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GENERAL INTRODUCTION AND OUTLINE OF THE THESIS
Chapter 1

**General Introduction**

Enterococci are gram-positive, facultative anaerobic bacteria that account for no more than 1% of the normal intestinal microflora of healthy adults. They are ubiquitous in nature and are also found in the intestines of other animals and in soil, water and food [1]. Enterococci are able to withstand harsh environmental conditions; they are able to grow in 6.5% NaCl, in a wide range of pH values and in temperatures between 10°C and 45°C. Furthermore, they can survive on inanimate objects for weeks – a feature that allows them to adapt well to any environment and that may have contributed to their nosocomial transmission [2, 3].

For years Enterococci were regarded as pathogens of low virulence, causing opportunistic infections in critically ill patients. Increased clinical and public interest was attracted since the first identification of vancomycin-resistant enterococci in 1986 [4]. Since then, rates of colonization and infection with vancomycin-resistant enterococci have risen steadily and currently *Enterococcus* spp. are the third most common pathogen isolated from nosocomial bloodstream infections and the most common pathogen in surgical-site infections reported from intensive care units, in the United States and some European countries [5-7]. Additionally, they are increasingly isolated from urinary tract infections (UTI), endocarditis and abdominal infections. Nowadays, more than 30% of all enterococci recovered from healthcare-associated infections are vancomycin resistant [5]. The rise in incidence has, in part, been attributed to changes in medical care, e.g. the growth in the numbers of immunocompromised and critically ill patients, the increased use of intravascular devices and the more prolonged hospital stays. But more important is their multiresistant nature to various classes of antibiotics that are used abundantly [8, 9] and their ability to acquire high-level drug resistance through horizontal gene transfer. In particular *Enterococcus faecium* has adapted to the abundant antibiotic use in hospitals by acquiring resistance to high dose aminoglycosides, β-lactam antibiotics and (more recently) vancomycin (Table 1) [8, 10, 11]. Beta-lactam and vancomycin-resistance has almost completely penetrated in *E. faecium*, with 90 and 80% of all *E. faecium* from nosocomial infections being resistant against ampicillin and vancomycin, respectively [5].
Table 1. Antimicrobial resistance of enterococci.

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<tr>
<th>Antibiotic</th>
<th>Species</th>
<th>Mechanism of resistance</th>
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<tr>
<td><strong>Intrinsic resistance</strong></td>
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<tr>
<td>β-Lactams</td>
<td>All enterococci</td>
<td>Low affinity PBP</td>
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<td>Penicillins (low level)</td>
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<td>Carbapenem (moderate level)</td>
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<td>Cephalosporins (high level)</td>
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<tr>
<td>Aminoglycides (low level)</td>
<td>All enterococci</td>
<td>Inefficient uptake</td>
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<tr>
<td>Aminoglycides (moderate levels)</td>
<td><em>E. faecium</em></td>
<td>Production of chromosomal AAC(6')II enzyme</td>
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<tr>
<td>Lincosamides and Streptogramins A</td>
<td><em>E. faecalis, E. avium,</em></td>
<td>Putative efflux</td>
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<tr>
<td>Glycopeptides (low level)</td>
<td><em>E. gallinarum, E. casseliflavus</em></td>
<td>Production of +1-Ala,-Ser ending peptidoglycan precursors</td>
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**Acquired resistance**

<table>
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<tr>
<th>Antibiotic</th>
<th>Species</th>
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<tr>
<td>Ampicillin (high level)</td>
<td><em>E. faecium, E. hirae, E. faecalis</em></td>
<td>Overproduction or alterations of PBPs β-lactamase (rare)</td>
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<tr>
<td>Aminoglycides (high level)</td>
<td><em>E. faecalis, E. faecium,</em></td>
<td>Modifying enzymes e.g. AAC(6')-APH(2‘)</td>
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<td>Macrolides</td>
<td>Most enterococci</td>
<td>Ribosomal methylation</td>
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<td>Chloramphenicol</td>
<td><em>E. faecium, E. faecalis</em></td>
<td>CAT encoding enzymes</td>
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<td>Tetracyclines</td>
<td><em>E. faecium, E. faecalis</em></td>
<td>Modification of ribosome protein</td>
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<td>Quinolones</td>
<td><em>E. faecium, E. faecalis</em></td>
<td>Alteration in DNA gyrase and Topoisomerase IV</td>
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<tr>
<td>Glycopeptides (high level)</td>
<td><em>E. faecium, E. faecalis</em></td>
<td>Precursor modification</td>
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<tr>
<td>Oxazolidinones</td>
<td><em>E. faecium</em></td>
<td>Mutation in 23S rRNA gene</td>
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**Clinical epidemiology**

