Enterococci and Their Interactions with the Intestinal Microbiome

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ABSTRACT

The Enterococcus genus comprises over 50 species that live as commensal bacteria in the gastrointestinal (GI) tracts of insects, birds, reptiles, and mammals. Named “entero” to emphasize their intestinal habitat, Enterococcus faecalis and Enterococcus faecium were first isolated in the early 1900s and are the most abundant species of this genus found in the human fecal microbiota. In the past 3 decades, enterococci have developed increased resistance to several classes of antibiotics and emerged as a prevalent causative agent of healthcare-related infections. In U.S. hospitals, antibiotic use has increased the transmission of multidrug-resistant enterococci. Antibiotic treatment depletes broad communities of commensal microbes from the GI tract, allowing resistant enterococci to densely colonize the gut. The reestablishment of a diverse intestinal microbiota is an emerging approach to combat infections caused by antibiotic-resistant bacteria in the GI tract. Because enterococci exist as commensals, modifying the intestinal microbiome to eliminate enterococcal clinical pathogens poses a challenge. To better understand how enterococci exist as both commensals and pathogens, in this article we discuss their clinical importance, antibiotic resistance, diversity in genomic composition and habitats, and interaction with the intestinal microbiome that may be used to prevent clinical infection.

BACKGROUND

The genus Enterococcus comprises over 50 species that can be found in diverse environments, from the soil to the gastrointestinal (GI) tract of animals and humans to the hospital environment (1, 2; http://www.bacterio .net/enterococcus.html). The first member of this Gram-positive genus was isolated in 1899 from a lethal case of endocarditis (3, 4). It was not until 1984 that enterococcal species were seen as genetically distinct from Streptococcus and assigned their own genus (3–5). Enterococci are Gram-positive facultative anaerobes that exist in chains or pairs and do not form spores. They grow optimally at 35°C, hydrolyze esculin in the presence of 40% bile salts, and are catalase negative (6, 7). Enterococcal species can be distinguished by phenotypic tests that rely on strains’ ability to form acid in mannitol and sorbose broth and to hydrolyze arginine (8, 9).

Enterococci are found in the fecal content of insects, birds, reptiles, and mammals (2, 10). Named “entero” to denote their intestinal residence, Enterococcus faecalis and Enterococcus faecium were first isolated in the early 1900s (11–13). Based on single nucleotide polymorphisms within 16S rRNA, enterococci are divided into seven evolutionarily distinct groups (14). E. faecalis is found in a host of different animals, suggesting that it was in evolutionary terms an early gut colonizer (14). In humans, E. faecalis and E. faecium are the most abundant species of this genus found in fecal content.
accounting for up to 1% of the adult intestinal microbiota (15–19).

Enterococci have recently emerged as a prevalent multidrug-resistant health care-related pathogen. Since the late 1970s and 1980s, enterococcal species have developed increased resistance to several classes of antibiotics (14, 20, 21). Resistant enterococci densely colonize the gut following antibiotic treatment, which can deplete the GI tract of large swaths of protective commensals (22–24). Antibiotic use has increased the spread of drug-resistant enterococci in the hospital setting, leading to enterococci becoming one of the most common causes of hospital-associated infections (25).

Restoration of the intestinal microbiota to a healthy state is a new and developing approach to counter the continuing emergence of antibiotic-resistant microorganisms. However, manipulating the intestinal microbiome to prevent the spread of antibiotic-resistant bacterial strains, while also supporting the sensitive ecosystem of which enterococci are constituents, is a delicate task. It requires that we understand the relationship of enterococci to their natural intestinal habitat in the context of their dual life as commensals and health care-related pathogens. To do so, we discuss the road enterococci have traveled to become multidrug-resistant hospital-associated infectious agents that possess diversified genomes that allow them to survive in the post-antibiotic intestinal niche. With that in mind, we can consider how best to manipulate or restore the enteric microbiota to benefit human health. In this article, we discuss the enterococci’s (i) clinical importance, (ii) development of antibiotic resistance, (iii) diversity in genomic composition and habitats, and (iv) interaction with the intestinal microbiome that may help limit its infectious spread.

