterococci. When compared, PFGE patterns of the *Enterococcus* isolates from fermented meat/food and clinical samples showed a high level of intra-species heterogeneity. Although a small number of food and clinical enterococci isolates were used in this study, data obtained established that the transfer of antibiotic resistance from enterococci in food to enterococci isolated from humans was feasible (Chapter 6).

Horizontal transfer of resistance gene determinants from these food enterococci was evaluated (Chapter 6). It was found that one *E. faecium* strain (S27) isolated from fermented dry sausages was able to transfer the tetracycline gene [*tet*(M)] to clinical *E. faecalis* and *E. faecium* strains with a frequency of  $1.1 \times 10^{-6}$  and  $2.1 \times 10^{-5}$  cfu/mL respectively (Chapter 6). The *E. faecium* S27 strain also transferred the streptomycin resistance gene, *aadA* to a clinical strain, *E. faecalis* 82916, which was confirmed by the presence of this gene in both donor and transconjugant strains. Sequencing of the *tet*(M) and *aadA* gene of the donor and transconjugant revealed 95-99% nucleotide homology. Moreover, the possible mechanism of resistance determinant transfer via



natural conjugation was suggested to involve an integron. Previously, class 1 integrons have only been detected in clinical isolates of *E. faecium* (Xu et al., 2010). This finding reinforces the need to understand how these food isolates transfer resistance determinants. Thus, this study confirmed the hypothesis that some of the antibiotic resistant enterococci from fermented sausage entering the food chain may enhance the pool of antibiotic resistant bacteria in the human gut microbiota.

Transfer of resistance genes from fermented sausage enterococci to starter cultures and pathogens associated with meat and fermented sausages were studied in Chapter 7. It was found that *Listeria monocytogenes* GLM-2 acquired the tetracycline resistance determinant *tet*(M) and streptomycin resistance through *in vitro* mating with *Enterococcus faecium* S27. Similarly, *L. innocua* became resistant to tetracycline but the genetic basis for this change was not confirmed. Involvement of enterococci in the evolution of acquired antibiotic resistance in *Listeria* spp. can be confidently predicted. *Pediococcus* and *Staphylococcus* species are used as starter cultures and *Lactobacillus* occur naturally in fermented sausages (Hugas and Monfort, 1997; Smith and Palumbo, 1983). Conjugation experiments were also conducted with strains of *Pediococcus*, *Staphylococcus* and *Lactobacillus* using enterococci as the donor. Several attempts were made using various donor-recipient ratios but these did not result in the generation of any transconjugants. Lactic acid bacteria typically encompass Gram-positive organisms belonging to the genera *Lactococcus*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc* are largely used in the production of fermented foods, and the development of enhanced antibiotic resistance in these organisms would be a concern (Mathur and Singh, 2005). It has been documented that some meat starter cultures used in dry sausage fermentations were multi-drug resistant and contained resistant determinants (Corderio et al., 2010). One explanation for the origin of their resistance might be the acquisition of resistance genes from enterococci.

The presence of multi-drug resistant enterococci was confirmed in commercially fermented dry sausages. Patterns of both expressed and silent virulence genes in these enterococci were also documented. It is of concern that these virulence determinants in *E. faecalis* and *E. faecium* strains may be significant in the evolution of pathogenic species. The transfer of antibiotic resistance determinants to human isolates and pathogens (*Listeria*) via natural conjugation was demonstrated in the present work. These results reinforce the concern expressed elsewhere about the safety of enterococcal strains present in foods. More study is needed to gain better understanding of the mechanism of antibiotic resistance development and gene transfer phenomena.

## Chapter 9

### Future Direction

It will be important to understand the extent to which exposures of *Enterococcus* strains to stressful environments influences expression of antibiotic resistances. In addition, it would be valuable to assess whether environmental stress can alter the coordinated expression of virulence and antibiotic resistance.

The mechanisms involved in biofilm formation by enterococci isolated from raw and fermented meat remain unclear. In future studies it will be interesting to discover more about the roles of factors responsible for biofilm formation and whether they interact with other genetic factors. For this, extensive genetic and biochemical analysis will be required. By understanding the process of biofilm formation at the molecular level, it should provide insight enabling more effective control of enterococci, particularly those from fermented food, as it is clear that biofilms are an important component in the pathogenicity of this group of organisms.