Historically, 80 to 90% of clinical isolates of enterococci were *E. faecalis*, whereas *E. faecium* accounted for 5-10% [13]. Currently in the US, the proportions of *E. faecium* isolates among all enterococcal infections are steadily increasing and exceed 30%, which almost equals the incidence of invasive isolates of vancomycin-resistant *E. faecium* (VRE) [5]. In Europe, VRE prevalence rates in hospitals have been increasing since the year 2000 [14, 15]. In most European countries prevalence rates are not as high as in the United States, although over the past six years, vancomycin resistance in *E. faecium* causing invasive infections increased significantly in 6 countries, namely Germany, Greece, Ireland, Israel, Slovenia, and Turkey (EARSS annual report 2007). In the Netherlands the prevalence of VRE among bloodstream isolates has been consistently low (<1%) over the years, which is probably due to the prudent use of antibiotics and a “search and destroy” policy in Dutch hospitals (for both VRE and methicillin-resistant *Staphylococcus aureus* (MRSA)). Importantly, the global VRE epidemic was preceded by the emergence of ampicillin-resistant *E. faecium* (ARE) in the US in the early 1980s, followed by the rapid emergence
of VRE in the 1990s [16, 17]. In several European countries, a similar increase in ARE has been observed, but with a 10-year delay [18]. Although VRE prevalence rates are low, data from a recent nationwide study revealed a significant increase in invasive ARE in the Netherlands [19, 20]. Furthermore, among all enterococcal bacteremias, the proportion of ARE increased from 4% in 1994 to 20% in 2005 and a study to determine the intestinal ARE reservoir revealed carriage rates of 35% in hematology and geriatric wards [20].

Different genotyping methods have been used to study enterococcal epidemiology, such as pulsed field gel electrophoresis, amplified fragment length polymorphism analysis and multilocus sequence typing. Interestingly, a clear dichotomy between isolates from hospitalized and non-hospitalized persons was detected [11]. Defined clonal groups of *E. faecium* show an enhanced capacity to disseminate in the nosocomial setting and are called epidemic or hospital-acquired [21, 22]. VRE outbreaks in single centers tend to be polyclonal suggesting a highly diverse population of hospital-acquired *E. faecium* strains and a highly mobile resistance determinant, capable of spreading widely among suitable recipient strains [15]. Hospital acquired *E. faecium* are mostly ampicillin-resistant, partly high-level ciprofloxacin-resistant and share an important part of their accessory genome content, which includes putative virulence traits such as a gene for an enterococcal surface protein, *esp*, genes encoding different cell wall-anchored surface proteins, a putative hyaluronidase gene, *hyl*<sub>6m</sub> and a gene encoding collagen-binding protein, *acm* [22-25]. Interestingly, mixed whole-genome microarray analysis based on comparative genome hybridization of 97 *E. faecium* strains isolated from different epidemiological niches worldwide revealed a distinct hospital clade [23] enriched in over hundred hospital-clade specific genes, including mobile elements like insertion sequence (IS) elements, phage genes and plasmid sequences, hypothetical and membrane proteins and antibiotic resistance and regulatory genes [23].
Pathogenesis of VRE infections: A) colonization and B) infection

A. Colonization

Infection with VRE typically follows VRE colonization, predominantly of the gastrointestinal tract [26]. Within hospitals, widespread colonization with VRE may occur with a relatively small number of documented infections [27]. This is of concern as colonized patients, which may remain colonized for long periods, serve as a silent reservoir for transmission of VRE to other patients [28]. In addition to the intestinal tract, colonization has been identified from the groin, axilla, oropharynx, and gastric and endotracheal aspirates [2]; VRE has been isolated from virtually everything in the health care environment, including monitoring devices, furniture, toilet seats, doors, floors, linen and other medical equipment and is capable of prolonged survival (at least 1 week) on fabric seat cushions [29]. Transmission takes place by direct contact, the most likely vectors being the hands of health care workers and contaminated equipment [30]. Once VRE colonization has become endemic, it is extremely difficult to effectively control the spread of infection. Colonization is contingent on exposure to VRE and on being a “susceptible” host and is predominantly found on intensive care, nephrology, oncology, transplantation and long-stay wards [2, 7, 21, 31-34]. Risk factors include advanced age, renal and hepatic failure, hematological malignancy, severe comorbid illness, invasive procedures and devices, gastrointestinal surgery, transplantation, enteral feeding, proximity to another VRE-positive patient, colonization pressure, prolonged hospital stay and antimicrobial therapy [2, 32, 35, 36].

