


Model systems for the study of Enterococcal colonization and infection

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ABSTRACT

Enterococcus faecalis and *Enterococcus faecium* are common inhabitants of the human gastrointestinal tract, as well as frequent opportunistic pathogens. Enterococci cause a range of infections including, most frequently, infections of the urinary tract, catheterized urinary tract, bloodstream, wounds and surgical sites, and heart valves in endocarditis. Enterococcal infections are often biofilm-associated, polymicrobial in nature, and resistant to antibiotics of last resort. Understanding Enterococcal mechanisms of colonization and pathogenesis are important for identifying new ways to manage and intervene with these infections. We review vertebrate and invertebrate model systems applied to study the most common *E. faecalis* and *E. faecium* infections, with emphasis on recent findings examining Enterococcal-host interactions using these models. We discuss strengths and shortcomings of each model, propose future animal models not yet applied to study mono- and polymicrobial infections involving *E. faecalis* and *E. faecium*, and comment on the significance of anti-virulence strategies derived from a fundamental understanding of host-pathogen interactions in model systems.

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Epidemiology of enterococcal infections

Enterococcus species are ubiquitous organisms present in dairy and fermented food products, natural environments (i.e. plants, soil and water bodies), and the gastrointestinal (GI) tract of humans, other mammals, reptiles and insects.¹ This broad distribution is likely due to its ability to survive and persist in a broad range of environments, such as pH, temperature, hyper- and hypotonic conditions.¹ In susceptible hosts, Enterococci can cause opportunistic infections. Enterococci are the second most common nosocomial pathogen causing up to 14% of all hospital-acquired infections (HAIs) in the US between 2011–2014 (Fig. 1).² Between 2006–2007 in the US, Enterococci caused 40% of device-associated infections in the medical intensive care unit (ICU), including central-line associated bloodstream infection (CLABSI), catheter-associated urinary tract infection (CAUTI), surgical site infection (SSI), and ventilator-associated pneumonia (VAP) (Fig. 2). These infections often lead to other clinical manifestations such as infective endocarditis (IE), urinary tract infection (UTI), bacteremia, peritonitis, prosthetic joint infection (PJI),

and endophthalmitis; all of which can be serious and life-threatening if left untreated.^{3,4} Furthermore, infection-associated Enterococci are often antibiotic resistant, making it more complicated to treat.

Among *Enterococcus* species, *E. faecalis* and *E. faecium* are the 2 most commonly identified species in the human GI tract, and *E. faecalis* is responsible for 80–90% of Enterococcal-associated nosocomial infections, followed by *E. faecium* (10–15%).⁵ This over-representation of *E. faecalis* among clinical isolates may be related to its natural abundance in the GI tract, where *E. faecalis* is approximately 100 times more prevalent than *E. faecium*. However, during the last 20 years, a major epidemiological shift has been noted in the incidence of *E. faecium* in both US and European hospitals where *E. faecium* has become increasingly prevalent. The reason for the ecological replacement of *E. faecalis* with *E. faecium* is unknown but it has been speculated to be due to the extensive use of antibiotics in hospitals. Currently 90% and 80% of *E. faecium* from HAIs are resistant to ampicillin and to vancomycin, respectively, while *E. faecalis* is still largely susceptible to both of these antibiotics.⁶ The reasons for the difference in antibiotic susceptibility

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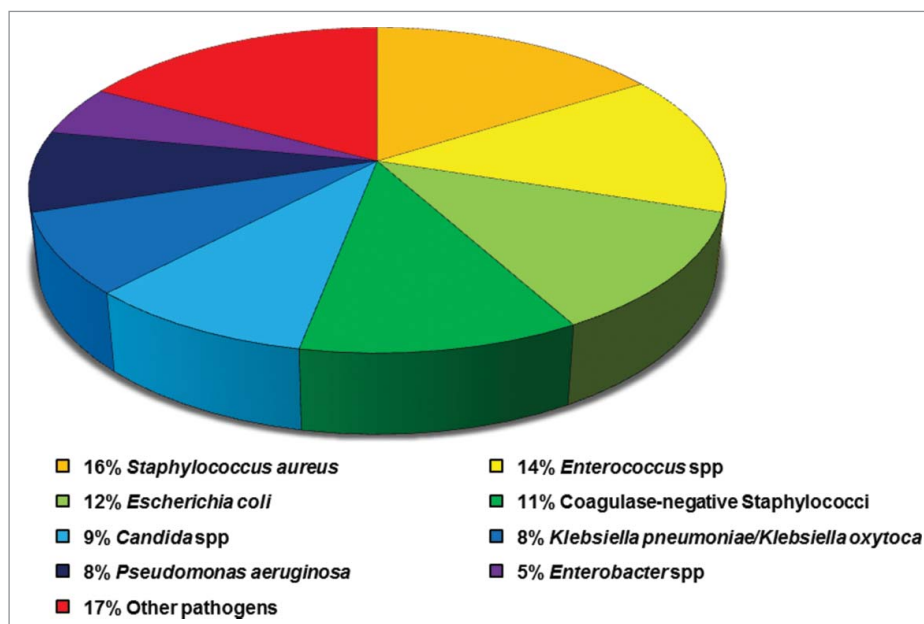


Figure 1. Eight common pathogens account for 83% of the reported HAIs in the United States. Data adapted from the summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014.²

between these 2 *Enterococcus* species are not well understood.

Many infections are polymicrobial, in which bacteria exist within mixed-species biofilms on host tissues or on medical devices and are more tolerant to antibiotic treatment or environmental stresses. Clustering of microorganisms within biofilms can facilitate and enhance horizontal gene transfer (HGT) of determinants that may increase the capacity of the organisms to colonize, infect, and persist in patients in the clinical setting.^{7,8} Emerging strains of multi-drug resistant *Enterococci* are a major medical problem, as its resistance profile has extended to include vancomycin and daptomycin, leaving limited options for treatment.

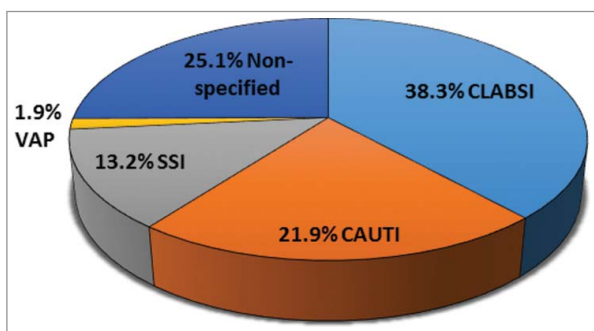


Figure 2. Prevalence of *E. faecalis* and *E. faecium* in device-associated HAIs. Data adapted from the summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, January 2006–October 2007.⁶

Enterococcal-associated polymicrobial infections

Polymicrobial infections involving several multidrug-resistant pathogens are implicated with increased mortality, hospitalization care, healthcare, and treatment costs.⁹ *Enterococci* can cause opportunistic, polymicrobial disease in immunocompromised hosts or in those with underlying health conditions.^{1,10,11} Since the 1980s, polymicrobial infections of the urinary tract, catheterized urinary tract, wounds, diabetic soft tissues, heart valves, bloodstream, and intra-abdominal and pelvic sites have been reported to be *Enterococci*-associated.^{9,11–19} Bacterial species that are frequently, but not always co-isolated with *Enterococci* in these infections include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp. and *Proteus* spp.^{9,15–21} Epidemiological reports describe the presence of *Enterococci* in polymicrobial infections, but there is limited literature defining the role of *Enterococcus* spp at infection sites, prompting the need for *in vitro* and *in vivo* studies to identify *Enterococcal* virulence factors, and their mechanism and contribution during interspecies interactions.

Multispecies biofilms rely strongly on interspecies interaction to successfully colonize a niche, either to cause disease (e.g. CAUTI) or to establish colonization resistance (e.g., in the gut).^{22,23} *In vitro* models can serve as a preliminary platform to recapitulate human infections for screening antimicrobial agents and anti-biofilm therapeutics, or simulate conditions to discover synergistic or antagonistic effects of interspecies interactions.^{23–26} In a recent study, Galván and colleagues found that

E. faecalis attachment during biofilm formation *in vitro* can be partially inhibited by uropathogenic *E. coli* (UPEC) but biofilm formation by *K. pneumoniae* or UPEC are not affected by *E. faecalis*.²³ Similarly, *E. faecalis* can promote *E. coli* biofilm biomass accumulation.^{27,28} Moreover, co-culture of an *E. faecium* probiotic strain with enteropathogenic *E. coli* increased the antibiotic susceptibility of *E. coli* to aminoglycosides, β -lactams and quinolones.²⁴ *In vitro* models have also proved significant in identifying virulence mechanisms such as *Fusobacterium nucleatum* Adherence Inducing Determinant 1 (*aid1*) mediating co-aggregation with *E. faecalis* and oral Streptococci but not *Lactobacillus casei* or *Staphylococcus epidermidis*,²⁶ and the growth promotion of *S. aureus* menaquinone biosynthesis-deficient variants in the presence of *E. faecalis* via menaquinone exchange.²⁵ Recently, Zackular and colleagues showed enhanced Enterococcal dissemination from the GI tract to the liver of *Clostridium difficile*-infected mice fed with a high-zinc diet, suggesting a possible synergistic relationship between the organisms during *C. difficile* infection.²⁹

Introduction of invertebrates (i.e., *Caenorhabditis elegans*) to model polymicrobial infections are an improvement to *in vitro* models as host survival can be assessed to measure lethality of co-culture combinations and can be useful in screening assays. The *C. elegans* worm model is a popular invertebrate to study pathogenesis (see section on *C. elegans* below). Lavigne and colleagues reported increased lethality of worms co-infected with *E. faecalis* and *E. coli*, associating enhanced mortality with polymicrobial infections.³⁰ On the other hand, *E. faecalis* is able to attenuate *Candida albicans* killing in the *C. elegans* model by antagonizing hyphal morphogenesis via the Fsr quorum sensing (QS) system.³¹ However, *in vivo* mammalian models are the most ideal in simulating human infections due to the presence of a more similar host immune response. In particular, mice offer the ready possibility of genetically modified animals to assess infections in immune deficient backgrounds, while rats are amendable to surgical procedures that make them suitable for modeling post-surgical infections (see Table 1 and later sections).

Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium*

The pathogenesis of Enterococci in opportunistic infections is achieved, at least in part, by their production of virulence factors and their resistance to antimicrobials. These virulence factors are involved in the attachment to host cells or extracellular matrix (ECM) proteins, as well as in immune evasion. Absence of these virulence factors

attenuate infection. Most Enterococcal virulence factors have been described in *E. faecalis*, whereas only a limited number of genes in *E. faecium* have been experimentally demonstrated to be involved in virulence. Many virulence factors of *E. faecalis* and *E. faecium* have been comprehensively reviewed by Garsin and colleagues;⁴ in this review we will highlight virulence factors of both *E. faecalis* and *E. faecium* that have been characterized using various vertebrate and non-vertebrate model systems since 2000 (Table 2).

Clinical manifestation and animal models for *Enterococcus faecalis* and *Enterococcus faecium* Infections

Animal models of infection that can mimic human diseases are crucial tools in establishing the microbial etiology for infectious diseases. Enterococci can colonize and infect the host at a variety of sites (Fig. 3). There is no single comprehensive model system for all Enterococcal infections. Models to study Enterococcal infections have been recently reviewed;⁴ here we focus on significant features and findings in Enterococcal pathogenesis derived from these model systems in recent years.

Vertebrates

Infective endocarditis

Endocarditis is a heart condition characterized by damage of the vascular endothelium due to high-pressure blood flow through narrowed openings into low-pressure regions of the heart causing platelet-fibrin (vegetation) formation; often prevalent in individuals with pre-existing cardiac conditions.³² In addition to formation of nonbacterial thrombotic vegetation, other endocarditis risk factors include those with medically implanted devices (e.g., artificial heart valves, pacemakers, or implantable defibrillators) that predisposes the individual to bacterial infection from the bloodstream, resulting in infective endocarditis (IE).³³ Vegetations serve as a substratum for adhesion and biofilm formation by the infecting bacteria, which limit clearance by host defenses necessitating intensive antibiotic therapy or surgical intervention for treatment.³³ IE can be categorized as nosocomial or community acquired IE, with coagulase-negative Staphylococci and Enterococci predominantly causing nosocomial IE.³⁴ Enterococci are the third leading cause of IE in North America, and fourth worldwide.³⁵ Enterococcal endocarditis accounts for 5–15% of all IE cases in the US.³⁶ As endocarditis rarely occurs in healthy hearts, catheters are implanted to induce aortic

Table 1. Advantages and disadvantages of animal models used for Enterococcal colonization and infection.

Model system	Advantages	Disadvantages
Vertebrates		
Mouse	<p>General advantages:</p> <ul style="list-style-type: none"> • Space saving (4–7 animals per cage, depending on cage size) • Easy to maintain • Inexpensive • Large number of animals can be used with reliable statistical evaluation • Small quantities of antimicrobials required due to small size • Share similarities with human immune systems, making it ideal for studying innate and adaptive immune responses²⁴⁶ • Susceptible to most pathogens that cause human infections²³¹ • Germfree, gnotobiotic, immunodeficient, and genetically modified mice are more readily available compared with larger animals • Diabetic or neutropenic condition can be induced using chemicals • Survival after infection and bacterial burden in tissues can be assessed • Bacterial burden and translocation can be determined quantitatively • Infecting organism or infectious dose can be manipulated to compare intensity of the inflammatory response and its dynamics <p>Peritonitis, bloodstream and systemic:</p> <ul style="list-style-type: none"> • Surgical or non-surgical infection protocols are available • Simple to perform non-surgical procedure for infection • Survival studies are possible • <i>In vivo-in vitro</i> infection model (i.e., peritonitis model) is available • Antimicrobial efficiency can be evaluated • <i>In vivo</i> drug interactions (e.g., synergistic or antagonistic) can be evaluated • Animal must be exsanguinated to collect large volumes of blood⁸⁰ • Reagents are readily available for immunologic studies in rodents⁸⁰ <p>Gastrointestinal:</p> <ul style="list-style-type: none"> • Non-surgical procedure for infection, simple to perform • Intestinal development is similar to humans with similar immune responses²⁴⁸ • Human and murine intestinal bacterial communities share common diversity²⁴⁹ • Ease of manipulation for administering inoculum or antibiotics supplemented in drinking water <p>Urinary tract (UTI and CAUTI):</p> <ul style="list-style-type: none"> • Non-surgical procedure for infection, simple to perform • Sensitive to most uropathogens that cause human UTI²⁵¹ • Mice do not exhibit vesicoureteral reflux (VUR), are more representative of human UTI compared with rats²⁵² • Many bacteria agents that cause human UTI infect mice, making it an ideal model for studying ascending UTI and pyelonephritis²⁵¹ • Different permutations for acute and chronic UTI and a protocol for CAUTI have been established, providing a well-studied model that offers comparison among different research groups • Well characterized bladder immune response <p>Surgical site (SSI):</p> <ul style="list-style-type: none"> • Protocols with or without foreign body implantation are available²⁵⁴ • Multiple permutations available (surgical wounds can be created on the back, abdominal, thigh, or the shoulder region)²⁵⁴ 	<p>General disadvantages:</p> <ul style="list-style-type: none"> • Insufficient bacterial burden in an infected tissue, blood or foreign body may require pooling of multiple samples, leading to increased cost and number of animals required • Small animal size limits the implementation of surgical procedures and use of foreign implants • Expression of TLR2, TLR3, TLR4 and TLR9 are different between humans and mice.²⁴⁷ <p>Peritonitis, bloodstream and systemic:</p> <ul style="list-style-type: none"> • A single, overwhelming dose may not be relevant to clinical scenario where infection often starts with a low inoculum with progressive pathogenesis over time.⁸⁰ • Animals can only be used once to harvest organs or fluid for bacterial enumeration.⁸⁰ • Lack of reproducibility compared with clinical scenario⁸⁰ • Poor surgical techniques may lead to post-surgical complications or reduced survival rates <p>Gastrointestinal:</p> <ul style="list-style-type: none"> • Chemical injury to induce intestinal lesions in mice are different from lesions observed in IBD patients.²⁵⁰ • Coprophagy can influence experimental outcome and interpretation of results.²⁵⁰ <p>Urinary tract (UTI and CAUTI):</p> <ul style="list-style-type: none"> • Overwhelming doses may not be clinically relevant • Difficult to correlate treatment times with human infection as stage of disease when patient seeks treatment is unknown • Short lifespan of mice compared with humans limits stratification of age-related studies.²⁵³ • Urine sampling is a common, non-invasive, but less reliable method of monitoring infection <p>Surgical site (SSI):</p> <ul style="list-style-type: none"> • Overwhelming doses may not be clinically relevant • Poor surgical techniques may lead to post-surgical complications
Rat	<p>General advantages:</p> <ul style="list-style-type: none"> • Size is amenable for implanting foreign devices and surgical procedures • Allows acquisition of larger sample sizes and less need to pool tissue samples • Blood and abscess fluid can be sampled over a time course without sacrificing animal • Bacterial burden and translocation can be determined quantitatively • Infecting organism or infectious dose can be manipulated to compare intensity of the inflammatory response and its dynamics 	<p>General disadvantages:</p> <ul style="list-style-type: none"> • Genetically modified animals are less common compared with mice • Higher cost compared with mice.²⁵⁵

