Enterococcus faecium infections: where bacterial virulence meets innate immunity
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SUMMARY AND GENERAL DISCUSSION
Systemic infections with multiresistant and vancomycin resistant *E. faecium* (VRE) are a major problem in hospitals worldwide [1, 2]. Little is known about either bacterial virulence factors or host defense mechanisms operative in enterococcal infections. The limited choice of antimicrobials still available for treatment of serious *E. faecium* infections underscores the importance of understanding the critical elements of immune control of infections caused by this pathogen. The general aim of this thesis was to gain more insight into the pathogenesis of *E. faecium* infection, in particular regarding the normal immune response to this pathogen, as well as the immune response of the immunocompromised host. We investigated the significance of several mechanisms at play during the normal host response during *E. faecium* infection: Toll-like receptors (TLRs), neutrophils, macrophages and the complement system. We then investigated the immune response toward *E. faecium* infection in the immunocompromised host and during polymicrobial infection. Furthermore, we examined intestinal colonization with or without subsequent infection and the role of the potential virulence factor Esp on colonization and infection.

In part I, chapter 2, a new *E. faecium* peritonitis mouse model is described. This model was used for most of the studies described in this thesis, to investigate several aspects of the normal or hampered immune response towards *E. faecium* infection. In these experiments a vancomycin resistant strain of *E. faecium* (VRE), E155, was used. This clinical isolate belongs to a genetic subpopulation of hospital-associated *E. faecium* that is responsible for the worldwide emergence of nosocomial multiresistant *E. faecium*, characterized by high-level quinolone and ampicillin resistance, a pathogenicity island, containing the variant esp gene, and the presence of five cell surface protein genes [3].

TLRs have been implicated in the recognition of pathogens and the initiation of an adequate innate immune response [4]. In chapter 2, we sought to determine the roles of MyD88, the common adaptor protein involved in TLR signaling [5], TLR2, TLR4 and CD14 in host defense against *E. faecium* peritonitis. MyD88 knockout (KO) mice demonstrated an impaired early response to *E. faecium* peritonitis, as reflected by higher bacterial loads in peritoneal fluid and liver accompanied by a markedly attenuated neutrophil influx into the abdominal cavity. *In vitro*, not only MyD88 KO macrophages but also TLR2 KO and CD14 KO macrophages displayed a reduced responsiveness to *E. faecium*. In line, transfection of TLR2 rendered HEK 293 cells responsive to *E. faecium*, which was enhanced
by cotransfection of CD14. TLR2 KO mice showed higher bacterial loads in peritoneal fluid after in vivo infection with \textit{E. faecium} and a diminished influx of neutrophils, whereas CD14 KO mice had an unaltered host response. \textit{E. faecium} phagocytosis and killing were not affected by MyD88, TLR2 or CD14 deficiency. TLR4 did not play a role in the immune response to \textit{E. faecium} in vitro or in vivo. These data suggest that MyD88 contributes to the effective clearance of \textit{E. faecium} during peritonitis at least in part via TLR2 and by facilitating neutrophil recruitment to the site of the infection.

**Chapter 3**, describes the essential role of neutrophils in the rapid clearance of VRE peritonitis. Infections with multiresistant \textit{E. faecium} are especially troublesome in cancer and neutropenic patients [6, 7]. In this study, neutrophils were depleted by intraperitoneal injections of MAb RB6-8C5. Neutropenic mice demonstrated a severe delay in enterococcal clearance from all cultured organs (peritoneal fluid, blood, lung and liver). In particular, neutropenic mice remained bacteremic up to 3 days, whereas all non-neutropenic mice had cleared the bacteria from the circulation by 2 days. Furthermore, neutropenic mice displayed elevated peritoneal cytokine and chemokine levels 1 day after the infection and attracted less macrophages into the peritoneal cavity. In the circulation, prolonged elevations of TNF-\(\alpha\), IL-6 and the acute phase proteins SAA and C3 were measured in neutropenic mice. Thus, attraction of neutrophils to the primary site of \textit{E. faecium} infection is important for a rapid clearance of this bacterium, thereby attenuating a systemic inflammatory response.