**CLINICAL IMPORTANCE**

**Infections**

Enterococci emerged as a leading hospital-associated pathogen in the late 1970s and 1980s (26). In the United States, enterococci cause roughly 66,000 infections each year (27). They are often cultured from mixed species infections of the pelvis, abdomen, and other soft tissues (28). Although the role that enterococci play in these infections is not often clear, they are frequently treated with antibiotics. Less commonly, enterococci can cause meningitis and septic arthritis in patients with comorbidities or who are immunocompromised (28).

Even more clinically important, enterococci are leading causes of hospital-associated bacteremia, endocarditis, and urinary tract infections (20, 26, 29). Enterococci are the second-most-common cause of health care-related bacteremia and are associated with an overall mortality of roughly 33% (25, 30). Enterococcal bacteremia is often preceded by dense colonization of the GI tract, from which enterococci can translocate into the bloodstream (23, 31). In addition, the loss of mucosal immunity and disruption of the GI barrier have been associated with enterococcal bacteremia; risk factors include mucositis, *Clostridium difficile* infection, and neutropenia (32–34).

Over 10% of infective endocarditis cases seen in North America are caused by enterococci, making it the second leading cause (35). Of the total cases of enterococcal endocarditis, more than 35% of infections are acquired in the hospital (36). Enterococci on damaged heart valves grow biofilms that grow into structures called vegetations. Prosthetic valves can also serve as a platform for enterococcal growth (36). As with bacteremia, enterococci that cause endocarditis are often former inhabitants of the GI or genitourinary tract that gained access to the bloodstream (37, 38). Over 10% of catheter-associated urinary tract infections are of enterococcal origin (29).

**Transmission and Sources of Infectious Enterococci**

Hospital-acquired enterococcal infections are of particular concern due to both their increasing prevalence and growing resistance to antibiotics. Enterococci can readily spread within hospital units (39–44). Transmission of enterococci in the clinical environment is aided by two key factors: the ability of enterococci to survive outside the GI tract and the potential for health care workers to inadvertently transfer bacteria to adjacent patients. Enterococcal species can survive for prolonged periods on hospital surfaces, such as medical devices and bed rails, creating fomites that are a major risk factor for further spread (45, 46). Enterococci are transferred from patient to patient via health care workers’ hands (47, 48). Contaminated hands of medical staff can transfer vancomycin-resistant enterococci (VRE) to roughly 1 out of every 10 clean surfaces that the health care workers touch (49).

The GI tract is the major site colonized by VRE and thus is an important source of hospital-associated infections. Hospital contamination is increased when colonized patients become incontinent (50). The density of VRE in patients’ fecal content is correlated with the number of VRE transmission events (47). For roughly every 10% increase in patients colonized with VRE,
the risk of additional hospitalized individuals acquiring VRE rises by 40% (46). A critical mechanism by which hospitalized patients become densely colonized with VRE is antibiotic treatment; how antibiotics allow for VRE expansion is detailed in the last sections of this article. The majority of antibiotic regimens with anti-anaerobic activity result in high-burden intestinal VRE density (22). Metronidazole increases the risk for high-density VRE colonization by 3-fold in allogeneic hematopoietic stem cell transplant (allo-HSCT) patient cohorts (24). Other risk factors for colonization include the use of catheters in the bloodstream or urinary tract, prior surgery, length of hospital stay, and exposure to VRE-colonized patients (40, 47, 51–54).