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Table 1. (Continued)

Model system	Advantages	Disadvantages
Rabbit	<p>Infective endocarditis (IE):</p> <ul style="list-style-type: none"> • Can be used to study bacterial virulence and response to antimicrobial agents²⁵⁶ • Represents a biofilm-associated infection in the presence of a catheter implant²⁵⁶ • Data is reproducible²⁵⁶ <p>Peritonitis, gastrointestinal (GI) and sepsis:</p> <ul style="list-style-type: none"> • Surgical or non-surgical infection protocols are available • Non-surgical infections are simple to perform • Ideal for studying polymicrobial peritonitis/sepsis from anastomotic leak or cecal ligation and puncture (surgical), with reproducible clinical manifestations of sepsis²⁵⁷ • Inoculum preparation is not required²⁵⁷ <p>Urinary tract (CAUTI):</p> <ul style="list-style-type: none"> • Larger animal allows application of urinary catheter¹⁷⁷ 	<p>Infective endocarditis (IE):</p> <ul style="list-style-type: none"> • Vegetations need to be artificially induced for bacterial infection, may not be clinically relevant • High-risk surgical procedure may be technically challenging, leading to reduced post-surgical survival rates • Labor intensive <p>Peritonitis, gastrointestinal (GI) and sepsis:</p> <ul style="list-style-type: none"> • Single application of a high bacterial dose results in a rapid clinical course, acute inflammatory response and sometimes, early death.²⁵⁷ • Poor surgical techniques may lead to post-surgical complications or reduced survival rates • Difficult to control amount of bowel leakage for anastomotic leak or cecal ligation and puncture studies, intestinal bacteria may differ among animals.²⁵⁷ <p>Urinary tract (CAUTI):</p> <ul style="list-style-type: none"> • Exhibits VUR.²⁵²
	<p>General advantages:</p> <ul style="list-style-type: none"> • Larger size is amenable for surgical procedures.²⁵⁸ • Blood and abscess fluid can be sampled over a time course without sacrificing animal <ul style="list-style-type: none"> • Bacterial burden and translocation can be determined quantitatively • Infecting organism or infectious dose can be manipulated to compare intensity of the inflammatory response and its dynamics 	<p>General disadvantages:</p> <ul style="list-style-type: none"> • Genetically modified animals are less common compared with mice • High cost, labor intensive and ethical considerations.²⁵⁵ • Rabbit pharmacokinetics of antimicrobial agents differ from humans.²⁵⁵ • Antibiotic half-lives are shorter in rabbits compared with humans.²⁵⁵
	<p>Infective endocarditis (IE):</p> <ul style="list-style-type: none"> • Appropriate for studying bacterial virulence and response to antimicrobial agents.²⁵⁶ • Represents a biofilm-associated infection in the presence of a catheter implant.²⁵⁶ • Data is reproducible.²⁵⁶ <p>Endophthalmitis:</p> <ul style="list-style-type: none"> • Large surface area for surgical procedure 	<p>Infective endocarditis (IE):</p> <ul style="list-style-type: none"> • Vegetations need to be artificially induced for bacterial infection, may not be clinically relevant • High-risk surgical procedure may be technically challenging, leading to reduced post-surgical survival rates • Labor intensive <p>Endophthalmitis:</p> <ul style="list-style-type: none"> • Requires significant surgical intervention • Poor surgical techniques may lead to post-surgical complications
	<p>Foreign body subdermal abscess:</p> <ul style="list-style-type: none"> • Bacterial burden and translocation can be determined quantitatively • Reproducible data 	<p>Foreign body subdermal abscess:</p> <ul style="list-style-type: none"> • Requires significant surgical intervention²⁵⁷ • Poor surgical techniques may lead to post-surgical complications
Dog	<p>General advantages:</p> <ul style="list-style-type: none"> • Larger size is amenable for surgical procedures.²⁵⁸ • Blood can be sampled over a time course without sacrificing animal 	<p>General disadvantages:</p> <ul style="list-style-type: none"> • Requires significant surgical intervention • Ethical concerns about using companion animal species for research • Genetically modified animals are less common compared with mice • Higher costs are associated with housing larger animals
Zebrafish	<p>Endodontic infection:</p> <ul style="list-style-type: none"> • Acquisition of larger samples for analysis compared with smaller animals.¹⁸⁴ <p>General advantages:</p> <ul style="list-style-type: none"> • Cheapest vertebrate organism • Amenability to scaling up • Transparent body allows <i>in vivo</i> imaging of host-pathogen interactions 	<p>Endodontic infection:</p> <ul style="list-style-type: none"> • Surgical procedures requires technical expertise <p>General disadvantages</p> <ul style="list-style-type: none"> • Difficulties in translating host response to mammalian infections due to high degree of evolutionary divergence. • Difficult to create truly inbred zebrafish lines

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Table 1. (Continued)

Model system	Advantages	Disadvantages
Invertebrates		
<i>C. elegans</i>	General advantages: <ul style="list-style-type: none"> • Smallest in size (adults are ~1mm) among the 3 invertebrates • Low cost • Rapid life cycle (~3.5 days at 20°C) • Transparent body and genetic tractability • Easiest to maintain in large-scale quantity • Excellent choice to investigate gut microbial-host infection 	General disadvantages: <ul style="list-style-type: none"> • Lack cellular and humoral system • Lack of a fully sequenced genome • Infection cannot be performed at 37°C • Not as readily available as compared with <i>G. mellonella</i>
<i>G. mellonella</i>	General advantages: <ul style="list-style-type: none"> • Have cellular and humoral immune system • Low cost • Readily available from commercial source • Infections can be performed at 37°C • Largest in size among the 3 invertebrates, allows direct injection of bacteria or drugs • Allows exact quantification of experiment inoculum or drug dosage administered 	General disadvantages: <ul style="list-style-type: none"> • Lack of a fully sequenced genome • Lack of molecular tools to genetically manipulate this organism
<i>D. melanogaster</i>	General advantages: <ul style="list-style-type: none"> • Genetically tractable • Fully sequenced genome • Availability genetic mutant libraries • Low cost • Easy to bred • Rapid life cycle 	General disadvantages: <ul style="list-style-type: none"> • Infection cannot be performed at 37°C • Not as readily available as compared with <i>G. mellonella</i>
Plants		
<i>A. thaliana</i>	General advantages: <ul style="list-style-type: none"> • No ethical issues 	General disadvantages: <ul style="list-style-type: none"> • May not represent eukaryotic systems.

valve damage in laboratory animals, followed by bacterial infection to mimic IE in humans.³²

An experimental model of endocarditis was first established in rabbits, before the development of a rat model in 1978 by Santoro and Levison.³⁷ Rabbits are the most commonly used experimental model of IE, although at times rats are selected for the advantage of housing more animals at a lower expense. After anesthesia, aortic damage is caused by inserting a polyethylene catheter through the right carotid artery into the left ventricle by surgical incision. Catheters can either be removed following intravenous (IV) administration of bacteria or left in place for the duration of the study.³⁸⁻⁴¹

Aggregation substance (AS) is a highly conserved family of surface-anchored polypeptides with adherence properties that enable *E. faecalis* to interact with host cells and ECM proteins. AS mediates *E. faecalis* intercellular clumping and is one of the best studied endocarditis virulence factors, although Enterococcal endocarditis is not dependent on the presence of AS.⁴² AS encoded on the *E. faecalis* pCF10 sex pheromone plasmid contributes to larger cardiac vegetations in rabbits, as well as complications such as liver necrosis and enlarged spleens.⁴¹ Plasmid pCF10 carriage confers a selective advantage to *E. faecalis* during Enterococcal endocarditis, as seen in mixed infections using varying donor (harboring pCF10)/recipient (no plasmid) ratios.⁴¹ Different chromosomal antibiotic resistance markers for differentiation showed consistent shifts in

favor of cells harboring pCF10 at 3 d post infection (dpi).⁴¹ Immunohistochemistry positively correlates AS expression with virulence *in vivo* using this model.³⁹ AS is a large 137 kDa protein that harbors an N-terminal signal sequence, lipoteichoic acid (LTA) binding aggregation domain, central aggregation domain (with 2 RGD motifs), and a C-terminal cell wall anchor.⁴³ AS is encoded by the *prgB* gene of the conjugative plasmid pCF10. Chuang and colleagues observed attenuated virulence in *E. faecalis* endocarditis upon substitution of glycine to alanine residues in the 2 RGD motifs of AS.⁴³ Active immunization using a purified AS₄₄₋₃₃₁ fragment is ineffective in protecting rabbits from IE due to formation of platelet/fibrin-rich structures formed in endocardial vegetations shielding infecting *E. faecalis* from host antibodies and possibly immune cells of the humoral immune system.^{39,44} A combination of studies showed that when challenged with AS-expressing (AS⁺) *E. faecalis*, neither active immunization against AS⁺ *E. faecalis* cells nor the N-terminal domain of the AS protein protects rabbits against IE; instead the disease is aggravated suggesting that IgG-mediated aggregation of AS⁺ *E. faecalis* promotes disease.^{39,42} Rabbits infected with AS⁺ *E. faecalis* following active immunization with AS⁻ *E. faecalis* showed lower mortality than rabbits actively immunized with AS⁺ *E. faecalis*.⁴² Likewise, Fab fragments of IgG from rabbit antibodies raised against purified AS partially protected AS⁺ *E. faecalis*-challenged rabbits (immunized

Table 2. *E. faecalis* and *E. faecium* virulence factors studied in various host model systems.

Pathogenesis/virulence assays: IE, peritonitis, bloodstream and systemic infection, GI infection and colitis, UTI, CAUTI, endophthalmitis, foreign body subdermal abscess, SSI, endodontic infection

Enterococcal virulence factors/ Experimental model	Rabbit	Rat	Mice	Zebrafish	C. <i>elegans</i>	G. <i>mellonella</i>	D. <i>melanogaster</i>	A. <i>thaliana</i>
<i>ace</i> (Collagen adhesin) ^a		63	71, 87, 160			71		
<i>acm</i> (Cell wall-anchored collagen adhesin) ^b		60	60					
<i>ahrC</i> (Transcriptional regulator) ^a	38	38, 47	38					
<i>ahp</i> (Alkyl hydroperoxide reductase) ^a			100					
<i>altA</i> (Autolysin) ^a	46							
<i>asrR</i> (antibiotic and stress response regulator) ^b			126			126		
<i>bepA</i> (Biofilm and endocarditis-associated permease A) ^b		61						
<i>bgsA/bgsB</i> (Glycosyltransferase) ^a		58	83, 110, 120, 121					
<i>capD</i> (Capsular polysaccharide protein) ^b			127					
<i>ccpA</i> (Global transcriptional regulator) ^b		65						
<i>cpsI</i> (Capsular polysaccharide I) ^a			182					
<i>cspI</i> (Serotype-specific carbohydrate) ^a			182					
<i>cspR</i> (Cold shock RNA-binding protein) ^a			123			123		
<i>cyl</i> (Cytolysin) ^a	88		87, 90, 141		206, 209		222	
<i>dltA</i> (D-alanylation of lipoteichoic acids) ^a			116, 166					
<i>ebp/emp</i> (Endocarditis and biofilm-associated pili) ^c		62, 76	74, 76, 159, 160, 163, 174, 176					
<i>ebrB</i> (Enterococcal biofilm regulator B) ^b			79					
<i>eep</i> (Membrane metalloprotease) ^a	47	38, 47	38					
<i>efaCBA</i> (Endocarditis-specific antigen) ^a			87, 91					
<i>efbA</i> (Enterococcal fibronectin-binding protein A) ^a		64	73					
<i>ef0149</i> (Predicted adhesin) ^a			87					
<i>ef0377</i> ^a			86			86		
<i>ef0685</i> (Parvulin family rotamase PPlase) ^a			118			118		
<i>ef1097</i> (Hypothetical protein) ^a							221	
<i>ef1534</i> (Cyclophilin family PPlase) ^a			118			118		
<i>ef_3196/7</i> (Two-component system) ^a						86		
<i>elrA</i> (Enterococcal leucine-rich protein A) ^a			82, 107					
<i>elrR</i> (Enterococcal leucine-rich protein regulator) ^a			108					
<i>epaA-epaR</i> (Enterococcal polysaccharide antigen operon) ^a			109					
<i>epaB</i> (Enterococcal polysaccharide antigen B) ^a			84, 132, 158	186	132	132		
<i>epaE</i> (Enterococcal polysaccharide antigen B) ^a			84					
<i>epaM</i> (Enterococcal polysaccharide antigen M) ^a			109					
<i>epaN</i> (Enterococcal polysaccharide antigen N) ^a			109					
<i>epaX</i> (Glycosyltransferase) ^a			140					
<i>ers</i> (PrfA-like regulator) ^a			98					
<i>esp</i> (Enterococcal surface protein) ^c		67	128, 129, 139, 150, 161					
<i>fnm</i> (Fibronectin-binding protein) ^b		66						
<i>fsrA</i> (Fsr system) ^a			51, 87		51, 53			224
<i>fsrB</i> (Fsr system) ^a	54, 55	56	51, 53, 87, 206	186	53, 206, 207	215	222, 221	224
<i>fsrC</i> (Fsr system) ^a			51, 87		53			224
<i>fsrD</i> (Fsr system) ^a			87					
<i>gelE</i> (Gelatinase E) ^a	40, 54, 55, 88	56,	133	51, 87	186	53, 209	215, 259	221
<i>gldA/glpK</i> (Glycerol dehydrogenase and kinase) ^a			87					
<i>gls24/glsB</i> (General stress protein) ^a		57	68					
<i>gls24-like/glsB-like</i> (General stress protein) ^b			111					
<i>grvR/etaR</i> (Global regulator of virulence/ Enterococcal 2-component system a) ^a			70, 93					
Heptapeptide pheromone ^a			147					
<i>hylEfm</i> (Putative hyaluronidase) ^b			7, 8					
<i>hypR</i> (HypR transcriptional regulator) ^a			99					
<i>ldh-1/ldh-2</i> (Lactate dehydrogenase) ^a			124					
<i>lgt</i> (lipoproteinsprolipoprotein diacylglycerol transferase) ^a			132		132			
<i>lrgAB</i> (LrgAB operon) ^a							221	
<i>lytRS</i> (LytRS operon) ^a							221	
<i>mafR</i> (Mga/AtxA-like <i>faecalis</i> regulator) ^a			95					
<i>menB</i> (Demethylmenaquinone synthetase) ^a		136						
<i>mprF1/mprF2</i> (Multiple peptide resistance factor) ^a			260					
<i>msrA/msrB</i> (Methionine sulfoxide reductase) ^a			102			102		
<i>npr</i> (NADH peroxidase) ^a			100					
<i>orfde4</i> (Putative glycosyl transferase) ^a			84					

(Continued on next page)

Table 2. (Continued)