The present study established the horizontal transfer of antibiotic resistance genes from *E. faecium* S27 isolated from fermented sausage to clinical enterococci isolates as well as to *Listeria* species. This phenomenon raised the possibility that contaminated food can serve as a vehicle for transfer of other resistance genes from enterococci to other pathogens. Further, although the present work showed that the resistance gene transfer mechanism likely involved an integron, this was not established. Numerous enterococci genomes from various sources have been sequenced so far. It would be valuable to determine the complete sequence of the *E. faecium* S27 genome and by comparing it with the existing database to facilitate understanding the gene transfer mechanism. The genome sequencing will also reveal numerous encoded stress response genes which can be

used to understand how this organism has adapted to the harsh environment of fermented dry sausage.

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**Appendix I****Chapter 3 (Manuscript 1)**

Organism name and GenBank accession numbers for 16S rRNA sequences

<i>Enterococcus</i> isolates	GenBank accession number
<i>E. faecalis</i> S5	KT027563
<i>E. faecium</i> S6	KT027564
<i>E. faecium</i> S9	KT027565
<i>E. faecalis</i> S10	KT027566
<i>E. faecalis</i> S11	KT027567
<i>E. faecalis</i> S13	KT027562
<i>E. faecium</i> S15	KT027568
<i>E. faecalis</i> S18	KT027569
<i>E. faecium</i> S22	KT072734
<i>E. faecium</i> S27	KT027561
<i>E. faecium</i> S28	KT027570
<i>E. faecium</i> S29	KT027571
<i>E. faecium</i> S31	KT027572
<i>E. faecalis</i> S36	KT027573
<i>E. faecalis</i> S39	KT027574
<i>E. faecalis</i> S40	KT027575
<i>E. faecium</i> S50	KT027576
<i>E. faecalis</i> <sup>c</sup> H1	KT027579
<i>E. faecium</i> <sup>d</sup> M1	KT027577
<i>E. faecium</i> M2	KT027578

## 16S rRNA sequences of enterococci isolated from raw and fermented meat

*E. gallinarum* S19

AACCTGCCCATCAGAAGGGGATAAACTTGGAAACAGGTGCTAATACCGTATAAACTATTTTCCGCATGGAAGA  
 AAGTTGAAAGGCGCTTTTGCCTACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCAC  
 CAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCAACTGGGACTGAGACACGGCCAGACTCCTAC  
 GGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCTGAGTGAAGAAGGT  
 TTTCGGATCGTAAAACCTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAGAACGTTTCATCCCTTGACGGTATCTAAC  
 CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGG  
 CGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAA  
 CTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAA  
 CACCAATGGCGAAGCGGCTCTCTGGTCTGTAAGTACGCTGAGGCTCGAAAGCGTGGGGGAGCGAACAGGGATT  
 AAATACCCTGGTAGTCCACGCCCTTAAACCAATGAGTGGCTAAAATGTTTGGGAGGGTTTTCCCGCCCTTCAGTT  
 GCTGGCAGCCAAAACGGCATTATGCACTTCCCGCCCTGGGGGAGTTACCAACCGCCAGGGTTGAAACTCCAAA  
 GGAAATTTGACGGGGGGGCCCCCCCCCAATCCGTGTGGAAGCAGTGGTTGGGTTTTTTAATTTTCATAAAGGC  
 AACCCCGGAAGAAAACCCATGTCCCGGGGCCCTGGGATAATCCCTTTTTGAAGCCCCCACC