**Treatment**

Severe cases of enterococcal infections, such as infections of heart valves, have relied on combination drug therapy (55, 56). Combined administration of penicillin and streptomycin (a beta-lactam and aminoglycoside, respectively) successfully cured 80% of enterococcal infective endocarditis cases, which previously had a mortality rate of between 20 and 50%, and became standard therapy by the 1950s (38, 57). Today, for infective endocarditis caused by ampicillin- and vancomycin-sensitive *E. faecalis* lacking high-level resistance to aminoglycosides, gentamicin is the preferred aminoglycoside used in combination with ampicillin. Ampicillin plus ceftriaxone is an alternative therapy for ampicillin-susceptible *E. faecalis*. This regimen has also been used to treat aminoglycoside-sensitive *E. faecalis* isolates, as it is associated with similar cure rates and less nephrotoxicity compared to ampicillin-gentamicin therapy (58, 59). Although *E. faecalis* isolates are intrinsically resistant to cephalosporins, the two beta-lactam antibiotics work synergistically by binding different penicillin-binding proteins (PBPs), the enzymes involved in bacterial cell wall synthesis (60). For ampicillin- and vancomycin-resistant isolates causing infective endocarditis, the majority of which are *E. faecium*, daptomycin or linezolid can be used, although clinical data about their efficacy are limited (61, 62).

There are few other examples of bactericidal synergy against enterococci, and novel antibiotic therapies are urgently needed for multidrug-resistant species (37). This clinical picture begs the question: how did commensal enterococci become such a challenging pathogen? The plasticity of the enterococcal genome is a key factor that has allowed the bacteria (i) to acquire traits that confer antibiotic resistance through mobile genetic elements, (ii) to diversify over time into lineages specifically adapted to the hospital environment, and (iii) to colonize the GI tract at greater densities following antibiotic exposure (37). We discuss each point in the following three sections.

**DEVELOPMENT OF ANTIBIOTIC RESISTANCE**

Roughly one-third of enterococcal infections in the United States are drug resistant, totaling 20,000 antibiotic-resistant cases per year, from which an estimated 1,300 patients succumb yearly (27). *E. faecalis* caused over 90% of clinical infections until the mid-1990s, at which point *E. faecium* became more clinically prevalent (63, 64). The rise of health care-related *E. faecium* strains has been attributed to the increased use of vancomycin and broad-spectrum antibiotics (20, 25, 37, 65). To date in the United States, *E. faecium* causes nearly a third of all enterococcal health care-related infections and constitutes over 75% of all health care-associated VRE strains (27, 29). The majority of *E. faecium* infections associated with medical equipment are vancomycin resistant and ampicillin resistant (80 to 87% and 90%, respectively) (25, 66).

VRE emerged in the mid-1980s, first in Europe among livestock and then in the United States in hospitals (67, 68). In the United States, glycopeptide resistance developed among hospital-adapted ampicillin-resistant isolates that were the predominant enterococci in hospital intestinal microbiota (21, 65). Vancomycin-resistant isolates have been associated with oral vancomycin used to treat antibiotic-associated diarrhea due to *C. difficile* in hospitalized patients. Of note, administration of vancomycin intravenously is not correlated with the development of VRE infection (22, 24, 69). Vancomycin by this route results in low intestinal concentrations (70). In Europe, the issue of VRE was initially confined to animal husbandry. VRE was seen in livestock regularly exposed to antibiotics. Avoparcin, a growth-promoting antibiotic that also provides cross-resistance to vancomycin, is thought to have contributed to the rise of VRE (71–73). Avoparcin was subsequently banned from use in 1996, and the prevalence of VRE in animals decreased (74–76). However, VRE has made a recent appearance in European hospitals with isolates closely related to health care-associated strains found in the United States (77).

Enterococci harbor resistance through two means: (i) resistance that is encoded in the core genome of all enterococcal strains (intrinsic) and (ii) resistance that is passed among isolates on mobile genetic elements by
horizontal transfer (acquired). Some of the mechanisms by which enterococci developed resistance to ampicillin, vancomycin, and daptomycin are briefly outlined in the following sections.