Enterococcal virulence factors/ Experimental model	Rabbit	Rat	Mice	Zebrafish	C. <i>elegans</i>	G. <i>mellonella</i>	D. <i>melanogaster</i>	A. <i>thaliana</i>
<i>orfde6</i> (Putative dTDP-rhamnose biosynthesis enzyme) ^a			84					
<i>oatA</i> (O-acetyl transferase) ^a			116					
<i>paiA</i> (Transcriptional repressor) ^a			207		207			
<i>Pcyl</i> (Cytolysin promoter) ^a			141			141		
<i>perA</i> (Pathogenicity island-encoded regulator) ^a			94					
<i>perR</i> (Peroxide regulator) ^a			106					
<i>pgdA</i> (Lysozyme-induced peptidoglycan N-acetylglucosamine deacetylase) ^a						217		
Phage03-like element ^a					209	209		
<i>phrB</i> (Deoxyribodipyrimidine photolyase) ^a			207		207			
<i>pmvE</i> (Polyamine N-acetyltransferase-like enzyme) ^a			216			216		
pPD1 conjugative plasmid (Bacteriocin 21) ^a			137					
<i>prgB</i> (Aggregation substance) ^a	39, 41–44		87, 92, 167					
<i>prkC</i> (One-component signaling protein) ^a			138					
<i>proB</i> (Glutamate-5-kinase) ^a	47							
Prophage 5 ^a		119	119					
<i>ptsD</i> (Phosphotransferase system IID enzyme subunit) ^b			142					
<i>recQ</i> (ATP-dependent DNA helicase) ^a			207		207			
<i>relA/rsh</i> (Bifunctional synthetase and hydrolase) ^a	179							
<i>relQ</i> (Small synthetase) ^a	179							
<i>scrB</i> (Sucrose-6-phosphate hydrolases) ^a					206, 207			
<i>sigV</i> (Extracytoplasmic function sigma factor) ^a			116					
<i>slyA</i> (Transcription regulator) ^a			122			122		
<i>sprE</i> (Serine protease) ^a	40, 54, 55, 88	51, 56, 133	51	186	53	259	221	224
<i>spx</i> (Spx global regulator) ^a			101					
<i>srtA</i> (Sortase A) ^a			164, 171					
<i>srtC</i> (Sortase C) ^a			164					
Tissue matrix metalloproteinase 9 (MMP9) ^a			133					
<i>tpx</i> (Thiol peroxidase) ^a			100					
<i>vanA/vanB</i> (Vancomycin resistance) ^a		85						
<i>wxl</i> (WxL proteins) ^b		75						
<i>zps</i> (Zwitterionic bacterial polysaccharides) ^b		81						

Notes. ^aDenotes study describing virulence factor of *E. faecalis*; ^bdenotes study describing virulence factor of *E. faecium*; ^cdenotes study describing virulence factor from both *E. faecalis* and *E. faecium*.

just before infection) by reducing endocardial lesion microbial counts.⁴²

Several other *E. faecalis* factors have been demonstrated as virulence factors in the rabbit IE model. AtlA is a major autolysin of *E. faecalis* that cleaves the β -1, 4 links between N-acetylglucosamine and N-acetylmuramic acid within the peptidoglycan. AtlA is a 3-domain enzyme composed of an N-terminal threonine- and glutamic acid (T/E) rich domain of unknown function, a central putative catalytic domain, and a C-terminal cell wall binding domain consisting of 6 LysM modules.⁴⁵ *E. faecalis* Δ atlA displayed reduced susceptibility to the bactericidal effects of amoxicillin compared with wild-type (WT) at 48 hours post infection (hpi) in rabbits, suggesting a role for AtlA during IE.⁴⁶

Recombinase-based *in vivo* expression technology (RIVET) during *E. faecalis* surgical site infection identified 2 genes highly expressed in rabbit subdermal abscesses: *proB* and *eep*.⁴⁷ *proB* encodes for glutamate 5-kinase, involved in proline metabolism, and proline

production has been shown to contribute to the survival and pathogenicity of other Gram-positive bacteria in selected animal models under an osmolyte-depleted environment.⁴⁸ *eep* encodes an intramembrane metalloprotease that is a member of the site 2 protease family, a conserved class of enzymes that performs regulated intramembrane proteolysis.⁴⁹ Characterization of these deletion mutants and their role in IE showed stronger attenuation of the *eep* mutant compared with Δ *proB*.⁴⁷ An ortholog of the ArgR family transcription factor, AhrC, was identified in a transposon screen for *in vitro* biofilm mutants, and subsequently shown to also be strongly attenuated in IE.³⁸ However, not all biofilm-impaired mutants are attenuated in IE, suggesting that biofilms grown on abiotic surfaces *in vitro* and biotic surfaces *in vivo* have different properties.³⁸ Consistent with this observation, Leuck and colleagues reported inconsistent adherence phenotypes by *E. faecalis* clinical strains comparing polystyrene dish biofilm assays and an *ex vivo* heart valve assay.⁵⁰ Data from both studies

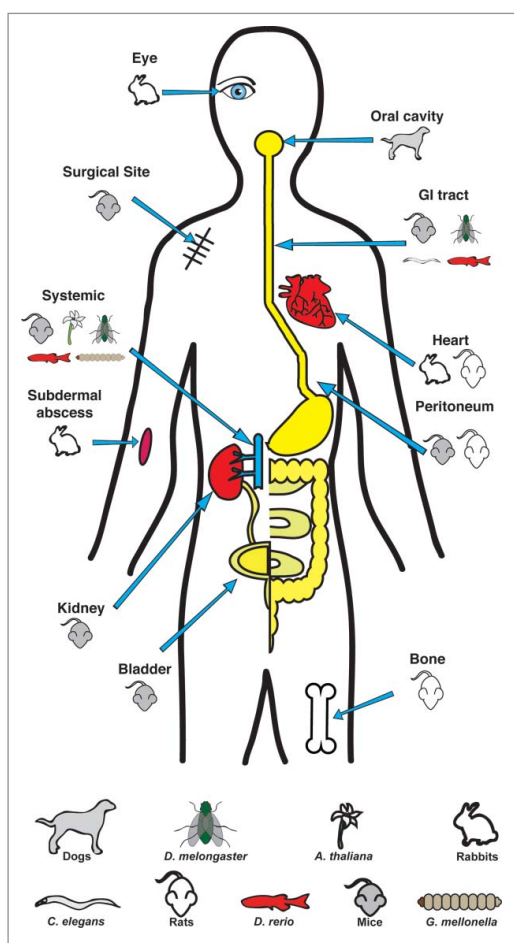


Figure 3. *In vivo* systems for modeling *E. faecalis* and *E. faecium* virulence. Cartoon depiction of the different host model systems that have been used to study niche-specific Enterococcal diseases.

suggest that biofilm formation *in vitro* and endocarditis development may not necessarily be linked.^{38,47}

Bacterial QS relies on 2-component systems involving a sensor transducer and response regulator for sensing cell density and regulates expression of virulence factors.⁵¹ The *S. aureus agr* QS locus mediates *S. aureus* dissemination during animal models of infection.⁵² *E. faecalis* expresses Agr-like proteins (FsrA, FsrB and FsrC) that are necessary for positive regulation of the virulence-associated proteases Gelatinase E (GelE) and serine protease (SprE).^{51,53} FsrC is a histidine kinase that senses extracellular accumulation of a peptide lactone encoded at the C-terminus of the FsrB protein. FsrC sensing leads to activation of the response regulator and transcription factor FsrA. GelE, encoded by *gelE*, aids in the subversion of host immune responses to *E. faecalis* during IE.⁴⁰ Even though the *fsr* system and its products are important for virulence in all the infection models discussed in this review, the absence of GelE alone can limit dissemination from the primary vegetation and results in lower bacterial burdens in IE.⁴⁰ In contrast, the

absence of SprE does not impact virulence in IE. Since gelatinase hydrolyzes fibrin to facilitate bacterial dissemination, aortic vegetations from rabbits infected with *E. faecalis* lacking GelE display more fibrin-rich matrices than WT-infected rabbits.⁴⁰ GelE also cleaves chemoattractants such as complement C5a resulting in decreased neutrophil migration *in vitro*.⁴⁰ In IE, GelE modulates the host immune response by reducing neutrophil-like cell migration to infected tissues.⁴⁰ Consistent with the rabbit model of IE and other infection models, rat cardiac colonization is attenuated in *E. faecalis* lacking *gelE* and *sprE*;^{40,51,53-55} requiring a higher ID₅₀ than WT to colonize rat heart valves and display reduced endocarditis induction rate.⁵⁶

E. faecalis and *E. faecium* virulence are also well studied using a rat model of IE. This model has been used to test the efficacy of new therapeutics and prophylactic countermeasures against *E. faecalis* and *E. faecium*.⁵⁶⁻⁶⁷ IE is initiated in the same manner as in rabbits, and disease severity can be assessed by mortality rates, embolism, colonization efficiency, 50% infective doses (ID₅₀), and mixed competitive infections.⁵⁷

E. faecalis glycosyltransferases, encoded by *bgsA* and *bgsB*, play important roles in synthesising cell wall glycolipid; mutants in an individual cell wall glycolipid display fewer endocarditic lesions and reduced bacterial colony-forming units (CFU) in vegetations.⁵⁸ Strains mutated in the general stress gene *gls24* require a higher ID₅₀ in both the mouse peritonitis and rat endocarditis model.^{57,68} *gls24* encodes a general stress protein involved in bile-salt resistance and its transcription was induced under glucose starvation and other stress conditions, including the presence of bile salts and cadmium chloride.⁶⁹ Two other *E. faecalis* virulence factors, Ace (collagen adhesion) and EfbA (Enterococcal fibronectin-binding protein) were studied by Singh and colleagues in the rat IE model. *ace* encodes a collagen-binding protein whose transcription is positively regulated by a 2-component system regulatory system, GrvRS (global regulator of virulence).⁷⁰ Another transcription regulator, Ers (PrfA-like regulator), can act as a repressor of Ace.⁷¹ Ace is an adhesin that binds to collagen (type I and IV) and laminin and belongs to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) protein family, sharing sequence homology to the ligand-binding region with *S. aureus* Cna.⁷² EfbA is encoded by the Enterococcal ortholog of *Streptococcus pneumoniae pava* that binds with strong affinity to immobilized fibronectin, collagen I and collagen V.⁷³ Independent abrogation of either *ace* or *efbA* resulted in significant attenuation in a mixed infection.^{63,64} Finally, rats pre-immunized with either Ace or EfbA were less susceptible to IE, highlighting the potential of Ace and

EfbA as immunotherapeutic antigenic targets.^{63,64} Another prophylactic treatment effective in protecting the host against IE includes passive protection of rats by injecting anti-EbpC (endocarditis and biofilm-associated pilus subunit protein C) monoclonal antibody (Mab), compared with control IgG, intravenously through the tail vein 24 h post-catheterization (hpc) and 1 h before bacterial inoculation. The endocarditis- and biofilm-associated pilus (Ebp) is encoded by a polycistronic gene locus which consists of 3 structural pilin genes, *ebpA*, *ebpB* and *ebpC*, and an adjacent downstream gene, *srtC* that is independently expressed from a second promoter.⁷⁴ Sortase C (SrtC) is a pilin-specific sortase that polymerizes the Ebp/Emp pili by a transpeptidation reaction before their attachment to the cell wall by Sortase A (SrtA). SrtA is a housekeeping enzyme that recognizes the LPXTG motif at the C-terminus of most cell wall protein precursors and is required for the attachment of these cell surface-anchored virulence factors on the cell wall. Rats passively immunized with anti-EbpC were less susceptible to IE by *E. faecalis*.⁶² Laverde and colleagues performed similar passive immunization experiments by introducing polyclonal antibodies against LTA fragments into rats over 3 doses: (i) at the time of catheterization, (ii) 24 hpc, and (iii) 4 hpi by *E. faecalis*. When compared with controls, passively immunized rats had fewer cardiac vegetations and greater bacterial clearance.⁵⁹

A number of *E. faecium* virulence factors have been examined in the rat IE model, including the cell wall-anchored adhesion for collagen from *E. faecium* (*acm*),⁶⁰ Enterococcal surface protein (*esp*),⁶⁷ global transcriptional regulator (*ccpA*),⁶⁵ fibronectin-binding protein (*fnm*),⁶⁶ novel cell wall binding proteins containing a WxL motif,⁷⁵ biofilm and endocarditis-associated permease A (*bepA*)⁶¹ and EmpA (previously *ebpA_{fm}*).⁷⁶ Like Ace in *E. faecalis*, Acn is also a homolog of Cna, a collagen adhesin shown to be important for *S. aureus* endocarditis.⁷⁷ Acn is important for early adherence as well as vegetation formation.⁶⁰ Enterococcal surface protein Esp is encoded on a pathogenicity island in both *E. faecalis* and *E. faecium*. It contains multiple repeat motifs, a characteristic found in many bacteria surface protein adhesins involved in host-ligand binding.⁷⁸ *esp* transcription is regulated by Enterococcal biofilm regulator B (*ebrB*) located upstream of *esp* in *E. faecium*.⁷⁹ Esp contributes to colonization of heart valves at 24 hpi but not at 3 hpi and is therefore not likely to play a role in initial colonization.⁶⁷ EmpA, the tip subunit of the Emp pili, is essential for vegetation colonization at 48 hpi.⁷⁶ *ccpA* that encodes CcpA, a global transcriptional regulator of carbon catabolite repression in Gram-positive bacteria, affects the growth of *E. faecium* in a rat IE model.⁶⁵ In competitive infections, *E. faecium* Δ *ccpA* is

outcompeted in aortic valve colonization compared with WT; however, this could be due to reduced fitness of the Δ *ccpA* strain as it has a growth defect when cultivated in brain heart infusion (BHI) broth.⁶⁵ The Fnm protein of *E. faecium*, is a homolog of *S. pneumoniae* PavA. Fnm binds to immobilized fibronectin in a concentration dependent manner, and can also bind to collagen type V and laminin.⁶⁶ Deletion of *fnm* resulted in reduced binding to fibronectin and this strongly attenuates cardiac colonization.⁶⁶ Cell wall binding proteins harboring a WxL sequence are involved in IE pathogenesis, since a strain defective in all 3 WxL (Δ *wxlABC*) genes was outcompeted in vegetations by WT at 48 hpi.⁷⁵ *E. faecium* *bepA*, encoding a carbohydrate phosphotransferase system (PTS) permease, is enriched in *E. faecium* hospital outbreak isolates; *bepA* mutants are also outcompeted by the WT strain in IE.⁶¹ A common observation from these competitive mixed infections is that genetic mutants usually fail to colonize as efficiently as their WT counterpart, suggesting that the competing WT strain is unable to compensate for loss of gene function in the mutant.

Peritonitis

Peritonitis is an infection of the abdominal lining and can progress in 3 stages: (i) primary peritonitis - arises via hematogenous spread of bacteria which often affects immunocompromised individuals; (ii) secondary infection - arises via damage to a visceral organ, which may be postoperative (for example perforation, trauma, postoperative complication, etc.); (iii) tertiary peritonitis - defined as persistent or recurrent intra-abdominal infection. Peritonitis may be mono- or polymicrobial and can lead to bloodstream infection, organ failure, or in more serious cases, death.¹¹

Rodents are preferred over other animals to study peritonitis owing to their small size and low cost.⁸⁰ Typically, rodents are challenged with an intraperitoneal (IP) injection of 0.2–1 ml bacterial inoculum mixed with sterile rat fecal extract.^{81–84} Modifications to rat infections may include making an abdominal incision before implantation of gelatin capsules harboring bacteria.⁸⁵ A combination of RIVET screens performed under different *in vivo* conditions (i.e., mouse bacteremia or mouse peritonitis) identified *E. faecalis* *ef_0377* transcriptional activation during peritonitis.⁸⁶ Other genes identified in the RIVET screen have yet been characterized *in vivo*. Additionally, genes induced during *E. faecalis*-mediated mouse peritonitis include cytolysin (*cyll_L*, *cyll_S*, *cyll_M*), endocarditis-specific antigen (*efaCBA*), *gelE*, QS 2-component system (*fsrABCD*), *ace* adhesin, AS (*prgB*), a predicted adhesin (*ef0149*) and genes involved in glycerol metabolism (*gldA* and *glpK*).⁸⁷ Cytolysin is made up of 2

subunits; the large subunit encoded by *cylL_L* and the small subunit encoded by *cylL_S*. Both subunits are post-transcriptionally modified by the *cylM* gene product. Once modified, the cytolysin subunits are secreted from the cell by an ABC transporter encoded by *cylB*. Activation and subsequent maturation of the cytolysin subunits is performed by a protease encoded by *cylA*.⁸⁸ The endocarditis-specific antigen (*efaCBA*) is a 3 gene operon, predicted to encode components of an ABC-type transporter, with EfaA as its putative substrate-binding lipoprotein component.⁸⁹ Virulence determinants cytolysin,⁹⁰ Efa,⁹¹ GeLE,⁵¹ FsrABC⁵¹ and AS⁹² were previously characterized and contribute to mouse peritonitis. Double deletion of *gldA* (glycerol dehydrogenase) and *glpK* (glycerol kinase) resulted in attenuated bacteremia in mice while single mutants of *gldA* and *glpK* were not attenuated.⁸⁷ Overall, screening methods have been highly informative for obtaining a global view of bacterial responses in a defined environment, and especially useful for identifying novel factors that are relevant to specific diseases. However, validation including reverse-transcription quantitative PCR (RT-qPCR), gene characterization, and mutational analyses must be performed to fully understand gene functions during infection.