In **chapter 4**, the importance of another major player in innate immunity was investigated, peritoneal macrophages (PM) [8, 9]. In an ex vivo setting, PM harvested from mice were responsive to, and able to phagocytose and kill \textit{E. faecium}. In vivo, PM were depleted by intraperitoneal injection of clodronate-encapsulated liposomes, prior to inducing \textit{E. faecium} peritonitis. Depletion of resident PM caused a clear delay in peritoneal clearance of \textit{E. faecium} with increased systemic dissemination. Mice depleted of PM were able to recruit macrophages and neutrophils to the peritoneal cavity after infection, comparable to control mice. Furthermore, increased levels of peritoneal cytokines and chemokines were found in PM depleted mice. This study indicates that PM are important in the early containment of \textit{E. faecium} peritonitis and for the regulation of the inflammatory response.

To complete our insight in the role of different players of the innate immune system during infection with \textit{E. faecium}, the role of the complement system was investigated in **chapter 5**. Previous in vitro studies (performed by others) demonstrated an important role for complement proteins in neutrophil-mediated phagocytosis of \textit{E. faecium} [10, 11]. We investigated the importance of complement in vitro and in vivo. Mice were
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rendered complement deficient by intraperitoneal Cobra Venom Factor (CVF) injections, whereas in supplementary experiments, C3 KO mice were used. In vitro, opsonisation by complement clearly enhanced phagocytosis by neutrophils and macrophages. In vivo, CVF treated and C3 KO mice were severely hampered in clearing *E. faecium* from all cultured organs (peritoneal fluid, blood, lung and liver). Higher peritoneal cytokines and chemokines were measured in decomplemented mice, whereas no differences in systemic or peritoneal cell kinetics were detected. Thus, complement is important in containing *E. faecium* peritonitis and reducing subsequent systemic infection.

In part II, the immune response towards *E. faecium* infection in the immunocompromised host is described. In general, *E. faecium* only causes serious diseases in patients with severe underlying illnesses. These underlying illnesses are often associated with an acute phase response (APR) that renders patients vulnerable to nosocomial infections [12, 13]. In chapter 6, the influence of the APR on host defence against *E. faecium* is described. By injecting either turpentine or casein subcutaneously, sterile tissue injury is induced that causes an APR, as reflected by increases in the plasma concentrations of IL-6, SAP and C3. A pre-existent APR in mice was associated with a strongly reduced capacity to clear *E. faecium*, resulting in prolonged bacteremia for several days. The inflammatory response to *E. faecium* was impaired in mice with an APR, as shown by reduced capacity to mount a neutrophilic leucocytosis in peripheral blood and by decreased local cytokine concentrations. These data indicate that the APR impairs host defence against *E. faecium*, suggesting that this condition may contribute to the increased vulnerability of critically ill patients to enterococcal infections.

In chapter 7, in stead of a sterile model, an infectious model was used to render mice immunocompromised; the model of cecal ligation and puncture (CLP)-induced sepsis. Mice were first subjected to CLP or sham surgery and 2 days later they were superinfected with VRE by peritoneal injection. Mice infected with VRE after CLP were severely impaired in eliminating VRE from the peritoneal cavity and distant body sites compared to sham surgery mice. These mice failed to mount an early inflammatory response at the primary site of VRE infection. VRE superinfection did not influence CLP induced organ damage or polymicrobial bacterial loads. We concluded that sublethal polymicrobial sepsis, induced by CLP, greatly facilitates infection and dissemination of VRE, but that VRE does not influence the course of CLP induced sepsis.
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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 [14, 15]. Enterococci are frequently isolated from polymicrobial infections originating from the intestines [16]. The impact of VRE on these infections and vice versa is not clear and this was investigated in chapter 8. Mice were first intestinally colonized with VRE during oral vancomycin treatment and after 14 days of colonization, CLP was performed to induce polymicrobial peritonitis in the presence or absence of VRE colonization. CLP resulted in systemic VRE infection in all VRE colonized mice, with high VRE loads in peritoneal fluid, blood, liver and lungs. Forty-eight hours after CLP, VRE infected mice had significantly lower bacterial loads in all organs tested when compared to mice not infected with VRE. Additionally, lower inflammatory parameters were measured in VRE infected mice. CLP induced transient liver and kidney damage, with a faster recovery in VRE colonized mice. From this study, we concluded that VRE infection, originating from a natural source (the intestinal tract), does not worsen the outcome of CLP-induced polymicrobial peritonitis and sepsis, but rather facilitates bacterial clearance and attenuates host inflammatory responses.