**Antibiotic Resistance: Ampicillin**

Beta-lactams, such as ampicillin, inhibit bacterial growth by modifying and thereby inactivating a group of enzymes called PBPs. PBPs cross-link side chains of peptidoglycan peptides during cell wall synthesis. Enterococcal strains harbor some intrinsic resistance to beta-lactams by producing PBP5, which is chromosomally encoded (78, 79). Given their low affinity to beta-lactam drugs, PBP5s can continue peptidoglycan synthesis as other PBPs are modified (80). Increased resistance to ampicillin is associated with mutations to the PBP5-encoding gene that further reduce the protein’s affinity for beta-lactam antibiotics, such as mutations that result in amino acid substitutions near the active site (81–83). Resistance is further amplified when multiple mutations are present in the pBP5 gene (83). Mutated alleles can be horizontally transferred to beta-lactam-susceptible strains in vitro (84). Altogether, the pBP5 gene differs in nucleotide sequence by about 5% between sensitive and resistant strains (85). The acquisition of specific pBP5 gene mutations contributed to the high-level ampicillin resistance that health care-related *E. faecium* isolates developed in the late 1970s and 1980s (21, 85, 86).

**Antibiotic Resistance: Vancomycin**

Glycopeptide antibiotics, such as vancomycin, prevent peptidoglycan cell wall synthesis by forming complexes with the D-alac-D-alac peptide terminus of peptidoglycan precursors, blocking enzymatic binding sites. Resistant isolates alter peptidoglycan precursors to form D-alac-D-lactate or D-alac-D-serine, with 1,000-fold to 7-fold lower drug-binding affinity, respectively (65, 87, 88). These modifications inhibit antibiotic binding while still allowing PBP enzymes to use these substrates to build a functional cell wall. In enterococci, nine gene clusters associated with resistance have been identified, with most being encoded on mobile elements (65). In response to glycopeptides, these resistance operons regulate the expression of a suite of enzymes that together create modified peptidoglycan precursors and remove those that are unaltered. The two major resistance operons are vanA and vanB (88). vanA gene loci are encoded on Tn1546 or related transposons, conferring high-level resistance to vancomycin and teicoplanin. vanB gene clusters are found on Tn5382/Tn1549-type transposons either on plasmids or in the chromosome, providing moderate resistance to vancomycin only. Variants of these vancomycin-resistance gene loci are found worldwide (89).

**Antibiotic Resistance: Daptomycin**

Daptomycin is a recently introduced antibiotic for the treatment of multidrug-resistant enterococci; however, its bactericidal mechanism of action is not fully understood. It is thought to alter the cytoplasmic membrane and cause depolarization in a calcium-dependent manner, leading to a release of potassium ions from the cell and subsequent cell death (90, 91). For enterococci, the ability to resist daptomycin results in part from alterations in the composition of their cell membrane and envelope. Whole-genome sequencing of a pair of sequentially isolated vancomycin-resistant *E. faecalis* clones, the first daptomycin-sensitive and the second resistant, from a single patient’s bloodstream identified in-frame deletions in three genes: cls, gdpD, and liaF (92). cls and gdpD encode proteins thought to play a role in phospholipid metabolism, and liaF is part of a regulatory system that coordinates the cell envelope response to antibiotics. Resequencing experiments found resistance-associated mutations that became fixed after only 2 weeks of in vitro serial passage with increasing concentrations of daptomycin (93). The transfer of the cls mutation to susceptible *E. faecalis* strains confers resistance to daptomycin (93). Comparative sequencing analyses were performed on five vancomycin-resistant *E. faecium* strain pairs, all initially susceptible and then later resistant to daptomycin, that colonized HSCT patients’ GI tracts (94). These intestinal VRE isolates were exposed to systemic daptomycin as it was partially excreted into the gut, highlighting the capacity of the GI tract to serve as a reservoir for the development of antibiotic resistance, even at low antibiotic concentrations (94). Point mutations in the cardiolipin synthase-encoding gene cls were detected in four out of five of these isolate pairs.