VRE colonization has been associated with multiple classes of antibiotics, most importantly with second and third generation cephalosporins and antibiotics with prominent anti-anaerobic activity (metronidazole, clindamycin, and imipenem) [37-39]. The association of vancomycin with VRE colonization remains controversial as several studies showed no effect [40, 41]. In the situation in which a patient colonized with VRE has been identified, efforts to eradicate carriage are often unsuccessful. No antimicrobial regimen has been effective in providing a more than transient clearance of enterococcal colonization, despite investigation of a number of agents and combinations targeting the gastrointestinal tract [42].

B. Infection

Severe invasive enterococcal disease after VRE colonization occurs most often in hematology and organ transplant patients [26, 28, 43, 44]. Portals of entry for VRE typically include the urinary tract, intra-abdominal (e.g., gastrointestinal, biliary tree) or pelvic sources, wounds (surgical wounds, decubitus ulcers), and intravascular catheters.
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Especially neutropenic patients colonized with VRE are at risk for bacteremia as a result of gut translocation (mucositis), central venous catheter infections, or UTI [43, 45]. Patients with VRE have a high prevalence of skin colonization [8], which may result in colonization of intravascular catheters and subsequent intravascular catheter-related sepsis. Other risk factors for VRE bacteremia include hemodialysis, organ transplantation, receipt of corticosteroids, chemotherapy, or parenteral nutrition, surgery, severe illness, long-term antibiotic administration, indwelling urinary catheters, and mucositis [7]. UTI caused by VRE include cystitis, pyelonephritis, prostatitis, and perinephric abscesses; the majority of these infections are nosocomial and associated with urinary instrumentation [46]. In liver transplant recipients, the most common types of VRE infection include intra-abdominal infections associated with biliary leaks, stenosis, hepatic or perihepatic abscesses, stenosis or thrombosis of the hepatic artery, or perforated viscera [47]. Meningitis, endocarditis, pleural space and skin or soft tissue infections have also been reported [7, 48].

Mortality rates for patients with VRE bacteremia vary depending on the population at risk. Recipients of autologous peripheral blood stem cell transplants have been shown to have mortality rates of 10% [44]. Patients with endocarditis caused by VRE have been reported to have mortality rates higher than 30% [49], those with solid tumors have death rates higher than 50% [43], and some studies of critically ill and liver transplant patients have shown more than 70% mortality [26, 43, 50]. Furthermore, VRE bacteremia increases hospital length of stay by an average of 2 weeks and has an attributable mortality in comparison to vancomycin-sensitive E. faecium approaching 30% [50, 51].

Linezolid is the antibiotic recommended during serious infections caused by VRE [52]. It penetrates well to various tissues (including cerebrospinal fluid) and is available in oral form. Yet, it is expensive and can cause bone marrow suppression after prolonged use. Quinupristin/dalfopristin, also has anti-VRE activity, but is only active against E. faecium, and not E. faecalis, and has frequent side-effects, especially myalgia, arthralgia and inflammation at the infusion site [53, 54]. Other antibiotics with anti-VRE activity are daptomycin and tigecycline [55]. However, resistance to all newer antibiotic has already been reported [56-58].

Virulence factors

The pathogenicity of enterococci is probably not related to virulence factors in the classical sense but rather to a combination of ‘fitness factors’, such as increased resistance to antimicrobials and environmental stresses, as well as specific and unspecific adhesive properties [21]. The changing epidemiology and genotypic distinction between E.
from hospitalized and non-hospitalized patients, suggest that *E. faecium* might have acquired different abilities, facilitating adaptation of a specific subpopulation to the hospital environment. The cumulative acquisition of resistance determinants and putative virulence genes may have contributed to adaptation and selective advantage of specific *E. faecium* clones in the hospital environment [11]. So far, little is known about the nature of adaptive mechanisms supporting hospital survival and spread. Recently, some potential virulence genes have been described for *E. faecium*: *esp*, *hyl*, *acm*, *scm*, *sagA*, genes encoding additional surface-exposed LPXTG-like cell-wall-anchored proteins and genes required for the biogenesis of pili. They were all found more frequently in clinical isolates than in fecal isolates or non-human isolates [25, 59-67]. Although the exact role in the pathogenesis of *E. faecium* infections is not well understood, most are found (or thought) to enhance adherence to extracellular structures and biofilm formation, which might be the first step in colonization of the host.