Individual deletions of *fsrA*, *fsrB* and *sprE* delay mortality in mice suffering peritoneal infection while *fsrB* complementation restores killing.^{51,53} A homology search for putative 2-component systems led to the identification of an Enterococcal 2-component system *a* (*eta*), with the *etaR* regulator mutant requiring a higher LD₅₀ (50% lethal dose) compared with WT in the mouse peritonitis model.⁹³ Pathogenicity islands (PAIs) are large genomic regions containing a collection of virulence-related genes acquired via HGT. PAI-encoded virulence factors contain regulators that control virulence gene expression. A putative AraC/XylS-type transcriptional regulator, *perA*, encoded on a PAI of *E. faecalis* mediates persistence in spleens up to 72 hpi during mouse peritonitis.⁹⁴ PerA expression is variable and depends on the insertion of IS1191 in the *perA* promoter sequence; in strains where IS1191 is absent, PerA expression is not disrupted.⁹⁴ Low bacterial burden in liver and spleens at 24 and 48 hpi of *perA* mutants may be a result of sensitivity to oxidative stress, although dissemination was not hampered since both mutant and WT CFU were comparable in the blood in the mouse model of peritonitis.⁹⁴ Although attenuated in the peritonitis model, inactivating *perA* resulted in stronger biofilm formation *in vitro*.⁹⁴ PerA may negatively regulate expression of biofilm factors. The MafR (Mga/AtxA-like *faecalis* regulator) regulator of *E. faecalis* influences transcription of genes related to carbon source utilization and positively influences the transcription of genes during the growth

of *E. faecalis* in blood and urine. Although a *mafR* deletion did not result in attenuated colonization of the peritoneal cavity, a reduction of IL-6 and neutrophil infiltration in the peritoneal fluid implied that MafR modulates the host inflammatory response during this infection.⁹⁵

It is worth highlighting that some studies have used the mouse model of peritonitis to study *E. faecalis* virulence using an *in vivo-in vitro* infection model. Infections are performed as described in the *in vivo* model but peritoneal macrophages are harvested from peritoneal lavage fluid 4 h following infection.^{51,96} Giard and colleagues showed that the *E. faecalis* Ers (Enterococcal regulator of survival) mutant strain did not survive within murine peritoneal macrophages and were completely eliminated by 48 hpi.⁹⁶ Ers is a member of the Crp/Fnr family and showed 69% amino acid similarity to Srv, a PrfA-like regulator of *S. pyogenes* implicated in virulence. The Ers protein is important for survival within macrophages, in relation to oxidative challenge in *E. faecalis*.⁹⁷ Mice infected via peritoneal injection with Δ *Ers* survived for up to 100 hpi whereas mice infected with WT survived for only 70 hpi.⁹⁸ *E. faecalis* expresses oxidative stress mechanisms such as *hypR*,⁹⁹ *tpx*,¹⁰⁰ *spx*,¹⁰¹ *msrA*,¹⁰² and *msrB*¹⁰² that allow them to survive the intracellular environment of macrophages. The oxidative stress response transcriptional regulator HypR (hydrogen peroxide regulator) protects *E. faecalis* from oxidative challenge caused by hydrogen peroxide.⁹⁹ HypR directly controls the transcription of *hypR* itself, the *ahpCF* (alkyl hydroperoxide reductase) operon as well as *tpx* (thiol peroxidase). AhpC is a peroxide-reducing protein and is representative of a very large ubiquitous family of cysteine-based peroxidases, now designated as peroxiredoxins. AhpF is a flavoprotein and acts as the AhpC reductase.¹⁰³ Tpx, also a member of the peroxiredoxin family, plays an important role in protection against the oxidative burst produced by mouse peritoneal macrophages.¹⁰⁰ Besides intracellular macrophage survival, HprR and Tpx both contribute to virulence by causing lethality in peritoneally infected mice.^{99,100} The major global stress regulator Spx of *E. faecalis* is highly conserved among low-GC Gram-positive bacteria and was first identified as a suppressor of ClpP and ClpX phenotypes in *Bacillus subtilis*.¹⁰⁴ It promotes colonization of the peritoneum and spleen, contributing to mortality in a mouse model of foreign body-associated peritonitis.¹⁰¹ During the host immune response to an infection, *E. faecalis* that are engulfed by phagocytes must deal with reactive oxygen species within phagosomes and neutralize the environment for survival. *E. faecalis* survives by biosynthesizing superoxide dismutases¹⁰⁵ and peroxidases,¹⁰⁰ upregulating oxidative stress response pathways,⁹⁹ or by using oxidative repair

strategies.¹⁰² Methionine sulfoxide reductases A and B (encoded by *msrA* and *msrB*) are antioxidant repair enzymes synthesized to reduce oxidized methionine residues (MetSO) to methionine.¹⁰² Improved killing of *E. faecalis* $\Delta msrA\Delta msrB$, compared with $\Delta msrA$ or $\Delta msrB$, by activated mouse peritoneal macrophages demonstrates the essentiality of both *msrAB* for surviving phagocytosis.¹⁰² The PerR (peroxide regulator) of *B. subtilis* functions as a transcriptional regulator involved in oxidative stress tolerance and homologs have been found in other Gram-positive bacteria including *E. faecalis*.¹⁰⁶ PerR exerts weak regulation on oxidative response mechanisms and does not play an important role in *E. faecalis* survival within macrophages.¹⁰⁶ In the mouse model of peritonitis, 60% of mice survived infection with *E. faecalis* $\Delta perR$ at 70 hpi compared with 0% survival after WT infection.¹⁰⁶ The Enterococcal leucine-rich operon (*elrA-E*) encodes proteins that interact with host cells and is positively regulated by an upstream *elrR* gene product.^{107,108} During mouse peritonitis infection, *E. faecalis* $\Delta elrR\Delta elrA$ was attenuated the most compared with $\Delta elrA$ followed by $\Delta elrR$ ($\Delta elrR\Delta elrA > \Delta elrA > \Delta elrR$), displaying reduced dissemination into liver and spleen and decreased IL-6 pro-inflammatory cytokine production in peritoneal fluid.^{82,107,108} Deletion of *elrA* did not have any impact on macrophage phagocytosis but intracellular survival decreased at 24 hpi.¹⁰⁷ Overexpression of Elr inhibits adhesion and internalization into macrophages and increases killing of mice infected intraperitoneally, representing a mechanism for evading phagocytosis while promoting disease pathogenesis.⁸² Altogether, *elrR* not only regulates *elrA* expression, as seen with the additive attenuation of virulence by $\Delta elrR\Delta elrA$, but ElrB-E may contribute to virulence even in the absence of *elrA*.¹⁰⁸ Further work is required to define the role of combined or individual ElrB-E proteins in host pathogen interactions.

Bacterial polysaccharides confer adhesion, invasion, resistance to phagocytosis, and invoke host inflammatory responses.^{84,109} *E. faecalis* Enterococcal polysaccharide antigen (*epa*) encodes a locus of 18 genes (*epaA-R*) responsible for polysaccharide biosynthesis.¹⁰⁹ Each mutant of *E. faecalis* *epaB*, *epaE*, *epaM* and *epaN* was attenuated in a mouse peritonitis model by assessing survival rates at 100 hpi.^{84,109} Future studies to understand the implication of *Epa* in host pathogen interaction should include peritoneal macrophage survival assays as well as assessing infected tissue burden to determine dissemination. Cytokine profiling may also provide information about immunogenic properties of these proteins during infection.

The host immune system functions to recognize and activate responses to foreign molecules associated with invading bacteria, commonly termed as pathogen-associated molecular patterns (PAMPs). PAMPs from Gram-positive bacteria including peptidoglycan, lipoproteins, bacterial DNA, and LTA activate host immune defenses via the Toll-like receptor (TLR) pathway.⁸³ *E. faecalis* defective in *bgsA* does not synthesize the major cell membrane glycolipid diglycosyldiacylglycerol (DGlcDAG), resulting in a higher lipoprotein content in the cell membrane.⁸³ Mice challenged with $\Delta bgsA$ displayed increased lethality without a difference in bacterial burden (in blood, kidney and peritoneal lavage fluid) compared with WT.⁸³ Induction of proinflammatory cytokines (TNF- α and IL-6) and chemokine MIP-2, accompanied with increased TNF- α concentrations and increased leukocyte influx into the peritoneal lavage fluid, are indicative of an acute innate response detected in the plasma of infected mice 1 h following infection by $\Delta bgsA$.⁸³ Absence of *bgsA* enhances kidney colonization in the mouse model of UTI and reduces colonization in the rat model of IE.^{58,110} Overall, *E. faecalis* BgsA maintains lipoprotein levels on the cell membrane and subverts host responses by inhibiting immune activation *in vivo*.⁸³

Glucose starvation of *E. faecalis* induces general stress protein Gls24 expression, causing high mortality in the mouse peritonitis model.^{68,69} Loss of *gls24* resulted in a reduced LD₅₀.⁶⁸ Antibody protection using anti-Gls24 rabbit serum was achieved by immunizing mice before and during infection. Therefore, Gls24 is expressed during the mouse model of peritonitis and is a potential target for immunotherapy. Homologs of *E. faecalis* *gls24* and *glsB* present in *E. faecium* (*gls24*-like and *glsB*-like) displayed redundancy and had no role in virulence when inactivated individually, although disruption of both loci reduced mortality of mice during peritonitis.¹¹¹

E. faecium clinical strains belonging to clonal cluster 17 isolated in the US and Colombia harbor a large plasmid, pHyl_{Efm} (> 145 kb) that transfers readily from clinical strains to *E. faecium* hosts via conjugation and which promotes intestinal colonization in a mouse GI model.⁷ Infection with a strain that acquired pHyl_{Efm}, encoding *hyl_{Efm}*, results in increased mortality in a mouse peritonitis model.^{7,8} The *hyl_{Efm}* gene encodes a putative glycosyl hydrolase predicted to be a virulence determinant.¹¹² Strains bearing a deletion of the *hyl_{Efm}* region on the pHyl_{Efm} plasmid (pHyl_{Efm}TX16 Δ 7,534) are attenuated in peritonitis.¹¹² Complementation of *hyl_{Efm}* or *hyl_{Efm}* plus its downstream gene did not restore virulence, indicating that *hyl* alone nor in combination with its downstream gene are responsible for peritonitis virulence.¹¹² Therefore, the precise genes encoded on

pHyl_{EfmTX16} that mediate virulence in murine peritonitis remain unknown.

Bloodstream and systemic infection

Bloodstream infections are the 10th leading cause of death in the US. Incidence of bloodstream infection ranges from 1% in ICU patients to 36% in bone-marrow transplant patients.¹¹³ Bloodstream infections caused by Gram-positive bacteria are highly prevalent and 45% are Enterococci-associated.^{114,115} Bloodstream and systemic infections are primarily modeled using mice due to the ease of handling small animals. Mice are infected by administering the bacterial inoculum via IV injection into the tail vein; and at the time of sacrifice, bacteremia is determined by bacterial burden in the blood, whereas systemic infection or bacterial translocation is assessed by CFU in the liver, kidneys and spleen.

Enterococcal survival and *in vivo* colonization rely on stress response mechanisms such as *E. faecalis* sigma factor SigV that regulates gene expression in response to stress conditions such as heat, acid, ethanol and lysozyme resistance.^{116,117} *In vitro*, *E. faecalis* O-acetyl transferase (*oatA*) and D-alanylation of LTA (*dltA*) both had additive effects to lysozyme resistance, while the absence of *sigV*, *oatA* and *dltA* results in greater lysozyme susceptibility. Absence of *sigV* during systemic infection in mice resulted in attenuation of bacterial translocation, reducing colonization of kidneys and livers, while $\Delta dltA$ and Δoat did not.¹¹⁶ A slight additive effect in attenuation was observed in Δoat -*dltA*-*sigV*. Oxidative stress mechanisms mediated by *msrA* and *msrB* mentioned above for peritonitis have also been assessed in the context of systemic infections.¹⁰² In the systemic infection model, *E. faecalis* attenuation in kidneys and liver were observed for single and double mutants with slightly more pronounced attenuation in livers for the double mutant. The greater attenuation in livers may be associated with higher oxidative stress in that infected organ environment, resulting in lower bacterial burden in the absence of oxidative stress response pathways. In addition, extracytoplasmic foldases EF0685 and EF1534 of *E. faecalis* are peptidylprolyl *cis/trans* isomerases (PPIases) predicted to participate in folding extracellular proteins and the absence of either gene results in sensitivity to high salts, resistance to quinolones, ampicillin susceptibility, resistance to oxidative stress, and attenuated persistence in kidneys of mice displaying bacteremia.¹¹⁸ Moreover, adhesion to Caco-2/TC7 is not altered, although cytotoxicity is reduced only in the $\Delta ef0685$ mutant.¹¹⁸ Absence of *ef0685* or *ef1534* reduced kidney, but not liver, colonization in the mouse model of bacteremia.¹¹⁸ Lastly, nutrient adaptation is crucial for *E. faecalis*

survival in the host environment. Muller and colleagues showed induction of metabolism-related genes during peritonitis. Genes associated with glycerol metabolism were examined in the systemic mouse infection model to assess organ colonization.⁸⁷ The *E. faecalis* $\Delta gldA\Delta glpK$ double mutant, but not single gene mutants, were attenuated for kidney and liver colonization in mice at 7 dpi, suggesting that *E. faecalis* requires the activation of glycerol metabolism during bacteremia.⁸⁷

QS plays an important role in detecting signaling molecules and contributes to density-dependent virulence traits of pathogenic bacteria during adaptation to the environment. Signaling molecule autoinducer-2 (AI-2) disperses *E. faecalis* V583 Δ ABC (a derivative of V583 cured of plasmids A, B and C) biofilm and upregulates prophage 5-related genes in *E. faecalis*.¹¹⁹ A probiotic *E. faecalis* strain exposed to culture supernatants from *E. faecalis* V583 Δ ABC generated virulent transduced strains, inducing TNF- α release in macrophages, increased Caco-2 cell adherence, and virulence in a mouse bacteremia model and rat endocarditis model.¹¹⁹ This finding suggests that virulence genes can be shared through phage-mediated transfer, and that AI-2-mediated dispersal may generate a population with increased virulence. Ciprofloxacin-mediated phage release is also relevant in the GI environment which may be relevant after antibiotic treatment. Antibiotic-mediated phage transduction generates virulent Enterococci from commensal strains and could explain commensal Enterococci transition into opportunistic pathogens within the GI tract. Other virulence factors that demonstrate a role in more than one *in vivo* infection model include BgsA and BgsB which mediate kidney colonization of the mouse model of UTI, colonization of endocarditic lesions during IE in rats, and virulence in the mouse model of bacteremia.^{58,110,120,121} Mutants deficient in either gene are impaired in colonization and more readily cleared from the bloodstream.^{120,121} Both gene products are involved in LTA biosynthesis by synthesising major cell membrane glycolipids; $\Delta bgsA$ is deficient in DGlcDAG while $\Delta bgsB$ is deficient of both monoglucosyldiacylglycerol (MGlcDAG) and DGlcDAG.^{120,121} BgsA and BgsB do not compensate each other, and MGlcDAG alone is insufficient for complementing $\Delta bgsA$ virulence defects.^{120,121} BgsA and BgsB are potential drug targets to induce cell membrane instability for treatment of Enterococcal infections. The *E. faecalis* transcriptional regulator SlyA suppresses virulence in a mouse model of bacteremia and survival within peritoneal macrophages.¹²² SlyA regulates more than 100 genes, and absence of this protein could influence virulence expression. Increased virulence of $\Delta slyA$ indicates a role in mediating transition between commensal to pathogenic

state. Furthermore, post-transcriptional processing by the *E. faecalis* RNA-binding protein CspR (cold shock protein RNA binding protein) mediates persistence within murine peritoneal macrophages and kidneys of systemically infected mice.¹²³