*E. faecalis* S25

CTATACATGCAAGTCGAACGCCTTCTTTTCTCCCGGAGTGCTTGCACTCAATTGGAAAGAGGAGTGGCGGACGG  
 GTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAAACTTGGAAACAGGTGCTAATACCGCATAACAGT  
 TTATGCCGCATGGCATAAGAGTGAAGGCGCTTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGT  
 TGGTGAGGTAACGGCTACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCAACTGGGACTGAG  
 ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAACAAACGC  
 CCCGTGAGTGAAAAAAGTTTTCGGATCGTAAAACCTTTTTGTTAAAAGAAAAACAAAGGACTTTTAGTAACTGAAC  
 CTTCCCCTGACCGTATTCTAAACCCAAAAGGCCACGGCTTAACTTCTTCCCACCACCCCGGGTTTATTACCGTAAG  
 GTTGGGCCAACCGTTTTTCCCGGATTTTTTTGGGGGCTTAAATCTAACCTCCCGGGCGGGTTTTCTTAAATTTTT  
 AAGTTTAAAAACCCCCCGGCCACCCCGGGGAAGGGTTCTTTTGTGAAAACCTGGGAAAAAAGTTGGGAGTTG  
 CCCAAAAAAGGGAAAATTGGGAATTCTCCCGTTGTTTTACCGGGCTTGAAAATTCG

## Appendix II

### Chapter 6 (Manuscript 3)

#### Antibiotic resistance gene sequences

Organism	Gene	FASTA sequence
	(bp)	
<i>Enterococcus faecium</i> S27	<i>tetM</i> (1513)	ATGGGNNATTNTATTGATTTATGCNAAGATGGNGTCAAGNNNNNNTT NTGNTTATTTTCATGCACTNAAGAAAATGGGGATTCCCACAATCTNTTT TATCAATAAGATTGACCAAAGTGCCATTGATTTATCAACGGTTTATCA AGATATTAAGAGAAAACTTTCTATGGTAATTATAATCCAACACAAAGT ACAGCTGCTCCCTACTATGTGTGTGATGAGCTGACGGAACCTGAGCAA TGGGANNNNTCCCNAGAAGAAATGATGACCTTTTAGAGAATNTATGT CCGGNAATCNTTANAAGCATTAGAACTCGAANAAGAGGAAANNNA AGATTCAGAATTGCTCCTTGTACCCTGTTTATCATGGAAGCGCAGAA AGTACCATAGGGATTGAGCACTTTATATAAGTGATAACGAATAAATTT TATTCGTCTGCATACAGAAAGAAGTCTGACCTTTGCGGAAATGTCTTC NGAATTGAATATTCGGAAGAAAGACAACGTCTTGATATGTACGCCTT TATGGCGGAATCCTGCATTTGCGGGACTCGGTTAGAATATCAGACAAG GAAAAAATAANAATTACAGAAATGTATACTTCAATACATGGCGAATT ATGGAATAATTGATCAGGCTTATCCGGGGAAATTGTTATGTTGCAAN TGAGNTTTTGAAGCTNATTAGNGNTCTTGGAGATACANAGCTANNGN CACANAGAGAGAGAATTGANATCCGCTCCCTCTGCTGCACACNACTGT TGAACCGATCAAACCTCCACAAAGGGAAATGTTACTTGATGCACTTTT AGAAATCTCCGACAGTGACCCGCTCTACNATATTATGNNATCCTACGA CACNGNAATCTACNTTCTTGCTACGGAAAGNACAAATGGAANTGACT TCNGCTCTNTNGCAGAAAGCATNTGNNGNAGGTNAAAAACANANAAN CCNACGTCATTNANNNNAAAAGACCGTTAAAAAANGCAAANNNNNCC NTTNNCNCNAAGNGCCNCNTANTCCTCTCTGNNTACCAGTNGCCGCA
T1 (E. faecium S27::E. 83056)	<i>tetM</i> (1513)	ATGTNGNNTTCTATTGATTTCTGTCAAGATGGGGTAGAGCNNNNNTCN NATATGAGTTCATGCACTTAGGAAAATGGGGATTCCCACCATCNGTT ATCAATTAGATTGACCATTGTGCCATTGATTTATCAACGGTTTATCAA GATATTAAGAGAAAACTTTCTAGGGAAATTATTATCAAACACAAAGT ACAGCTGTCCCTACTATGTGTGTGATGAGCTGTNCGGAACCTGAGCNA TGGGATGTGGCAATAGAAGGAAAGGATGACCTTTTAGAGAAATATAT GTCCGGTAAATCNCTANAAGCATTAGAACTCGAANAAGAGGAAATCA GAAGATTTCATACTTGCTCCTTGTACCCTGTTTATCATGGAAGCGCAG AAAGTACCATAGGGATGGAGCACTTTATTTAAGTGATAACGAATAAA TTTTATTCGTCTGCATACAGAANGAAGTCTGCCCTTTGCGGAAATGTC TTCNAAATTGAATATTCNGAAGAAAGACNACGTCTTGATATGTACGC CTTTATGGNNGAATCCTGCATTTGCNNGATTCCGNTAGAATATCGGAN AAGGAAAAAATNANAATTACNGAAATGTATACTTCAATACATGGNGA ATTATGNANGATTGATCAGGCTNANTCCGGGNAAATTGTTATGTTGCA