The enterococcal surface protein Esp, located on a putative pathogenicity island [25, 65], is specifically enriched in hospital-acquired *E. faecium*. Esp is involved in biofilm formation [68] and its expression is affected by changes in environmental conditions, being highest in conditions that mimic the microenvironment of the human large intestines: 37°C and anaerobiosis [69]. Enterococcal Esp has also been shown to be involved in increased conjugation frequencies [70]. This suggests that either Esp plays a direct role in cell-cell interaction or that Esp may serve as a marker for strains with enhanced potential to acquire new genetic elements. The expression of collagen-binding adhesion Acm was found to be involved in adherence of collagen type I and IV. More importantly, Acm was found to contribute to the pathogenesis of *E. faecium* endocarditis and antibodies to Acm were present in sera of patients suffering *E. faecium* endocarditis [71, 72]. Recently, *E. faecium* surface expression of a second collagen adhesin, Scm, was described; with adherence specificity for collagen type V [67]. Secreted antigen A (SagA) appears essential for *E. faecium* growth and exhibits broad-spectrum binding to extracellular matrix proteins [66]. A similar protein expressed by *E. faecalis*, SalB, was found to be important for resistance to various stress conditions, including bile salts, ethanol, heat shock and alkaline and acid pH [73, 74].

Using *Caenorhabditis elegans*, it was shown that *E. faecium* produces hydrogen peroxide at levels that cause cellular damage [75]. Additional studies are necessary to investigate the relevance of hydrogen peroxide production by *E. faecium* in the human host. Comparative genomic hybridizations of 97 *E. faecium* nosocomial, commensal and animal isolates identified more than 100 genes that were enriched in nosocomial strains, including genes encoding putative adhesions, antibiotic resistance, IS elements, phage sequences, and novel metabolic pathways [23].
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Host response against enterococcal infections

Hardly anything is known about host defense mechanisms against enterococcal infections, and only a few studies have attempted to investigate this area systematically. In order to survive in the host, enterococci must successfully avoid specific and non-specific host defense mechanisms.

Certain strains of enterococci can survive within phagocytes, which might serve as vehicles for enterococci to translocate across the intestinal wall and disseminate into distant organs [76]. The failure of phagocytic cells to kill intracellular enterococci might lead to systemic spread [77]. Whether phagocytosis of enterococci represents a successful host defense mechanism or a means of immune response evasion for enterococci remains to be demonstrated.

Arduino et al. [78, 79] studied the resistance of *E. faecium* to neutrophil-mediated phagocytosis in vitro. While all *E. faecalis* strains were internalized, only 50% of the *E. faecium* strains were phagocytosed. Probably, a carbohydrate structure was responsible for the resistance to phagocytic killing. By electron microscopy, small electron-dense clumps, immediately adjacent to the cell wall of *E. faecium*, were identified, which could be consistent with capsular material. Whether this material is related to the resistance of phagocytosis is unclear as several strains of *E. faecalis* displayed this as well, while they were sensitive to phagocytosis [78].

Host defense against invading microorganisms

When a microorganism crosses the protective barriers of epithelia and mucous membranes it encounters innate defense mechanisms. These mechanisms are called innate because they do not require previous contact with the microorganism, mediate the first line of defense against invading pathogens and contain the infection prior to the induction of the adaptive immune response. It is regulated by a coordinated action of leukocytes, most importantly neutrophils and macrophages, which are able to phagocytose pathogens and to secrete a vast array of cytokines and chemokines, thereby coordinating additional host response mechanisms. The first task of innate immune cells is to recognize and discriminate potential pathogens from self. Microorganisms share several highly conserved molecular structures called pathogen-associated molecular patterns (PAMPs) that are recognized by phagocytes via certain pattern recognition receptors (PRRs). A major subset of these PRRs belong to the class of Toll-like receptors (TLRs), that have relatively recently been discovered. TLRs recognize distinct microbial patterns and subsequently initiate the production and secretion of cytokines and chemokines that are important for an effective host defense [80]. At present, 10
human TLRs are known, all recognizing different PAMPs. Examples of PAMPs include lipopolysaccharide (recognized by TLR4) from the outer membrane of gram-negative bacteria, peptidoglycan (TLR2), present in most bacteria, lipoteichoic acid (TLR2-TLR6), in many gram-positive bacteria, and zymosan (TLR2-TLR6), in the yeast cell wall. After stimulation of TLRs, the adapter molecule Myeloid Differentiation protein 88 (MyD88) is recruited, which finally results in the nuclear translocation of nuclear-factor-κB (NFκB), causing the transcription of a whole range of inflammatory genes [81].