It was previously shown that *S. aureus* can express lactate dehydrogenase (*ldh-1*) to withstand nitrosative stress, establishing a relationship between lactate metabolism and oxidative stress, which confers resistance of *S. aureus* to host innate immune defenses. Rana and colleagues found that *E. faecalis* lactate dehydrogenase gene paralogs (*ldh-1* and *ldh-2*) protect *E. faecalis* against environmental stresses and also contributes to virulence in the mouse model of bacteremia, exhibiting persistence in liver and kidney colonization.¹²⁴ Gene redundancy is observed *in vivo* where inactivation of *ldh-1* or *ldh-2* does not alter virulence, whereas the double mutant is attenuated.¹²⁴ The *ldh-1* gene, but not *ldh-2*, was essential in survival against stress conditions tested *in vitro*.¹²⁴ However, LDH-1-mediated survival of *E. faecalis* under hydrogen peroxide stress should be recapitulated in a mouse peritoneal macrophage assay or a mouse model of chronic wounds. As impaired wound healing correlates with an oxidatively stressed microenvironment with enhanced concentrations of reactive oxygen and nitrogen species, this could be appropriate for assessing LDH-1 function.¹²⁵

Similar to studies with *E. faecalis*, the pathogenesis of *E. faecium* has also been examined during mouse systemic infection. The global regulator AsrR (antibiotic and stress response regulator) represses virulence during systemic infection in mice by reducing colonization of kidneys and livers. AsrR is a stress-sensor that is inactive in the presence of hydrogen peroxide. The AsrR regulon is composed of 181 genes involved in pathogenesis, antibiotic and antimicrobial peptide resistance, oxidative stress, biofilm formation, adhesion to epithelial cells, and adaptive responses via AsrR-mediated deregulation of *uvr* and *mutS2* which promotes DNA mutation and increased transfer frequency of conjugative transposon Tn916, respectively.¹²⁶ AsrR represses virulence traits such as biofilm formation, adhesion to intestinal epithelial cells, and deletion of *asrR* leads to persistence in tissues at 7 dpi during mouse systemic infection.¹²⁶ The putative capsular polysaccharide biosynthesis protein (*capD*) is made up of 336 amino acids and it putatively catalyzes N-linked glycosylation.¹²⁷ Deletion of *capD* resulted in decreased bacterial burden in blood and livers. Esp of *E. faecium* is a key virulence factor for persistence in murine UTI and bacteremia, perhaps due to its ability to mediate immune evasion.^{128,129} *E. faecium* Esp also mediates kidney colonization.^{126,127,129} However, passive immunization using antibodies against *E. faecium* Esp do not protect mice from bacteremia, but may

prove to be effective during other infections where Esp is highly expressed.¹²⁹

Gastrointestinal infection and colitis

GI microbiota consisting of a consortium of bacterial species including Enterococci, *E. coli*, Streptococci, Lactobacilli, Bifidobacteria, *Bacteroides*, *Eubacterium*, and *Clostridium* plays an important role in disease prevention by mediating colonization resistance (CR), and dysbiosis of this consortium is associated with GI conditions ranging from cancer, obesity, malnutrition, diabetes and inflammatory bowel disease (IBD).¹³⁰ Modifications in dietary habit, health status or intake of medication influences the intestinal microbial composition.¹³⁰ IBD can result from (i) an aberrant mucosal immune response to gut commensals, (ii) dysbiosis alone, (iii) disruption of intestinal barrier function, or a combination of (ii) and (iii).¹³¹ In these situations, commensal Enterococci may transition into opportunistic pathogens to cause chronic IBD after acute *E. faecalis* infection, as reported in germ-free and IL-10 knockout mice but not in humans.¹³² Causes of other GI-related Enterococcal infections include bacterial translocation into organs and systemic infection from complications following colorectal surgery,^{133,134} and development of colorectal cancer from high concentrations of extracellular superoxide emitted by *E. faecalis* in the colon.^{135,136}

Mice are the most frequently used animal model to assess Enterococcal virulence in the GI tract, and colonization is initiated by administering Enterococci via oral gavage or in the drinking water.^{8,22,137-144} In contrast, rats have been used for studying post-surgical complication-induced Enterococcus infections, as their larger size is more amenable for surgical manipulation in these studies.^{133,134} Infection model systems include immunocompromised mice (e.g., germ-free, gnotobiotic or IL-10 knockout), as well as the administration of antibiotics (e.g., streptomycin, ceftriaxone, clindamycin) to conventional mice before infection.^{140,142} The utility of germ-free mice in mimicking any natural condition in humans is debatable. In mice, streptomycin perturbs many indigenous microbiota including *E. coli*, Enterococci, Streptococci, Lactobacilli and Bifidobacteria while *Bacteroides* spp, *Eubacterium*, and *Clostridium* are not affected.¹⁴¹ Ceftriaxone antagonizes *E. coli*, Lactobacilli, Bifidobacteria, Clostridia and *Bacteroides* spp, while clindamycin decreases Streptococci and anaerobic bacteria in humans; neither ceftriaxone nor clindamycin inhibit Enterococci.^{145,146}

Bioluminescence imaging (BLI) is a powerful tool for monitoring disease spread in intact animals during infection. Colonization by *E. faecalis* variants expressing the *luxABCDE* cassette under the control of either the

cytolysin or gelatinase promoter (detected using BLI) in a streptomycin pre-treated GI mouse model suggested greater expression in the lower GI tract (i.e., cecum and colon) than the upper GI tract measured by BL intensity.¹⁴¹ Cytolysin promoter activation, but not gelatinase promoter activation, was detected at high levels during GI infection, suggesting that expression of the former may be important for successful intestinal colonization.¹⁴¹ Drawbacks of using BLI in this setting include detection limit (10^5 CFU/fecal pellet), lower BL signal in intact animals compared with dissected single organs, low signal emission from tissues/cavities with limited oxygen concentrations, and plasmid stability. A more complete understanding of these virulence factors in GI colonization will require more sensitive methods of expression analyses.

Vancomycin-resistant Enterococci (VRE) contributes to a third of multidrug-resistant Enterococcal systemic infections in clinical settings.^{22,147} In healthcare settings, patients pre-colonized with VRE can experience VRE overgrowth following antibiotic-induced disruption of the gut microbiota, giving rise to translocation of VRE from areas of high bacterial load in the gut to the bloodstream.^{22,137} To prevent translocation of VRE to the blood, much research have focused on understanding the effects of antibiotic therapy on gut microbiota. In healthy individuals, resident bacteria of the GI tract stimulates the secretion of RegIII γ , a C-type lectin secreted by intestinal epithelial and Paneth cells that is important for eliminating Gram-positive bacteria, via the TLR-MyD88 signaling pathway.^{148,149} Antibiotic treatment results in RegIII γ downregulation.¹⁴³ Antibiotic disruption of gut microbiota and mucosal innate immune response via RegIII γ alteration allows VRE (*E. faecium*) proliferation by compromising CR.¹⁴³ Administrating resiquimod (R848), a synthetic ligand for TLR7-mediated induction of IL-22 and IL-23, to reactivate RegIII γ secretion or introducing obligate anaerobic commensal bacteria containing *Barnesiella* species can re-establish CR against *E. faecium* (the presence of *Barnesiella* correlates with prevention of *E. faecium* gut colonization and bacteremia).^{22,144} To gain a competitive edge for Enterococci in the intestinal niche, both *E. faecalis* and *E. faecium* are particularly receptive to taking up transferable elements, resulting in evolved strains gaining bacteriocin, pheromone, antibiotic resistance, and other virulence traits.^{8,137,147} Commensal *E. faecalis* that harbor the pPD1 plasmid can express bacteriocin 21 can displace other *E. faecalis* colonizing the gut.¹³⁷ Gilmore and colleagues found that commensal *E. faecalis* of the intestinal environment secrete a heptapeptide pheromone that can cause lethal crosstalk between inherited mobile elements within *E. faecalis* V583, mediating CR by preventing

incompatible Enterococci from inhabiting the GI tract.¹⁴⁷ These studies suggest multiple therapeutic strategies to combat intestinal colonization of resistant Enterococci, from introduction of probiotic *E. faecalis* harboring non-conjugative plasmids encoding bacteriocins, transferable colonizing ability among *E. faecium* recipient strains, pheromone-induced killing of multidrug-resistant *E. faecalis* strains, administrating resiquimod to reactivate RegIII γ secretion, and introducing commensals belonging to the *Barnesiella* genus to make use of the host microbiota or host immune defenses to limit Enterococcal proliferation.^{8,137,143,144,147}

Antibiotic-mediated depletion of gut microbiota is often used in intestinal colonization studies to eliminate susceptible bacteria, in turn promoting colonization and outgrowth of resistant organisms. A mannose family PTS highly prevalent in clinical isolates of *E. faecium* aids in intestinal colonization in ceftriaxone-treated specific-pathogen-free (SPF) mice.¹⁴² *ptsD* is predicted to encode the enzyme IID subunit of the PTS, and the absence of *ptsD* significantly impairs *E. faecium* colonization of the murine intestinal tract. *In vitro* growth of *E. faecium* Δ *ptsD* was not affected when tested under a range of different conditions (65 different carbohydrate substrates, sensitivity to cefoxitin and ceftriaxone, or in BHI or BHI-supplemented with cefoxitin).¹⁴² However, competitive infection in the mouse model of intestinal colonization revealed fewer mutant bacteria load in feces, small intestines, cecum, and colon of mice at 10 dpi compared with WT.¹⁴² The carbohydrate substrate for PtsD in the GI tract was not identified; however, identification of this carbohydrate may inform future therapeutic strategies to manage infections associated with drug-resistant *E. faecium*. The *E. faecalis* genome also encodes a one-component signaling system encoded by the *prkC* gene that maintains cell integrity/morphology; resistance to cell wall-targeting antibiotics, sodium dodecyl sulfate, and bile-related components *in vitro*; as well as contributing to cecum persistence after intestinal colonization of conventional mice.¹³⁸ The selective advantage for *E. faecalis* survival and persistence in the intestine is likely PrkC-mediated bile resistance.¹³⁸ Using an intestinal model of clindamycin-treated mice that promotes Enterococci overgrowth, *E. faecalis* glycosyltransferase (*epaX*) encoded within the *epa* locus resulted in attenuated intestinal colonization.¹⁴⁰ Loss of EpaX function impairs sugar incorporation into cell wall polysaccharides, resulting in alteration of cell wall architecture and sensitivity to sodium deoxycholate (bile salts).¹⁴⁰ *In vivo* functionality of EpaX is crucial for intestinal colonization,¹⁴⁰ and the *epa* locus (*epaA-epaR*) also mediates virulence in a murine peritonitis model.¹⁰⁹ Colitis is an outcome of IBD that causes serious medical implications

in immunocompromised patients. Recapitulating this human infection, *E. faecalis*-associated colitis in IL-10^{-/-} mice showed that GelE partially compromises the intestinal barrier through E-cadherin degradation.¹³² The absence of the virulence factors glucosyltransferase (*epaB*) and prolipoprotein diacylglyceryl transferase (*lgt*) did not impact on GelE activity, implying that EpaB and Lgt do not cause colitis via E-cadherin cleavage in the IL-10^{-/-} mouse model.¹³² Although they did not differ in colonization in the IL-10^{-/-} mouse model of colitis, *epaB* and *lgt* mutants are defective for causing colitis, with a stronger attenuation seen in the absence of *lgt*. The absence of *lgt* is independent of colonic mucus penetration; however, innate immune cell activation is dependent on the presence of cell surface-associated lipoproteins. In contrast, absence of *epaB* leads to impaired penetration of the intestinal mucus layer and EpaB is less immunogenic than Lgt, mediating partial intestinal inflammation in the host.¹³² While *E. faecalis* and *E. faecium* Esp do not appear to be essential for intestinal colonization in mice,^{139,150} EbrB is important since *E. faecium* Δ *ebrB* displays attenuated persistence in feces, small intestines, cecum of infected mice, as well as reduced biofilms *in vitro*.⁷⁹ RT-qPCR of *in vitro* grown *E. faecium* and its isogenic *ebrB* mutant demonstrates EbrB-mediated regulation of 3 genes downstream of *esp*, suggesting EbrB-mediated *esp* expression may indirectly involve their regulation.⁷⁹

Bacterial translocation due to post-surgical complication from surgical intervention often results in bacteremia, sepsis and in severe cases, death.¹³⁴ Colorectal anastomotic leak is the most significant post-surgical complication of intestinal anastomosis (reconnection of intestines following removal of an intestinal segment) where intestinal content leakage leads to mortality and morbidity. High collagenase-producing *E. faecalis* strains were associated with anastomotic leak via GelE- and SprE-mediated intestinal collagen-depletion followed by activation of tissue matrix metalloproteinase 9 (MMP9) that cleaves host ECM.¹³³ These models mimic complications arising from GI disorders (such as cirrhosis, hepatic ischemia and intestinal stasis or IBD) causing gut-associated bacteria to translocate into systemic organs and tissues.

Iron-rich diets have been correlated with increased incidence of colorectal cancer due to exposure to high iron leading to accelerated catalysis of superoxide to hydroxyl radical, inducing oxidative stress on the colon epithelium.¹⁵¹ Accelerated oxidative stress from high concentrations of hydrogen peroxide and reactive oxygen species can arise via the generation of extracellular superoxide by intestinal bacteria.^{135,136} Most intestinal bacteria produce small amounts of

intracellular superoxide but Enterococci produce superoxide extracellularly.¹⁵² Menaquinones are involved in superoxide production and *E. faecalis* appear unique among Enterococci and Streptococci in their ability to produce both menaquinone and extracellular superoxide.¹⁵³ Demethylmenaquinone (DMK) synthesis and superoxide production are dependent on the function of the *menBEDF* operon whereby inactivation of the *menB* gene in *E. faecalis* reduced hydroxyl radical levels in the rat intestinal colonization model.¹³⁶ High oxidative stress on the colonic epithelium is related to genomic instability of intestinal tumor cells, as more than 80% of sporadic colon cancers were due to genetic mutations.¹³⁵ Further studies are required to establish the association between oxidative stress within the intestinal tract milieu with colorectal cancer.