The increased prevalence of colonization with multidrug-resistant *E. faecium* is not only associated with a higher incidence of infections caused by enterococci, but also by infections with other nosocomial pathogens [17]. In chapter 9, we investigated the causality of this observed relationship, by determining the influence of intestinal colonization with *E. faecium* on pulmonary defense against *Pseudomonas aeruginosa*. Three groups of mice were tested; 2 groups of mice were pre-treated with vancomycin, of which one group was subsequently colonized by VRE and the third group did not receive any pre-treatment. *P. aeruginosa* pneumonia was induced in all mice. Vancomycin treatment resulted in intestinal gram-negative bacterial overgrowth and VRE treatment resulted in colonization throughout the intestines. All 3 groups of mice were able to clear *P. aeruginosa* from the lungs and circulation, with comparable lung cytokine responses and lung damage. Mice treated with vancomycin without VRE colonization displayed modestly increased plasma levels of TNF-α and IL-10. We concluded that intestinal overgrowth with *E. faecium* and/or gram-negative bacteria does not impact importantly on pulmonary defense against *P. aeruginosa* pneumonia.
In part IV, the role of enterococcal surface protein Esp, which has been implicated as a potential virulence factor involved in biofilm formation, was investigated [18]. Esp is specifically linked to nosocomial clonal lineages that are genetically distinct from indigenous *E. faecium* strains [19]. In chapter 10, we investigated whether Esp facilitates bacterial adhesion and intestinal colonization of *E. faecium*. No differences in adherence to human colorectal adenocarcinoma cells (Caco-2 cells) were found between an Esp expressing strain of *E. faecium* (E1162) and its isogenic Esp-deficient mutant (E1162Δesp). Mice, kept under ceftriaxone treatment, were inoculated orally with either E1162 or E1162Δesp and with both strains simultaneously. Both E1162 and E1162Δesp were able to colonize the murine intestines with high and comparable numbers. No differences were found in the contents of cecum and colon. Both E1162 and E1162Δesp were able to translocate to the mesenteric lymph nodes. These results suggest that Esp is not essential for cell adhesion and intestinal colonization or translocation of *E. faecium* in mice.

In the subsequent study the influence of Esp expression on urinary tract infection (UTI) and peritonitis was studied, as described in chapter 11. Esp expression by *E. faecium* enhanced *in vitro* binding to bladder and kidney epithelial cells. In mice, higher numbers of E1162 were cultured from kidneys and bladders one and three days after induction of UTI compared to E1162Δesp. This was accompanied by a higher frequency of bacteremia, higher levels of proinflammatory cytokines in kidney tissue, and renal insufficiency. No differences in urine cultures were found. Esp expression had no impact on the course of, or inflammatory response during a model of *E. faecium* peritonitis. Thus, differences in levels of attenuation of the Esp mutant *E. faecium* in a model of UTI and peritonitis suggests a niche specific role of Esp in the pathogenesis of *E. faecium* infections.

**General discussion**

Multidrug resistant strains of *E. faecium* are increasingly found colonizing and infecting hospitalized patients [1, 14, 15]. These strains belong to a genetically distinct population that has acquired resistance determinants and putative virulence genes that may have contributed to adaptation to the hospital environment [20]. Because of the growing resistance to almost all antibiotics used in the hospital setting, infections with this multidrug resistant pathogen are difficult to treat. To improve therapeutic options, further knowledge of the pathogenesis of these infections and the interaction with host defense systems is needed.
In this thesis we describe a mouse model of *E. faecium* peritonitis with subsequent systemic infection. Using this non-lethal model, we surveyed the normal host immune response towards *E. faecium* infection and subsequently, we systematically investigated several distinct components and conditions of the immune system. The healthy immune system reacts to *E. faecium* infection with a rapid neutrophil influx into the primary site of the infection, the peritoneal cavity, causing a consecutive rapid decline in peritoneal and systemic enterococcal load. Healthy mice clear an infection with a large inoculum of $10^8$ CFU *E. faecium* with only a moderate cytokine response and without showing important signs of illness. This resembles the scenario in healthy humans who are unlikely to develop severe infections and disease by *E. faecium*. Clearly, the healthy innate immune system is able to control *E. faecium* infections and prevent development of severe disease. The absence of mortality and distant organ injury is in line with the fact that the intact immune system can mount an effective response against this opportunistic pathogen.