**Antibiotic Resistance: Genetics**

In some enterococcal strains, such as vancomycin-resistant *E. faecalis* V583, acquired genetic elements make up 25% of the genome (95). There are two major types of plasmids in enterococci: pheromone-responsive and transposon-type. The pheromone-responsive plasmid pMG2200 encodes VanB-type vancomycin resistance (96). VanA-encoding pheromone-responsive plasmids can be transferred between *E. faecium* and *E. faecalis* (97). Large regions of the *E. faecalis* genome
can be shuttled between isolates in vitro via conjugative plasmids, involving up to a quarter of the chromosome \(98\). Crossover between chromosomal and plasmid DNA can occur through insertion sequences (also known as IS elements). In \(E. faecalis\), pheromone-responsive conjugative plasmids that contain IS256 copies can integrate into the chromosome of recipient strains in vitro and transfer chromosomal DNA from donor isolates, creating hybrid genomes \(98\). This plasticity of the enterococcal genome has important clinical implications. For example, the transfer of DNA among enterococci has led to multiple lineages of mutated \(pbp5\) genes conferring ampicillin resistance in hospital-associated strains \(84, 85\).

Transposons occur throughout the enterococcal genome and are of three types: conjugative, \(Tn3\)-family, and composite (flanking IS sequences). The \(van\) gene cluster is encoded by a \(Tn3\)-derivative transposon, \(Tn1546\) \(99\). \(Tn916\)-family conjugative transposons include \(Tn5382\) and \(Tn1549\), which are the main genetic elements that contain the \(Van\)B resistance operon \(100–102\). The gene encoding PBP5 can also be transferred between enterococcal isolates with the \(Tn916\)-family conjugative transposon \(Tn5386\) that carries the \(Van\)B cluster \(103\).

**DIVERSITY IN GENOMIC COMPOSITION AND HABITATS**

The genomic diversity seen among enterococcal strains has been well characterized by application of high-throughput whole-genome sequencing. The first enterococcal genome, published in 2002, belonged to \(E. faecalis\) V583 \(95\). Now, hundreds of completed or draft genomes are available \(104\). The GC content of enterococcal species can vary from 37 to 45%, and genome sizes can range from 2.7 to 3.6 Mb \(105–107\). Compared to commensal enterococcal strains, multidrug-resistant clinical isolates possess larger genomes, through the acquisition of foreign genetic material \(107\). Hospital-associated \(E. faecalis\) strains generally lack clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems that help block phage infections and cleave plasmid-encoded DNA \(107, 108\). In 48 \(E. faecalis\) strains, the absence of a CRISPR-Cas system was significantly correlated with resistance to two or more antibiotics \(108\). Multidrug-resistant \(E. faecium\) isolates are also generally CRISPR-Cas deficient, although this relationship has been demonstrated in smaller studies \(108, 109\). IS elements, such as IS16, drive genomic variation across isolates and likely aided hospital adaptation of enterococci as they transitioned from antibiotic sensitive to resistant \(110–112\). Additionally, recombination has been an important mechanism for generating diversity \(89, 98, 113\). By contrast, commensals are far less diverse; for example, \(E. faecalis\) OG1RF does not contain any laterally acquired mobile elements and harbors a CRISPR locus \(114\).

**Population Genetics**

Phylogenetic analyses have found considerable genomic differences between human commensal enterococci and endemic hospital strains. Health care-related strains are more closely related to animal isolates than human commensals \(107, 115–117\). Whole-genome sequencing of \(E. faecium\) isolates has revealed two major clades, one composed of community-derived isolates from healthy humans (clade B) and the other a complex cluster of animal-derived as well as hospital-associated strains (clade A). This split between clades occurred an estimated 3,000 years ago, which coincides roughly with the development of agriculture and animal domestication that conceivably separated animal and human commensals into distinct lineages \(117\). A second bifurcation occurred almost 75 years ago within clade A between modern health care-related strains and animal-derived isolates \(117\). Ampicillin-resistant strains are seen more frequently in pets than in healthy humans \(118\). Enterococcal strains of animal origin can act as a reservoir of antibiotic-resistance elements that can be shared with human isolates \(119, 120\). For example, \(van\) genes from animal-derived enterococci can be laterally transferred to human commensals in the gut \(121, 122\).