Urinary tract infection and catheter-associated urinary tract infection

Women are at a higher risk of UTI for several reasons, including an anatomically shorter urethra and proximity of the urethral opening to the microbial communities of the GI and vaginal tracts. In addition, age and sexual activities can increase a woman's susceptibility to UTI.^{154,155} UTI is one of the most common bacterial infection and affects individuals from all age groups, accounting for approximately 8.1 million doctor visits per year.¹⁵⁶ According to the Centers for Disease Control and Prevention, an estimated 93,300 UTIs were reported in 2011 and the majority of these cases were associated with instrumentation of the UT.¹⁵⁷ A variety of uropathogens can cause both UTI and CAUTI. Uropathogens that cause UTI include UPEC (75%) *Klebsiella oxytoca* (6%), *Staphylococcus saprophyticus* (6%), *E. faecalis* (5%), group B *Streptococcus* (GBS) (3%), *Proteus mirabilis* (2%), *Pseudomonas aeruginosa* (1%), *S. aureus* (1%) and *Candida* spp (1%). Organisms associated with CAUTI include UPEC (65%), Enterococcus spp (11%), *K. oxytoca* (8%), *Candida* spp (7%), *S. aureus* (3%), *P. mirabilis* (2%), *P. aeruginosa* (2%) and GBS (2%).¹⁵⁴

Urinary tract infection

To investigate Enterococcal uropathogenicity, variations of a mouse model of ascending UTI for studying Gram-negative UTI has been used. Typically, female mice are inoculated transurethrally with 10⁵–10⁸ bacteria in a 50–200 μ l volume, the higher volumes giving rise to vesicoureteral reflux (VUR) of bacteria into the kidney.^{70,71,73,74,102,128,158-164} Among vertebrate models, mice have been favored for studying UTI as they are the smallest animal to possess similar anatomy and

immunological responses to humans for an *in vivo* recapitulation of the infection.⁴

Since 2000, several virulence factors that mediate UTI by *E. faecalis* have been reported. Individual deletion mutants of the following genes result in attenuation compared with isogenic parental strains: *esp*,¹⁶¹ *srtC*,¹⁶⁴ *ebpA* and *ebpC*,^{74,163} *ace*,^{71,160} *epaB*,¹⁵⁸ *msrA* and *msrB*,¹⁰² *sigV*,¹¹⁶ *efbA*,⁷³ and *grvR/etaR*.⁷⁰ Unlike the *srtC* mutant, deletion of *srtA* did not indicate a strong role in colonization of the UT. Moreover, a double mutant of both *srtA* and *srtC* showed no further reduction of colonization when compared with a single *srtC* deletion mutant suggesting that polymerized pili are essential for UTI, whether they remain retained on the membrane in a *srtA* mutant or become wall-associated in WT strains.^{164,165}

In contrast, the expression of several factors appears to limit UTI colonization, including the *dltA* and *oatA*, such that their deletion promotes colonization of the UT.^{116,166} *DltA* was found to suppress UT colonization in one study, while no contribution was found in another. Reasons for these disparate observations may include differing expression of *dltA* in different *E. faecalis* strains used as well as protocol variations.^{116,166} The actual role of D-alanylated LTA within the bladder or with urothelial cell remains to be determined. In addition, *E. faecalis* deficient in either *bgsA* or *bgsB* also display enhanced colonization of mouse kidneys.¹¹⁰

A noteworthy aspect of this model is that *E. faecalis* has greater tropism for the kidneys, making it useful to study factors implicated in Enterococcal-mediated pyelonephritis.^{4,73,110,162} Inoculum volumes of 100–200 μ l are used to induce VUR; both Kau *et al.* and Singh *et al.* reported inconsistent recovery of *E. faecalis* from infected organs of mice administered with a 50 μ l inoculum.^{162,163} Therefore, studies of virulence factors where no contribution to upper or lower UT colonization was found, such as for *E. faecalis* AS and *E. faecalis* Enterococcal binding substance (Ebs) should be revisited by increasing inoculum volumes.¹⁶⁷

A variation of this model, designed to recapitulate UTI pathogenesis in Type I diabetics, was described by Rosen and colleagues in which pancreatic islet cells are depleted by the β -cell toxin streptozocin in C57BL/6 mice. Streptozocin-treated diabetic mice are more susceptible to infection by *E. faecalis* which is consistent with higher prevalence of Enterococcal-associated UTI in diabetic women compared with nondiabetics.¹⁶⁸

Although *E. faecium* is commonly isolated from nosocomial UTIs, very few UTI studies have been performed in comparison to *E. faecalis*.^{76,159} Like *E. faecalis* UTI, *E. faecium* surface proteins Esp and EmpABC pili (previously EbpABC_{fm}) mediate colonization of the mouse

UT,^{76,128,159} and *E. faecium* also display kidney tropism.¹⁵⁹

Catheter-associated urinary tract infection

Enterococcal species are among the top 3 most common causes of HAI,¹⁶⁹ and CAUTI accounts for more than 40% of all nosocomial infections arising from acute-care hospitals and extended-care facilities.^{169,170} Among CAUTI, Enterococcal species are the second most common bacterial species detected.⁶ CAUTI in mice is achieved by first transurethraly placing a 0.5 cm piece of silicone tubing into the bladder, before subsequent transurethral inoculation of bacteria in a 50 μ l volume harboring 10⁴–10⁷ CFU.¹⁷¹ In this model, the catheter serves as a substrate for biofilm formation by uropathogens and acts as a reservoir for continual re-seeding of the infection.

Sterile catheterization of the bladder elicits a marked increase in mammalian gene expression associated with defense responses and cellular migration, production of cytokines and chemoattractants that induce immune cell maturation, and recruitment of neutrophils and monocytes into the bladder.¹⁷² Infection at the same time as catheterization, before catheter-dependent sterile inflammation and immune infiltration, occurs at an *E. faecalis* ID₅₀ of 10⁴ CFU.¹⁷² In contrast, infection after catheterization and in the presence of catheter-mediated inflammation requires a 3 log-fold higher ID to establish UTI.^{172,173} Furthermore, major histological changes including edema in the mouse bladder and secretion of host fibrinogen occur in response to a urinary catheter.^{173,174} Fibrinogen released during the inflammatory response accumulates on the catheter, mediating adhesion of *E. faecalis* via EbpA.¹⁷⁴ Microscopic examination of urinary catheters from hospitalized human subjects with indwelling catheters ranging from 1 h to 59 d demonstrated that fibrinogen accumulation on urinary catheters is associated with dwell times, independent of catheter material.¹⁷⁵ Moreover, *E. faecalis* utilizes fibrinogen as a carbon source to replicate and form catheter-associated biofilms *in vitro* in female human urine.¹⁷⁴ *E. faecalis* biofilms formed on the catheter surface contribute to persistence in the bladder because they are recalcitrant to immune clearance, shear force from urination, and antibiotic treatment.^{154,171,173,176} *E. faecalis* CAUTI is preceded by adhesion to the catheter via *ebpA*,^{174,176} *ebpB*,¹⁷⁶ *ahrC*,³⁸ *eep*,³⁸ *srtC*,¹⁷⁶ and *srtA*.¹⁷¹ Importantly, an EbpA-based vaccine can limit or prevent *E. faecalis*-associated CAUTI by inhibiting the interaction between EbpA and fibrinogen.¹⁷⁴ Therefore, this model has proved to be a valuable tool for the identification of virulence determinants and subsequent development of targeted therapeutics against Enterococcal infections.

Despite the many advantages of using mice to study the role of *E. faecalis* virulence factors in CAUTI, one limitation of this experimental model does not recapitulate typical human urinary catheters, which extend from the bladder to the urethral opening. However, this can be achieved in rats by inserting a 24 gauge polyurethane IV catheter (0.8–1 cm) into the urethra whereby one end of the catheter tip is located inside of the bladder and the distal end located outside of the urethral meatus, with the catheter secured in place by suturing it to the vaginal opening and hiding the knot inside the vagina.¹⁷⁷ Although this model has not been used to study the role of *E. faecalis* virulence, it would be clinically relevant for studies of bacterial ascension during CAUTI.

Endophthalmitis

Endophthalmitis is a vision-threatening nosocomial infection emerging from postoperative procedures in hospitals. Enterococci are only rarely associated with endophthalmitis, accounting for only 1.23% of acute endophthalmitis cases.¹⁷⁸ Using Enterococci as the infectious agent in endophthalmitis models is deemed useful in elucidating Enterococcal virulence factors contributing to endophthalmitis, with added advantages of assessing retinal function without sacrificing animals and correlating outcomes with disease progression.⁴ Rabbits and rats have been used for modeling Gram-positive bacterial endophthalmitis but rabbits are preferred due to technical reasons.^{4,129} In the rabbit model, New Zealand White rabbits receive general anesthesia and their eyes are dilated to expose the injection site. Subsequently, the ocular surface and its surrounding tissues are disinfected before aspirating fluid from the anterior chamber below the cornea to relieve intraocular pressure. *E. faecalis* are introduced via injection into the mid vitreous cavity of the eye 3 mm behind the limbus, avoiding damage to the lens.^{54,55,178}

In 2 separate studies, individual mutants of *fsrB*, *gelE*, or *sprE*, and a double mutant of *gelE* and *sprE* were compared with WT during endophthalmitis in rabbits.^{54,55} Virulence was evaluated by loss of retinal function and the study showed that $\Delta fsrB$ was the most attenuated followed by $\Delta gelE \Delta sprE$. Single mutant strains $\Delta gelE$ or $\Delta sprE$ were as virulent as their WT counterparts, suggesting redundancy of these genes in the pathogenesis of endophthalmitis.^{54,55} Severity of infection caused by the *fsrB* mutant was the lowest with retinal layers still intact and an absence of immune infiltration, whereas WT infected eyes suffered the most severe outcomes with compromised structural integrity and cellular infiltrate.^{54,55} Surprisingly, intraocular bacterial burdens for all deletion mutants were similar to WT levels suggesting

that these genes do not impact infectivity or fitness in this model but that disease severity is dependent on specific virulence traits.^{54,55} The contribution of FsrB to rabbit endophthalmitis is consistent with observations on the role of Fsr in other mammalian models, suggesting it is important for *E. faecalis* virulence in both local and systemic infections.^{51,53,56}

Foreign body subdermal abscess

To mimic abscess formation in the presence of a foreign material (e.g., a splinter), the first *in vivo* application of a RIVET system was performed by Frank and colleagues using a subdermal abscess model in rabbits to investigate *E. faecalis* adaptation to the host environment during infection.^{47,179} Subdermal chambers were created by aseptic implantation of a sterilized hollow, perforated polyethylene golf ball into a surgically created subcutaneous pocket on the flanks of anesthetized rabbits. During 6 weeks of post-surgical recovery, the perforated golf ball surface becomes encapsulated with fibrous tissue and filled with serous fluid containing immune cells.⁴⁷ To perform the infection, a small volume of serous fluid from the subdermal chamber is replaced with the same volume of bacteria. During the course of infection, *E. faecalis* co-exists with a stable immune cell population encompassing approximately 10% granulocytes (or PMNs) and 90% mononuclear cells (lymphocytes and monocytes), while being subjected to a changing host response from additional immune cells that enter the chamber during the course of infection.⁴⁷ This *in vivo* model is highly amenable to time course experiments since bacterial gene expression studies, metagenomic analyses during polymicrobial infections, and changes in host immune responses can be followed in the same animal.

Using this model, 249 putative promoters were identified that were induced during subdermal abscess infection by *E. faecalis* including promoters for genes encoding transport and binding proteins, energy metabolism, cell envelope, DNA metabolism, regulatory functions, protein fate, hypothetical proteins, and others. More than one-third of these promoters are predicted to generate antisense transcripts and this was the first report of *in vivo*-expressed antisense RNA in *E. faecalis*.⁴⁷ Overlapping putative virulence genes from the *in vivo* RIVET screen with genes associated with *in vitro* biofilm formation were subsequently validated in a rabbit IE model and showed that both the *eep* and *proB* mutants were both attenuated.⁴⁷ This work led Frank and colleagues to assess differential gene expression using microarray, whereby cellular adaptation was

induced at 2 hpi while cellular pathways required for growth and replication were downregulated at 8 hpi.¹⁷⁹ Downregulated genes were consistent with bacteria entering a stringent response state, similar to stress conditions such as nutrient deprivation and antibiotic treatment.¹⁷⁹ Upon activation of the stringent response, accumulation of the metabolite (p)ppGpp modulates transcription of stress-related genes, some of those identified at 8 hpi were consistent with genes reported from the transcriptome of *E. coli* response to amino acid starvation and *E. faecalis* exposure to mupirocin.¹⁷⁹ Subdermal chambers infected with deletion mutants in the bifunctional synthetase/hydrolase RSH (or RelA) and small synthetase RelQ, involved in stress responses and maintenance of (p)ppGpp levels, were evaluated for survival in subdermal chambers up to 96 hpi and both of $\Delta relQ$ and $\Delta rsh\Delta relQ$ were attenuated compared with WT. These findings suggest that persistence *in vivo* is dependent on intracellular (p)ppGpp concentrations rather than activation of stringent response.¹⁷⁹

Surgical site infection

SSI occurs in 2–5% of patients undergoing inpatient surgery, arising either at the incision site or in deeper tissue or surrounding organs at the incision site.²² SSIs are common in immunocompromised patients and are associated with up to 7–11 additional postoperative hospital-days, which carries a large economic burden.¹⁸⁰ Patients with SSIs are at 2–11 times higher risk of death compared with surgical patients without SSIs.²² Enterococci were the 2nd most commonly isolated organism reported for SSIs in the US between 2006–2007.^{6,181} A mouse cutaneous infection that models SSI and evaluates phagocyte-mediated bacterial clearance from the infection site was used to examine the role of *E. faecalis* capsular polysaccharides in SSIs.¹⁸² *cpsI*, encodes capsular polysaccharide I, is one of the genes in the *cps* locus necessary to generate the carbohydrate for capsule polysaccharide.¹⁸² At 4 dpi, persistence within draining abdominal lymph nodes of subcutaneously-injected mice are dependent on the *cpsI* capsule of *E. faecalis*.¹⁸²

Endodontic infection

Although an uncommon dental pathogen, *E. faecalis* has been isolated from persistent root canal and periapical infections.¹⁸³ The link between *E. faecalis* and periodontal disease has been investigated using change-mediated antigen technology (CMAT) and a canine experimental animal model of endodontic infection to identify proteins upregulated in *E. faecalis* during endodontic infection. CMAT involves generation of hyperimmune

antisera against *E. faecalis* harvested from infected clinical samples of pulp and endodontic infection from humans to identify *in vivo*-induced proteins or antigens during the course of infection. One of the limitations of this approach for human studies is the low number of *E. faecalis* cells that can be recovered. Hence, a beagle dog was used in a pilot study to screen for proteins expressed by *E. faecalis* specifically under *in vivo* conditions. In an anesthetized canine, perforations in the dental pulp tissue are made and *E. faecalis* is injected into the pulp cavity to induce endodontic infection with radiography monitoring. Infected premolars are extracted and hemisectioned, infected pulp and periapical tissues removed, and bacterial cell pellets are saved for hyperimmune antisera production in rabbits.¹⁸⁴ This study identified 16 *in vivo*-induced protein antigens involved in housekeeping functions, metabolism, and cellular processes. Potential virulence factors include a copper resistance protein that could confer resistance to metallic copper-coated antimicrobial surfaces and several putative membrane proteins of unknown function.¹⁸⁴ Given the lack of sensitivity in this pilot screen due to an underrepresentation of *E. faecalis* proteins thought to be induced *in vivo*, a more sensitive approach such as RNA-sequencing may better identify Enterococcal-related endodontic virulence factors in the future.

Zebrafish

While vertebrate models of Enterococcal infection have been informative with regards to pathogenic strategies, they are limited by the fact that they are not practical to large-scale studies and are costly. Over the past decade, Zebrafish (*Danio rerio*) have gained popularity as an alternative vertebrate model to study human bacterial pathogens due to their affordability and amenability to scaling up. In addition, due to their physical transparency, *in vivo* imaging of complex host-pathogen interactions, including developmental and dissemination studies can be easily performed *in vivo*.¹⁸⁵ Zebrafish are infected by inoculating bacteria into the bloodstream of embryos at varying hours post fertilization (hpf). The study of *E. faecalis* in zebrafish was first performed by Prajsnar and colleagues, where they found that 2 *E. faecalis* strains, OG1RF and V583, were virulent in zebrafish in a dose-dependent manner.¹⁸⁶ Mutants lacking virulence factors, *epaB*, or *gelE*, *sprE* and *fsrB* (Fsr-regulon) displayed attenuated virulence in the zebrafish larvae.¹⁸⁶ In another study, zebrafish were used in parallel with mice in a colitis model. They found that colonic heme oxygenase-1 (HO-1) protects mice from colitis by degrading heme and produces carbon monoxide as a by-product. The production of HO-1 enzyme can be

induced by enteric microbiota to enhance bactericidal activity of macrophages against *E. coli*, *E. faecalis*, and *S. typhimurium*.¹⁸⁷ Despite the numerous advantages that the zebrafish model can provide, due to high degree of evolutionary divergence, translating the host response to mammalian infection can be difficult. In addition, successive inbreeding often leads to infertility in the lower vertebrates, making it difficult to create truly inbred zebrafish lines.¹⁸⁸