			AAATGAGNNTTCGAAGCTNAATANNGTTCTTGGAGATACAAAGCTAT TGCCACANANAGAGAGAATTGAAAATCCGCTCCCTCTGCTGAAACNA CTGTTGNACCGATCAAACCTCCACAAAGGGAAATGTTACTTGATGCAC TTTTAGAAATCTCCGACAGNNACCCCTTCTACAATATTATGNNGATNC TACGACNCATGAAATTATNCTTTCTNNCTTANGGAAAGNACANATGG AAGTGNNTTGTGCTCTATTGTANAAANNCATCATGNGAAGTTAAAAAT NANANANCCNACNGTCATTATANGGAAAGACCGTTAANAANGCANNAN NANNNCATTGAAGTTGCCNNCTNANNCCNNTNCNTGGNNNTNCNNG NCTNNNCAGNNT
T2	( <i>E. faecium</i>	<i>tetM</i>	ATTTATGTCAAGATGGGGTCGTGANNNNNTTCGTGNTGAGTATATGCA CTTAACAAAATGGGGACTNNANCNTCTCTGTATCAATTAGATTGACCA
S27::E.	<i>faecalis</i>	(1513)	TTGTGCCATTGATTTATCAACGGTTTATCAAGAATTTGAAGAGAACT TTCTAGGGTAATTATACCCACACAAAGAACAGATGCTCCCTACCATGT GTGTGATGAGCTGNCCGGAACCTGAGCNCATAATGNGGCCCTTCAA GAAATGATGACCTTTTGAAGAAATATATGTCCGGTAAGAATTAGAAG CATCAGAACTCGAACACTTCCCAATCANAAGATTCATANTTGCTCCT TGTACCCTGCCCATCATGGAAGCGGAGGAACCTCCATAGGGATGGAG CACTCTGTTTAATTGATCCGATTAACCTTTTATTTCGTCTGCATGCTTAAA GATTTCTGCACTTTGCGGATATGTCTTCTGCATTGAATATCCGGAAGAT AGACTACGTCTTGCNTATGNACNCCTTTATGGCGTANTCCTGCNTTTG CAGGATTCGGNTAGAATATCAGACAAGGAACCTGATNNAANATNCCGA AATGTGTACTTCATTACATGGCGAATTATGGAAGATTGATCAGGCTTA TTCCGGGNAAATTGTTATTTTTGCAAATGACANTTCGATCTNAATAGN GNTCTTGGAGATACNAAGCTATNGNCACANANAGAGAGAATTGTCAA TCCGCTCCCTCTGNNGCNCNCAACTGTTGAACCTATCAAACCTCCACA ATNNNCCATGTTACTTGATGCACNTTTAGAAATCTNNNACAGTGACCC GCTTCTACAATANTATCNGGATNCNACNACACATGAAATNATNCNTTN CTNTCTTACGTAAGTACACATGGAAGTGNCTTGTGATCTANANATCN TCNGNAGGTNAAAAACANANATCCCNACNGTCATTANNNNNNNANGA CCNTTAAACAGCNNANAANNNCATCCNNGANNTGCCNCNNNNCNCN CCNNNTGGNCNTNCAGTGGNNTAAGCNGCA
82916)			
<i>E. faecium</i>	S27	<i>aadA</i>	CGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATCTCCGC GCTGTAAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTT (284) ATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGAC ATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCT ATCTTGCTACAAAAGCAAGAGAACATATCGA
T2	( <i>E. faecium</i>	<i>aadA</i>	AGCTTTGATCACGACGTTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGC GAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATC S27::E.
<i>faecalis</i>	(284)		ATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGG CAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGAC ATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGCGAAT
82916)			