After gaining an overview of the normal immune response, we used this model of systemic *E. faecium* infection to investigate the involvement of key players of the immune system during this infection, separately. TLRs are pattern recognition receptors (PRR) and are widely expressed in most tissues. Activation of TLR signaling is considered important for mounting an effective innate immune response. We found that recognition by TLR2 and subsequent signaling through MyD88, the common TLR adaptor protein, contributes to an effective clearance of *E. faecium* during the early phase of infection, most likely by stimulating the recruitment of neutrophils to the primary site of infection. Interestingly, MyD88 and TLR2 KO neutrophils and macrophages failed to respond to *E. faecium* in vitro. In vivo, deficiency of these molecules did not result in an overwhelming infection and the differences in bacterial loads compared to those of WT mice were relatively modest. In line, in vitro, CD14 (another PRR) KO cells were markedly less responsive to *E. faecium* than WT cells. Nonetheless, in vivo, CD14 did not play a detectable role in *E. faecium* infection. The apparent discrepancy between the in vitro and in vivo results suggests that other components of the immune system, not present in the in vitro systems may compensate for the absence of these molecules. This underscores the additional value of the parallel use of both in vitro as in vivo (animal) studies, in investigating the (patho-) physiologic response to specific infections.

In the subsequent studies we depleted neutrophils, macrophages or the complement system. In the early phase of peritonitis residential macrophages and the lymphatic system are important for containing the infection and neutrophil influx serves to eliminate those bacteria that have eluded the first line of defense [21]. Complement plays a role in opsonizing the bacteria, thereby facilitating the phagocytosis of bacteria...
by leukocytes. Neutrophils, as well as macrophages and the complement system were all found to be important in containing the infection. Depletion of these cells and complement proteins caused prolonged high peritoneal loads of *E. faecium*, with subsequent more severe systemic infection, lasting several days and with sustained elevated levels of inflammation markers. Interestingly, all mice were able to clear the bacteria, and the models did not convert to a lethal infection, although in previously healthy mice i.p. infection with 10 times more bacteria of the same *E. faecium* strain resulted in 30% lethality. Apparently, in the otherwise healthy host, deficiency of one part of the immune system is counterbalanced by other components of the immune system.

In particular severely ill, immunocompromised patients are vulnerable to infections with enterococci, whereas in healthy humans *E. faecium* only rarely causes infections. To resemble this scenario we induced an immunocompromised state before inducing *E. faecium* peritonitis. In one study, we induced a sterile APR (by turpentine or casein induced tissue damage), in another study we induced a (polymicrobial) sepsis, and caused a superinfection when the immune response was in an immunoparalytic state. The APR is an aspecific systemic response to tissue harm, also caused by surgery, major burns or malignant processes. This response was previously thought to be primarily beneficial. Recently, our laboratory demonstrated that the host with a pre-existent APR is more vulnerable to infections with nosocomial respiratory pathogens like *Acinetobacter baumanii* and *Pseudomonas aeruginosa* [22, 23]. In our study we demonstrated that the APR also renders the host more vulnerable to peritonitis, in our case caused by *E. faecium*.