Polymicrobial infections

The prevalence of polymicrobial *E. faecalis* infections highlights the need for more *in vivo* studies to recapitulate clinical scenarios. Risk factors for polymicrobial infection include extremes of age, being immunocompromised, having pre-existing health conditions, experiencing prolonged hospital stays (especially in ICUs), or undergoing surgical intervention.^{9,14,15,189,190} More often than not, the etiology of these infections and the virulence mechanisms involved are largely unknown and although clinical reports may associate polymicrobial infections with poor outcomes, studies by Lagnaf *et al.* and García-Granja *et al.* dispute this claim.^{9,15,19,191} Early Enterococcal polymicrobial infection studies found that co-infecting mice with *P. aeruginosa* and *E. faecalis* in the model of ascending UTI aggravates pyelonephritis and persistence of *P. aeruginosa* in the kidneys despite β -lactam antibiotic treatment during co-infection.¹⁹² An experimental polymicrobial wound infection in mice showed impaired wound healing at 8 dpi using a mixed inoculum of *E. faecalis*, *P. aeruginosa*, *S. aureus*, and *Finegoldia magna*. Polymicrobial biofilms from 4 day old wounds were less susceptible to bleach and gentamicin treatment compared with their monomicrobial counterparts.¹⁹³ It was also demonstrated recently that *E. faecalis* metabolic cues augment *E. coli* virulence and growth in a mouse model of polymicrobial wound infection.²⁷ Proximity to *E. faecalis*-within biofilms results in the induction of the *E. coli* siderophore enterobactin, enabling *E. coli* to overcome iron limitation *in vitro*. *E. faecalis*-mediated *E. coli* biofilm growth augmentation in a mixed species wound infection suggests a role for *E. faecalis* ornithine and *E. coli* enterobactin siderophore biosynthesis during polymicrobial wound infections.²⁷

Peritonitis-associated abscess formation is often a result of strong host immune response to implanted foreign bodies, postoperative procedures, or bacterial infection.^{47,194} Bacterial-induced intra-abdominal abscesses are usually polymicrobial, and *Bacteroides fragilis* is the most common anaerobe isolated from abscesses associated with intra-abdominal sepsis together with *Enterococcus* spp, *E. coli*, *S. aureus*, *Peptostreptococcus* spp,

Clostridia, and *Prevotella* spp.¹⁹⁴ While most peritonitis infections are modeled as monomicrobial infections, here we describe 2 reports of polymicrobial peritonitis infection studies, which are more representative of human infections. *B. fragilis* capsular polysaccharides strongly invoke immunologic responses to cause abscess formation in rats.¹⁹⁵ Abscess formation is mediated by T-cell activation and may be blocked by a CTLA4Ig fusion protein.⁸¹ Rats infected with *B. fragilis* alone, *Bacteroides distasonis* plus *E. faecium*, or *S. aureus* alone showed lower incidence rates of abscess formation when administered with CTLA4Ig, indicating that abscess formation is mediated by the activation of T-cells via the CD28-B7 pathway.⁸¹ Together with *in vitro* data, the pathway by which T-cells are activated during abscess formation was confirmed to be via CD28-B7-2.⁸¹ *E. faecium* expresses surface polysaccharides (zwitterionic bacterial polysaccharides; Zps) while *B. distasonis* does not; hence, it remains to be tested whether synergistic interactions in polymicrobial infections may be an outcome of abscess caused by *E. faecium* Zps.⁸¹ Recent emergence in glycopeptide antibiotic resistance (i.e., vancomycin) renders vancomycin-resistant *E. faecalis* infections difficult to treat. Vancomycin resistance is encoded by *vanA* or *vanB* genes and their influence on *E. faecalis* virulence is not known. A polymicrobial inoculum of *E. coli*, *B. fragilis*, plus each strain of *E. faecalis* (susceptible, VanA⁺ or VanB⁺) introduced in a rat peritonitis model demonstrated stronger colonization by *E. faecalis* strains at 24 and 72 hpi.⁸⁵ Immune cell infiltration and proinflammatory cytokine (IL-6 and TNF- α) levels in peritoneal fluid at 6 hpi were similar in all infections; this immune response has been suggested to be contributed by both *E. coli* and *B. fragilis*.⁸⁵ Nevertheless, increased serum α 1-acid glycoprotein concentrations at 72 hpi detected in vancomycin-resistant *E. faecalis* infections reflects inflammation and immunomodulation by resistant *E. faecalis*.⁸⁵

In the hospital setting, both pre-colonization by VRE and surgery are risk factors for VRE colonization and infection in critical care patients.^{14,189,190} Polymicrobial peritonitis is one of the common infections originating from a VRE colonized GI tract; that may or may not lead to poorer prognosis.^{189,196-203} Interestingly, mice colonized with VR *E. faecium* for 14 d in the GI tract followed by intestinal perforation (CLP; cecal ligation and puncture), suffered leakage of intestinal contents into the peritoneal cavity, but were able to accelerate bacterial clearance at 48 hours post-CLP compared with those not pre-colonized by VR *E. faecium*.²⁰⁴ This was accompanied by diminished peritoneal and plasma inflammatory response (TNF- α , IL-6 and MCP-1), and lower neutrophil-attracting and -activating chemokines (KC, MIP-2

and LIX) at 48 hours post-CLP in VR *E. faecium* colonized mice.²⁰⁴

Invertebrates

In the last decade, invertebrate models have been increasingly used to study Enterococcal infections and have been developed to complement mammalian host models. *C. elegans*, the Greater wax moth caterpillar *Galleria mellonella*, and *Drosophila melanogaster* provide advantages for pathogenesis studies because they are smaller in size, easy to maintain, economical, ethically expedient, and are generally inexpensive. Furthermore, as they are amenable to large-scale screening, both *C. elegans* and *D. melanogaster* have been used for high-throughput screening (HTS) of antimicrobial compounds; or mutant libraries for pathogenic determinants of a variety of microbes, including *E. faecalis* and *E. faecium*.

Caenorhabditis elegans

C. elegans is a well-characterized and common invertebrate model organism that is used to study a variety of host-pathogen interactions. *C. elegans* are the smallest in size (adults are ~1mm) among the 3 invertebrate models and has a rapid life cycle (~3.5 d at 20°C). Its transparent body and genetic tractability make the organism well-suited for experimental observation under the microscope and observation of host-pathogen interactions *in vivo*. The ease of scaling up for biochemical analyses, and for HTS, makes *C. elegans* a favorable option over *G. mellonella* and *D. melanogaster* because they are easiest to maintain in large-scale quantities. As infection is performed *ad libitum*, *C. elegans* are an excellent choice to investigate gut microbial-host interaction. However, a limitation of using *C. elegans* as an invertebrate model is their lack of a cellular and humoral response system. Although *C. elegans* express Toll pathways homologous to the mammalian system, there is no clear evidence that they play a role in defense response in this organism.²⁰⁵ Hence, most *C. elegans* infection studies are often paired with a mammalian model organism.^{53,206,207}

Studies describing Enterococcal virulence using the *C. elegans* infection model are assessed by monitoring the amount of time taken to kill the host. Garsin and colleagues noted that even though both *E. faecalis* and *E. faecium* can accumulate to high titers in the intestinal lumen causing gut distention, only *E. faecalis* is able to kill the nematodes efficiently when they are infected at high titers, while causing persistence when infected with low titers. Despite the inability of *E. faecium* to kill adult

hermaphrodites, both *E. faecalis* and *E. faecium* can kill eggs and hatchlings of *C. elegans*.²⁰⁶ However, Moy and colleagues recently showed that certain strains of *E. faecium*, when grown anaerobically, are able to kill adult hermaphrodites due to the high level of hydrogen peroxide production.²⁰⁸

Two well-established Enterococcal virulence factors, cytolysin and the QS Fsr system, are important in killing adult *C. elegans*.^{52,53,206,207,213} However, together these factors only account for approximately 50% of host killing, indicating that additional bacterial factors must contribute to *E. faecalis* virulence in *C. elegans*. A recent study revealed that phage03-like gene clusters are associated with significant nematocidal activity in non-cytolytic and gelatinase-negative *E. faecalis*. This phage03-like element acts as an efficient vehicle for HGT and is enriched in nosocomial and clinical isolates. Absence of the phage03-like element results in attenuation of *E. faecalis* virulence in both *C. elegans* and *G. mellonella* larvae.²⁰⁹ Other *E. faecalis* virulence-associated genes validated using *C. elegans* include *paiA*,²⁰⁷ *epaB*,¹³² *lgt*,¹³² *scrB* (sucrose-6-phosphate hydrolase),^{206,207} and genes encoding enzymes likely involved in DNA damage control and repair such as *recQ* and *phrB*.²⁰⁷

Taking advantage of the genetic tractability of *C. elegans*, gene orthologs whose functions were known to be involved in metabolism and IBD in humans were selected for knock-down by RNAi and screened for their roles in gut colonization, fat metabolism and maintaining epithelial junction integrity. *C. elegans* genes identified as having such protective roles include *nhr-49*, *dlg-1*, and *ajm-1*. This study also shed light on the association between fatty acid metabolism, innate immunity, and maintenance of epithelial junction integrity that can affect nematode survival rate.²¹⁰ In addition to acting as a pathogen to *C. elegans*, a recent study showed that *E. faecalis* Symbioflor[®] can be used as a probiotic to increase worms' lifespan when co-infected with enterohemorrhagic *E. coli* (EHEC). *E. faecalis* Symbioflor[®] was able to downregulate several virulence-associated genes of EHEC when they are co-fed in *C. elegans*.²¹¹ Another study by King and colleagues showed that a mildly pathogenic, resident strain of *E. faecalis* OG1RF evolved rapidly within the *C. elegans* host to protect *C. elegans* against infection by pathogenic *S. aureus*.²¹²

Galleria mellonella

The *G. mellonella* larvae infection model has recently been used to investigate Enterococcal infections. Unlike *C. elegans*, *G. mellonella* has a more complex immune system, based on both cellular and humoral systems that correlate to mammalian systems.⁴ Similar to other

invertebrate models, *G. mellonella* can be reared at low cost and are readily available from commercial sources. The main advantage of *G. mellonella* over *C. elegans* or *D. melanogaster* is that infections can be performed at 37°C, thus more effectively simulating human body temperatures, whereas 25°C is the maximum growth temperature for *C. elegans* and *D. melanogaster*.²¹³ Among the 3 invertebrate models, *G. mellonella* is the largest in size and can be directly injected with bacteria or drugs, allowing exact quantification of the experimental inoculum or drug dosage administered.²¹³ Thus, this model organism has been used to assess the safety and efficacy of antimicrobial compounds against several microbial infections including *Enterococcus* spp, and may have potential in future HTS studies.²¹⁴

Infection studies are performed with *G. mellonella* caterpillars at the final-instar stage of development and infected with bacterial culture or saline as a negative control.²¹⁵ The larvae are kept on petri dishes without food at 37°C, and survival can be monitored over time. Like in *C. elegans*, presence of *E. faecalis* GelE and the Fsr system correlates with killing of *G. mellonella* larvae, whereas *E. faecium* displayed limited virulence.²¹⁵ Absence of Ace in *E. faecalis* also limited the ability of *E. faecalis* to kill *G. mellonella* larvae.⁷¹ After 24 hpi, fewer than 10% of *G. mellonella* infected with WT *E. faecalis* survived, whereas 80% of larvae infected with the Δ ace mutant strain survived.⁷¹ PPIases encoded by *ef_0685* and *ef_1534* play a role in bacterial fitness and, by extension, in the virulence of *E. faecalis*. Δ ef0685 mutant displayed strong attenuated virulence traits while Δ ef1534 mutant displayed weak attenuated virulence traits in *G. mellonella*.¹¹⁸ *asrR*, encoding an antibiotic and stress response regulator, promotes greater persistence of *E. faecium* in *G. mellonella* colonization. Other *E. faecalis* virulence factors that contribute to *G. mellonella* killing include MsrA and MsrB,¹⁰² SlyA,¹²² EpaB,¹³² the cytolysin promoter (*Pcyl*),¹⁴¹ polyamine N-acetyltransferase-like enzyme (PmvE),²¹⁶ *ef_3196/7* 2-component system,⁸⁶ lysozyme-induced peptidoglycan N-acetylglucosamine deacetylase (PgdA),²¹⁷ phage03-like element,²⁰⁹ *ef0377*,⁸⁷ and *cspR*.^{123,126} Significant limitations of using *G. mellonella* include the lack of a fully sequenced genome or molecular tools to genetically manipulate this organism.^{213,218,219}

Drosophila melanogaster

The GI tract of *D. melanogaster* is naturally colonized by several Enterococcal species, including both *E. faecalis* and *E. faecium*, which makes *Drosophila* an excellent model to study Enterococcal gut infections. In addition to a fully sequenced genome, the availability of

Drosophila genetic mutant libraries makes manipulation of the host achievable. Unlike *C. elegans*, inoculation of bacteria into *Drosophila* is achieved via pricking the animal with a needle previously dipped into a concentrated culture of bacteria.²²⁰

Infection studies using *D. melanogaster* are routinely performed using anaesthetized male flies followed by injection of the bacteria inoculum with a pulled glass capillary needle using a micro-injector. The first study reporting *E. faecalis* virulence in *D. melanogaster* was in 2001.²²⁰ Since then, Teixeira and colleagues used *D. melanogaster* to examine virulence associated with the *fsr* quorum sensing system. *D. melanogaster* infected with *E. faecalis* mutants in the Fsr system and/or in Fsr-regulated genes *gelE* and *sprE* all survived longer than flies infected with the wild type parental strain.²²¹ In response to GelE and SprE induction, *lrgAB*, the 2-component system genes *lytRS*, and genes encoding proteins involved in cell wall metabolism, transport, and regulatory functions are all induced. Differential expression of any of these Fsr regulon genes may explain attenuated phenotypes of *fsr* and protease mutants in *D. melanogaster*.²²¹ Enterococcal cytolysin is also an important virulence factor in *D. melanogaster*. Additionally, Cox and Gilmore demonstrated the importance of cytolysin by showing lethality after high-level colonization as compared with a noncytolytic control.²²²

Plants

In addition to vertebrate and invertebrate models, plant models have been used to analyze the virulence of Enterococcal infections. Despite the evolutionary distance between plants and animals, there is evidence that some virulence mechanisms and factors involved in bacterial pathogenesis may be similar between these 2 kingdoms.²²³ Enterococci have been reported to cause diseases in plants.²²⁴ A range of plants have been used as models to study bacterial pathogenesis. *Arabidopsis thaliana* is commonly used and was first introduced to examine *P. aeruginosa* virulence in phyto-pathogenesis. *A. thaliana* infections can be initiated via the root, leaf, or soil. In root pathogenesis assays, bacteria are added into solid medium containing 25 day old *A. thaliana* plants and incubated at 30°C with a photoperiod of 16 h light and 8 h dark. To assay pathogenicity in the leaf, bacteria are injected directly into the leaf using the blunt end of the hypodermic needle. For soil infiltration, soil is flooded with bacteria culture and disease symptoms, such as leaf lesions, can be observed both macro- and microscopically.²²⁴

The first study of *E. faecalis* infection using *A. thaliana* was performed on a leaf infection model by Jha and colleagues in which they discovered that absence of the

E. faecalis FsrB QS system results in attenuated virulence in *A. thaliana*.²²⁴ *A. thaliana* infection begins with bacterial attachment to the leaf, followed by entry through the stomata or wounded surface. Attachment, followed by replication takes place shortly after entry into the intercellular space, causing damage to the plant cell walls and membrane, ultimately leading to rotting and eventual cell death. Three different *E. faecalis* strains (OG1RF, V583 and FA2-2) were capable of infecting the leaves and roots of *A. thaliana*, causing plant mortality by 7 dpi.²²⁴ Strains lacking either *fsrB* or *sprE* resulted in an inability to colonize the root.²²⁴ Unlike zebrafish and *C. elegans*, *E. faecalis* strains lacking *gelE* are not attenuated in the Arabidopsis model of infection. Although this study is the only report examining *E. faecalis* virulence using *A. thaliana*, it shows that *E. faecalis* can use a similar subset of virulence determinants that are used to elicit disease in animals, invertebrates in plants as well. Therefore, the results of plant infection studies can be relevant and useful to animal pathogenesis. Screening for putative virulence factors of animal pathogenic bacteria can be easily achieved in plant as it is less time-consuming and less tedious. Other plant models, such as rice, maize, eucalyptus, wheat, tomato have not been used to study Enterococcal infections but may be exploited in future for differential infection by *E. faecalis* that may provide leads to novel antimicrobial drugs. These plant models may also potentially be useful as a surrogate to overcome inherent limitations of other eukaryotic systems.²²⁵

Conclusion

In this review we (i) summarized the current vertebrate and invertebrate host systems that are available to model *E. faecalis* and *E. faecium* virulence (Fig. 3), (ii) examined conserved and niche-specific virulence mechanisms for *E. faecalis* and *E. faecium* *in vivo*, and (iii) presented evidence that polymicrobial infections involving *E. faecalis* and *E. faecium* are prevalent in some niches (e.g., intestinal tracts and peritoneal abscesses) and are understudied due to the historical assumption that most infections are monomicrobial.