Like the family of TLRs, the complement system comprises one of the major groups of pattern recognition molecules that are activated by several pathways following direct or indirect contact with invading microorganisms. The most abundant and most central component is C3; cleavage of this molecule is essential for all complement-mediated phenomena, and directly results in release of the soluble anaphylatoxin C3a and deposition of C3b and iC3b on the surface of the bacterium. Bacteria opsonized by C3b and iC3b will be recognized and phagocytosed by leukocytes bearing specific complement receptors [82].

**Immunoparalysis and the acute phase response**

Animal studies have shown that in spite of initial activation of immune responses, immunoparalysis (sooner or later) occurs in peritonitis and decreases systemic defense mechanisms [83]. Immunoparalysis is probably a later effect in the immune response, when pro-inflammatory cytokines, like TNF-α and IFN-γ, are down regulated but anti-inflammatory cytokine levels, like IL-10, persist [84, 85]. This has been shown for sepsis patients [86].

The acute phase response (APR) is a non-specific systemic inflammatory host response, that is induced after infection or sterile tissue injury, like trauma, major surgery, burn injury, tissue infarction, or during advanced cancer. The cytokines IL-1β and IL-6 play an essential role in the initiation of the APR. In particular IL-6 triggers the liver to produce so-called acute phase proteins, like C-reactive protein (CRP), serum amyloid A (SAA) and complement proteins [87]. Although considered initially beneficial for the host in removing pathogens and repairing injured tissue, the exact function of the strong rise of the plasma concentrations of acute phase proteins is still unknown. Evidence suggests that an ongoing APR causes an immune compromised state [88]. Subcutaneous injection of turpentine or casein in mice causes sterile inflammation and represent well-established experimental models to study the systemic acute phase response in response to sterile tissue injury [88, 89].
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Outline of this thesis

Relatively little is known about the pathogenesis of infections with *E. faecium*. The general aim of this thesis was to gain more insight into the normal immune response during *E. faecium* infection and into the immune response of the immunocompromised host. Furthermore, intestinal colonization with or without subsequent infection was investigated.

In part I, chapter 2, we describe a mouse model of *E. faecium* peritonitis, using a clinical isolate of *E. faecium*, harboring the putative pathogenicity island expressing Esp and with ampicillin and vancomycin resistance, to investigate the normal host response. This model was used throughout this thesis to investigate several components of the host defense system. In the same chapter, we used this peritonitis model and *in vitro* studies to investigate the role of the family of TLRs and CD14 in the innate recognition of *E. faecium* using mice deficient in TLR2, TLR4, CD14 and MyD88. In the subsequent chapters we investigated the role of neutrophils (chapter 3), macrophages (chapter 4) and the complement system (chapter 5) in host defense against *E. faecium* peritonitis. Part II describes the effect of an experimental *E. faecium* infection in an immunocompromised host. As a rule, *E. faecium* only causes serious disease in patients with severe underlying illnesses. In order to mimic several aspects of such comorbid conditions, mice were first rendered immune suppressed by previous turpentine or casein injection (inducing a sterile acute phase response) in chapter 6. In chapter 7 mice were first subjected to cecal ligation and puncture (CLP), inducing a polymicrobial (e.g. fecal) peritonitis. In both models, the course of the peritonitis and inflammatory response during subsequent *E. faecium* peritonitis were investigated. The focus of Part III was on *E. faecium* intestinal colonization and the effect of colonization on the susceptibility for infection. Hospitalized patients frequently become colonized with multidrug-resistant *E. faecium*, with high intestinal loads. In chapter 8, we investigated the course of *E. faecium* containing polymicrobial peritonitis, by performing CLP on *E. faecium* colonized mice. In chapter 9, we investigated the influence of intestinal *E. faecium* colonization on pulmonary defense against *Pseudomonas aeruginosa* pneumonia. In part IV, the role of the potential virulence factor Esp was investigated. In chapter 10 the role of Esp was investigated in intestinal colonization and in chapter 11, the influence of Esp expression on UTI and peritonitis was studied.
References


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Chapter 1

General introduction and outline of the thesis

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