The clinical importance of a pre-existing APR and the vulnerability for nosocomial infections is supported by a study in which patients who had an APR before surgery were at increased risk of developing infectious complications postoperatively [24]. Indeed, in one study 74 of 158 (48%) patients with enterococcal bacteremia had undergone recent major surgery or had sustained full-thickness burns or multiple traumatic injuries. In the second model we used the model of CLP-induced sepsis. Sepsis has been associated with a vigorous proinflammatory response and concurrent or sequential anti-inflammatory cascades, which are important to avoid detrimental inflammatory responses and achieve immunological homeostasis [13, 25]. Using the CLP model an endogenous polymicrobial infection with the host’s own feces is induced, resembling the clinical situation of intestinal leakage after surgery or intestinal perforation. CLP is considered a clinically relevant model for sepsis, since it mimics a common clinical scenario and is associated with an early hyper-inflammatory reaction followed by
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a subsequent hypo-inflammatory phase [26-28]. The anti-inflammatory response has been implicated in the inability of critically ill patients to eradicate the primary infection responsible for sepsis and their propensity to acquire secondary nosocomial infections. In this state, immune cells are less responsive to chemokines and cytokines and have a reduced capacity to release proinflammatory cytokines upon stimulation with bacterial agonists, which we also found when we induced a superinfection with \textit{E. faecium}. The mechanisms behind these immunoparalytic states either induced by sterile tissue damage or by sepsis are topics of intensive research. Down-regulation of the TLR signaling pathways appears to be important in this process [13, 25, 27, 29].

It is interesting that neither deficiency of solitary components of the immune system, nor manipulation of the immune system as a whole resulted in a complete inability to clear the infection. This, on the one hand, shows the comprehensiveness of the immune system, in that deficiency of one element is compensated for by other factors of the system. On the other hand this demonstrates the relative moderate virulent nature of \textit{E. faecium}, as this pathogen is not capable to take advantage of the debilitated and delayed immune response, and does not proliferate into an overwhelming infection. This parallels the clinical situation of \textit{E. faecium}, being an opportunistic pathogen, only causing severe infection and mortality in severely debilitated patients.

Intestinal colonization with nosocomial strains of \textit{E. faecium} appears to be an essential first step in the development of infections [14]. Subsequently, patients become infected either by direct translocation across the intestinal linings, e.g. in cancer patients treated with chemotherapeutics (causing mucositis), or by colonization of intravascular (or – urinary bladder) devices. Enterococci isolated from secondary peritonitis most commonly are part of a mixed flora, however, the pathogenicity of these bacteria during these infections is still not clear [30-33]. We developed a model in which we closely resembled the clinical situation in which patients are intestinally colonized by a nosocomial strain of \textit{E. faecium} and subsequently suffer a perforated intestine or leakage after surgery, by performing CLP. In this situation, the source of the infection remains \textit{in situ} causing a continuous infection. Thereby, we were able to cause a systemic infection with \textit{E. faecium} from an endogenous source and to investigate the course of an \textit{E. faecium} containing polymicrobial infection. Interestingly, we found a beneficial effect of \textit{E. faecium} on both the inflammatory response and on the clearance of the polymicrobial infection. It would improve our understanding of 1) enterococcal disease and 2) the (general) immune response, if the mechanisms behind these findings would be unraveled. The question is where does the beneficial effect come from? Is it the presence of \textit{E. faecium}
in the intestines, as a probiotic and potentially altering the environmental flora and/or the systemic immune response, or is it the presence of *E. faecium* in the polymicrobial infection? Previous studies have indicated that intestinal *E. faecium* colonization impacts on the inflammatory response [34, 35]. Furthermore, probiotics in the intestines can lead to inhibition of the growth of conventional organisms or potential pathogens through a variety of mechanisms. These include their capacity to decrease luminal pH, secrete bacteriocins, and inhibit bacterial adhesion to epithelial cells. In addition, there is evidence that probiotics interfere with the production of defensins in the intestinal crypts [36]. Certain *E. faecium* strains are known bacteriocin producers that can inhibit growth of, or have antibacterial activity against, other microorganisms [37, 38]. Potentially, colonization with hospital-acquired *E. faecium* alters intestinal microbial networks thereby reducing the number of pathogenic bacteria and/or creating a favourable environment for less pathogenic bacteria, with beneficial immunologic properties. Considering this, it would be of interest to know if colonization by *E. faecium* would alter the systemic immune response and has an influence on acute infection. Combining this with the fact that the increased prevalence of colonization with multidrug resistant *E. faecium* is associated with infections with other nosocomial pathogens [17], we wondered if a causative relationship exists. We therefore investigated the influence of intestinal colonization with *E. faecium* on pulmonary defense against *Pseudomonas aeruginosa*. Yet, we had to conclude that colonization with *E. faecium* does not impact importantly on pulmonary defense against *P. aeruginosa* pneumonia.