Emphasis on vertebrate host models to investigate Enterococcal virulence during mono- and polymicrobial infections has greatly advanced the elucidation of virulence factors relevant to infection and the consequence of virulence factor expression on host immune responses. Going forward, combining these model systems with high throughput 'omics approaches will increase the speed of discovery of new Enterococcal pathways important for colonization and infection. To date, screening for *in vivo* gene expression by

Enterococci has been most widely applied.^{47,86,179} RNA sequencing (RNA-seq) and transposon sequencing (Tn-seq) provide unbiased approaches to study large-scale gene expression and fitness profiling, and can be combined to probe bacterial virulence in different infection models.²²⁶

Understanding Enterococcal virulence factors and their mechanism of action in the host may help identify future antibacterial or anti-virulence therapeutic targets. The best-described virulence factors and mechanisms, identified using model systems for IE, GI colonization and CAUTI, are depicted in Fig. 4. During IE, vegetation formation is promoted by aggregation and colonization by AS-expressing *E. faecalis*. Host fibrin enveloping *E. faecalis* restricts host defenses, while GelE plays a role in digesting fibrin to accelerate dissemination (Fig. 4A). In the GI tract, CR and host immune responses are critical in combating Enterococcal infections. Synergistic interaction between commensals is key in preventing pathogenic bacterial overgrowth the intestinal tract (Fig. 4B). The intestinal environment is by far the best-described model for studying mechanistic interactions between infecting bacteria (VRE), the gut microbiome, and the host. The intestinal model can be used to examine how antibiotics increase host susceptibility to VRE infection, and how host CR can be re-established to combat VRE infection. In addition, applications on using bacteriocin or heptapeptide pheromone secretion to limit VRE can be studied. This model is also useful for determining the host and bacterial factors *E. faecalis* rely on to drive intestinal leakage, and whether high-iron levels in the gut mediate oxidative stress in the intestinal milieu as a consequence of *E. faecalis* superoxide production (Fig. 4B).^{22,137,143,144,147} During CAUTI, catheterization of the bladder results in cellular damage, a robust host response driving immune cell infiltration and host fibrinogen secretion that coats the catheter, ultimately forming the substrate for *E. faecalis* Ebp pilus adhesion (Fig. 4C).¹⁷¹⁻¹⁷⁵

Given the multi-factorial mechanisms of Enterococcal pathogenesis, new treatment strategies should similarly be multi-pronged. Currently, most UTI studies focus on bladder infection, but our understanding of Enterococcal-mediated kidney infection is poorly understood. Future studies should also include identifying commensals of the UT, whether the UT microbiome mediates CR to limit Enterococcal colonization, or whether changes of UT commensal populations after catheterization promote CAUTI. Since oral antibiotics complicate gut infections, alternatives to treat bacterial infections should be steered toward non-antibiotic approaches: triggering host responses or by introducing helpful members of the microbiota. To curb problems with colonic oxidative

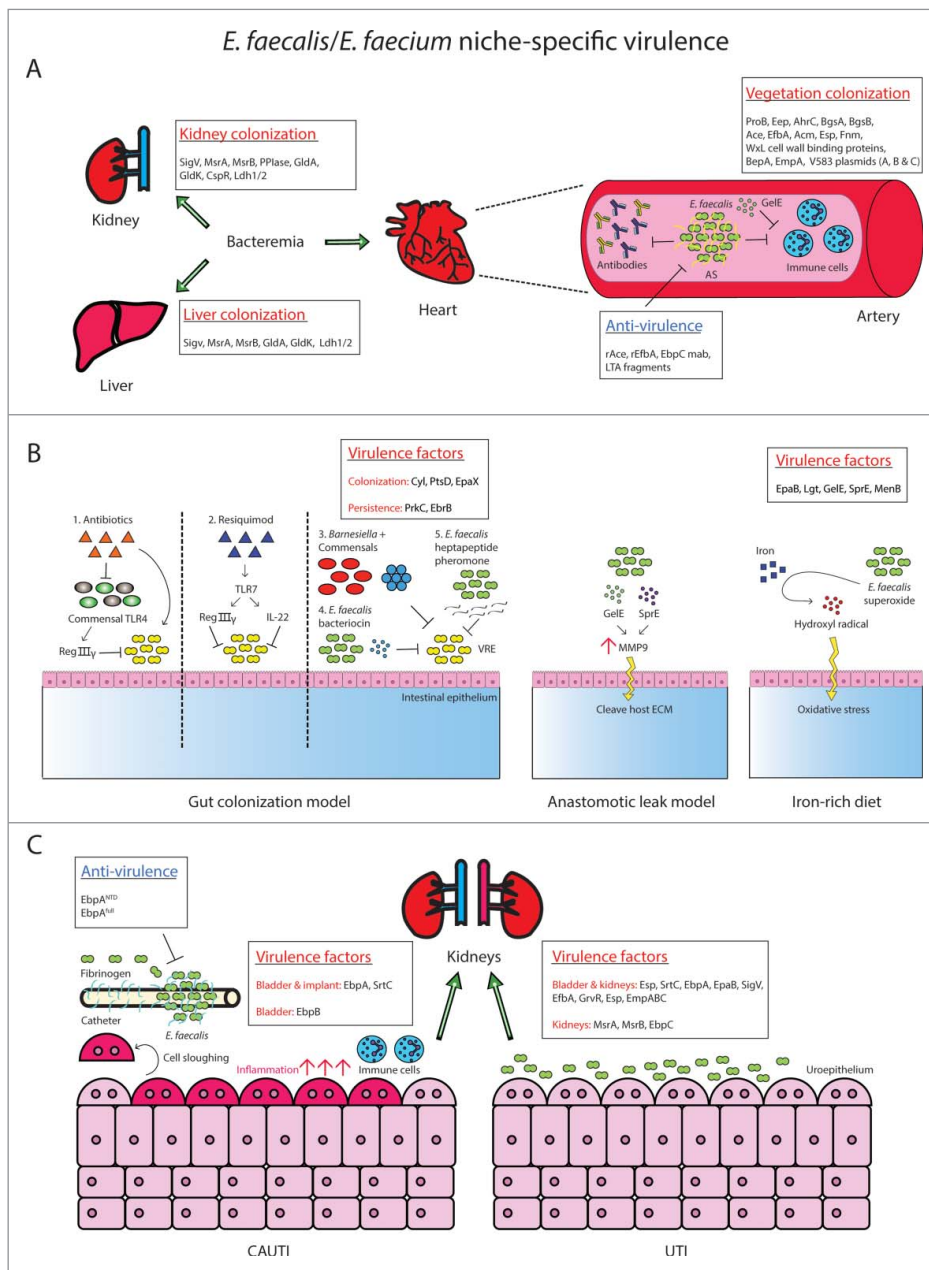


Figure 4. Role of *E. faecalis* and *E. faecium* virulence genes in pathogenesis. Cartoon depicting virulence factors involved during (A) systemic dissemination and infective endocarditis, (B) intestinal colonization, and (C) catheter-associated urinary tract infection and community-acquired urinary tract infection.

stress arising from high-iron diets, orally introduced *Lactobacillus rhamnosus* and *L. paracasei* may help in antagonizing *E. faecium* and *E. coli* already present in the gut, in turn relieving the oxidative stress and mucosal barrier injury observed in mice.²²⁷ In the UT, Wullt and Svanborg showed that *E. coli* 83972 (a prototype asymptomatic bacteriuria/ABU strain) protects patients with complicated recurrent UTI and reduced the frequency of symptomatic UTI by establishing protective ABU.²²⁸ Clearly, emerging antibiotic resistance calls for alternative therapies to treat bacterial infections. This is where

prior knowledge of virulence factors informs the design of anti-virulence strategies to limit, but not kill pathogenic bacteria, in turn relying on host immune defenses for bacterial clearance. For those that are immunocompromised, anti-virulence approaches combined with antibiotics could be an effective solution. These strategies are designed to reduce selective pressure on virulent bacteria to minimize evolution of multidrug tolerant bacteria, increasing success rates for disease treatment. Several examples of anti-virulence therapy targets include: recombinant Ace,⁶³ recombinant EfbA,⁶⁴ EbpA

monoclonal antibodies,⁶² LTA fragments reducing host susceptibility to Enterococcal IE,⁵⁹ partial protection when immunized with IgG Fab fragments (from rabbit antibodies raised against purified AS) against IE,⁴² and an EbpA-based vaccine that can limit or prevent *E. faecalis*-associated CAUTI.¹⁷⁴ Enterococcal SrtA that plays a crucial role in anchoring cell wall-associated virulence proteins (such as Ebp) is also an attractive candidate for anti-virulence therapy.²²⁹

Enterococci rarely cause disease in immunocompetent individuals. Instead, susceptible hosts include those who are immunocompromised or suffering comorbidities. In animals, predisposed states for infection are created to recapitulate clinical scenarios. For example, vegetation induction via heart valve catheterization (rabbits and rats), intestinal perforation (rats and mice), antibiotic-induced dysbiosis (mice), and CAUTI (mice). On the other hand, germfree, gnotobiotic, immunodeficient mice are readily available to facilitate studies that delve deeper into bacterial infections of immunocompromised hosts, making them valuable models for virulence studies.²³⁰⁻²³² Alternatively, when knockout mice are not easily accessible, immunodeficiency can be elicited in mice by administering cyclophosphamide (neutrophil depletion),²³³ anti-G-CSF antibodies (G-CSF cytokine neutralization),²³⁴ or antibodies specific for C-C chemokine receptor 3 (eosinophil depletion).²³⁵ Only by depleting immunological factors individually or in combination will we be able to identify key contributors of Enterococcal immune modulation. To better understand Enterococcal infections in hosts that are having or are susceptible to genetic diseases, we propose investigating mice with genetic disorders such as diabetes, IBD and even those with cancer susceptibilities to address virulence and therapeutic strategies for SSI (and wounds), GI complications (e.g., Crohn's disease), and iron-rich diet-associated colon cancer studies, respectively.^{236,237}

The future for *in vivo* polymicrobial Enterococcal studies should be expanded for models where mixed species infections are most common such as in chronic wounds in diabetic (db/db) hosts, similar to what has been used to study *P. aeruginosa* as well as CAUTI.²³⁸ *E. faecalis*, *P. aeruginosa* and *S. aureus* are the top 3 organisms found in polymicrobial surgical wounds, chronic foot ulcers, diabetic foot wounds, skin injury or burn wounds,²³⁹⁻²⁴² and these interactions should be modeled *in vivo*. Uropathogens commonly isolated with *E. faecalis* during UTI and CAUTI include UPEC, *P. aeruginosa* and *P. mirabilis*.^{15,21} These should be of main concern and the primary species of interest for studies on polymicrobial CAUTI. Other relevant models that should be examined include the use of males and aged hosts, as both groups display increased susceptibility to CAUTI.

E. faecalis is associated with a variety of medical device-related and biofilm-associated hospital infections such as CAUTI, central venous catheter bloodstream infections, VAP and orthopedic implant infections.¹¹ Osteomyelitis is a bacteria-mediated inflammation of the bone that can be acute or chronic. Infection of the bone is usually a post-surgical complication due to implantation of a prosthetic device. *E. faecalis* is associated with 2.5% of prosthetic joint infections (PJIs)³ but very limited work has been performed to understand these infections. The rat model of Enterococcal PJI is initiated by pre-incubating wire implants with *E. faecalis* to allow biofilm formation on the surface. Infected wires are then surgically implanted into the proximal tibia of rats and bacterial enumeration performed on the wires and bone at subsequent timepoints. One study has been reported since 2000 but found no role of *ahrC* or *eep* in biofilm-mediated acute foreign body osteomyelitis, suggesting that biofilm formation may not be sufficient for the pathogenesis of Enterococcal PJI.^{3,38,47} The well-established non-implant mouse model of osteomyelitis for Staphylococci may be more physiologically relevant for PJI and could be modified for Enterococcal studies such that a wire implant modeling the prostheses is implanted before bacterial inoculation.²⁴³ Larger animals such as rats and rabbits are more amenable to surgical procedures compared with smaller animals and are ideal for modeling device- and biofilm-associated infections, mimicking SSIs. However, the main drawback is the lack of genetically modified rats or rabbits available. Pigs and nonhuman primates better represent human anatomy and physiology to better mimic human disease,^{244,245} and can be suitable models to study IE and CAUTI, but cost and housing availability are often limiting factors. Larger animals allow large volume of blood sampling, echocardiography, and may be amenable to BLI for long-term studies in the same animal. Examination of experimental catheter coatings to prevent CAUTI are better performed in pigs or primates than in the mouse model, because Foley catheters with closed-system urine drainage bags are available for these species. Silicone catheters used in mice possess a limited surface area for antimicrobial coating and in low concentrations the coating may be ineffective. The advantages and disadvantages of each animal model discussed in this review are compared in Table 1.

The utility of non-mammalian models should not be underestimated as these can be important for large-scale anti-infection and therapeutic screens. These models are ideal for simple survival studies for mono- or polymicrobial infection which can be difficult to carry out in mammalian hosts due to ethical considerations. When selecting the appropriate non-mammalian model, incubation

temperature should be kept optimal for Enterococcal growth (37°C) as some virulence factors may require a physiologic temperature for gene expression, hence *C. elegans* and *D. melanogaster* may not be appropriate for these reasons. Although mammalian models (especially mouse models) are ideally positioned to address knowledge gaps during infections in a variety of infection niches, it is technically demanding to handle large numbers of animals for large-scale studies. Therefore, throughput combination of non-mammalian models for initial and large-scale screening, followed by confirmatory experiments in an animal model, may be ideal.

There remain many unanswered questions regarding mechanisms of Enterococcal colonization and pathogenesis. While there is a growing appreciation of Enterococcal virulence factors, the same factor may not necessarily play the same role in different animal models or in different niches within the same animal. Hence, there is no one-model-fits-all that can address all mechanisms of Enterococcal colonization and opportunistic infection. It is also clear that Enterococcal pathogenesis is multifactorial and there are virulence determinants that play redundant roles in certain infection sites. Moreover, some *in vitro* phenotypes meant as surrogate models for infection do not correlate with virulence *in vivo*, re-enforcing the need to utilize multiple host-based model systems to elucidate the contribution of specific virulence factors to infection.

Abbreviations

ABU	Asymptomatic bacteriuria
AI-2	Autoinducer-2
AS	Aggregation substance
BHI	Brain heart infusion
BLI	Bioluminescence imaging
CAUTI	Catheter-associated urinary tract infection
CFU	Colony-forming unit
CLABSI	Central-line associated bloodstream infection
CLP	Cecal ligation and puncture
CMAT	Change-mediated antigen technology
CR	Colonization resistance
DGlcDAG	Diglycosyldiacylglycerol
DMK	Demethylmenaquinones
Dpi	Days post infection
ECM	Extracellular matrix
EHEC	Enterohemorrhagic <i>E. coli</i>
GBS	Group B <i>Streptococcus</i>
GI	Gastrointestinal

HAI	Hospital-acquired infection
HGT	Horizontal gene transfer
Hpc	Hours post catheterization
Hpf	Hours post fertilization
Hpi	Hours post infection
HTS	High-throughput screening
IBD	Inflammatory bowel disease
ICU	Intensive-care unit
ID	Infective dose
IE	Infective endocarditis
IP	Intraperitoneal
IV	Intravenous
LD	Lethal dose
LTA	Lipoteichoic acid
Mab	Monoclonal antibody
MGlcDAG	Monoglycosyldiacylglycerol
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
PAMP	Pathogen-associated molecular pattern
PAI	Pathogenicity island
PJI	Prosthetic joint infection
PPIase	Peptidylprolyl <i>cis/trans</i> isomerase
PTS	Phosphotransferase system
QS	Quorum sensing
RIVET	Recombinase-based <i>in vivo</i> expression technology
RNA-seq	RNA sequencing
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
SPF	Specific-pathogen-free
SSI	Surgical site infection
TLR	Toll-like receptor
Tn-seq	Transposon sequencing
UPEC	uropathogenic <i>E. coli</i>
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
VRE	Vancomycin-resistant Enterococci
VUR	Vesicoureteral reflux
WT	Wildtype
Zps	Zwitterionic bacterial polysaccharides

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No potential conflicts of interest were disclosed.

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