The use of antibiotics with little or no anti-enterococcal activity is a predisposing factor to nosocomial colonization with enterococci. It is intriguing that the genetically distinct hospital population of *E. faecium* is able to outcompete the endogenous enterococcal flora in colonization. This suggests that these nosocomial enterococci pose traits that give them advantages in this environment. Currently, it is not known which factors facilitate intestinal colonization of nosocomial *E. faecium* strains; knowledge regarding the exact nature of these factors would improve our understanding of the pathogenesis of enterococcal disease. Potential virulence factor Esp is found to play a role in initial adherence to biotic and abiotic surfaces and biofilm formation. Considering that Esp expression is optimal under conditions resembling the intestinal environment, e.g., anaerobioses and 37°C, it was rather unexpected that we found no beneficial effect of Esp expression on adherence to colon cells in vitro and in intestinal colonization in vivo, although the hospital acquired strain exhibited higher adherence in the in vitro assay than a surveillance strain.
Previously, a significant relationship was found between *E. faecium* urinary isolates and the presence of *esp*, suggesting a role of Esp in UTI [39, 40]. In our study on the virulence potential of Esp, we indeed found that Esp enhances adherence to urinary tract epithelia and aggravates an urinary tract infection with subsequent invasion of the bloodstream. In the same study, Esp was not found to influence the course of a peritonitis. These data imply niche specificity for different virulence factors and indicate the importance of selecting an appropriate animal model in order to assess the impact of virulence factors.

As molecular studies are ongoing, more and more factors potentially participating in enterococcal pathogenesis are discovered. Recently, some potential virulence genes, additionally to *esp*, have been described for *E. faecium*: *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 [19, 41-49]. 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.

Our studies examining the pathogenesis of *E. faecium* infection were performed with two different strains. More studies should be performed with strains with other (and/or additional) virulence factors. In fact, some *E. faecium* strains are known to express polysaccharides, resembling capsular material. This might have implications in the efficiency with which leukocytes are able to phagocytose the bacteria and the efficacy of complement in opsonisation. Furthermore, it is described for the other clinically relevant enterococcal species, *E. faecalis*, to worsen the outcome of polymicrobial infection and to facilitate the formation of abscesses. Testing other strains of *E. faecium*, and their isogenic mutants, in the model of CLP after intestinal colonization would probably improve our insight in *E. faecium* pathogenesis importantly, and might explain the controversies found in literature on the outcomes of *E. faecium* containing polymicrobial peritonitis [30-33]. Using this model the influence of specific factors on colonization can be investigated and on the subsequent development and course of infection. Additionally, it would be of greatest interest to perform follow up studies, adjusting the model to other clinical scenarios, and by combining several of the topics investigated in this thesis, e.g., depleting neutrophils or inducing an APR during colonization, furthermore, mice could be treated with chemotherapeutics and aged (or neonatal) mice could be used.
In this thesis we used mouse models to investigate the pathogenesis of *E. faecium* infections. Although mice are the mainstay of *in vivo* immunological experimentation, one has to be cautious with extrapolating results obtained from animal studies to the human situation. In many respects mice mirror human biology remarkably well, yet significant differences in immune system development, activation, and response to challenge exist between mice and humans. Furthermore, we used KO mice to study the function of specific proteins; one should keep in mind that these genetically modified mice can have developed compensatory immunological mechanisms [50]. Most researchers that use animal models to investigate infections and the immune response to specific pathogens, use a single bolus inoculum to cause infection; so did we. In the human situation an infection most often develops gradually in time, yet these conditions are difficult to copy. Our model of *E. faecium* colonization and subsequent CLP, resembles the clinical situation rather well, in that there is an endogenous source of infection causing infection over time as the source remains *in situ*.

To conclude, in this thesis, several important issues regarding *E. faecium* pathogenesis were investigated. Combining molecular knowledge of hospital *E. faecium* traits and the mouse models described we can further improve our knowledge on the pathogenesis of *E. faecium* infections, e.g. by examining different potential virulence factors in the models of intestinal colonization, peritonitis with subsequent bacteremia, and urinary tract infection.

**References**

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