What is the evolutionary relationship between clinical enterococcal isolates? Numerous studies have employed multilocus sequence typing as a technique to resolve the enterococcal population structure \(123\). The process relies on sequencing amplified fragments of seven housekeeping genes \(113, 124\). Initial studies of \(E. faecium\) based on multilocus sequence typing found a distinct cluster of isolates that were enriched in hospitalized patients, named clonal complex 17 \(89\). \(E. faecalis\) isolates derived from the hospital environment also group together by multilocus sequence typing, namely into clonal complexes C2 and C9, which possess more resistance elements and pathogenicity island genes than other clusters \(113, 125–127\). However, clinical \(E. faecium\) isolates grouped in clonal complex 17 are not strictly clonal \(111\). In phylogenetic analyses that rely on the algorithm eBURST, spurious groupings can occur for species with high recombination rates, such as \(E. fae-
cium (128). Analyses of *E. faecium* strains employing Bayesian models found three major hospital-associated lineages, indicating that health care-related isolates do not stem from a single ancestral strain (116). Rather, adaptive traits that characterize clinical isolates were likely acquired independently in different genetic backgrounds. Evidence that hospital-associated isolates derived from multiple lineages can also be seen by analyzing the sequence of a single resistance element. Specific amino acid changes in the PBP5 protein are shared between isolates from different sequence types, and sequence variation was found within sequence types (85). These data indicate that antibiotic resistance developed on the background of multiple enterococcal strains that were poised for survival in the hospital setting.

**Habitats**

As previously stated, the GI tract is the primary habitat of enterococci. In animals, *E. faecalis*, *E. faecium*, *Enterococcus hirae*, and *Enterococcus durans* are the enteroococal species most commonly found in the gut microbiota (129). Comparisons of VRE in animals and humans have found strains to be host-specific (130). However, patient isolates have been detected in animals such as dogs and pigs, and as discussed above, hospital-adapted strains share a relatively recent close evolutionary relationship to animal isolates (76). While the GI tract represents the largest reservoir for enterococci, strains have also been found in the environment. It is thought that soil and water isolates are derived from fecal contamination (6, 131–133). Enterococci possess the ability to adapt to extraintestinal environments, as discussed with regard to hospitals. *E. faecalis* can survive in nutrient-poor environments, such as sterilized waste, for up to 12 days (134). Enterococci are frequently found in human sewage, particularly outside hospitals (135). Not surprisingly, enterococcal strains isolated from effluents are antibiotic resistant. Isolates cultured from sewage as early as the 1970s were resistant to tetracycline (136). In water, enterococci are used by the Environmental Protection Agency, in addition to total coliform bacteria, as a marker of fecal contamination, as the result of finding a correlation between swimmers’ risk of GI infection and the number of enterococci cultured from the water site (137). In 2012, 24% of bodies of surface water in the United States were classified as impaired, a number of them due to enterococci (133).

In the human GI tract, enterococci live in the small and large intestine. Enterococcal strains represent roughly 1% of human fecal microbiota, with *E. faecalis* and *E. faecium* being the most common inhabitants (15–19). Average enteroocci density in the GI tract is between $10^4$ and $10^9$ bacteria per gram wet weight, with *E. faecalis* found at a somewhat higher abundance than *E. faecium* (138, 139). However, in one study, *E. faecalis* was found in over 75% of fecal samples, while *E. faecium* was detected in 100% (140).

Intestinal commensals thrive in a finely tuned microbial ecology that has evolved over millennia, aiding in nutrient breakdown and the development of mucosal immunity (141, 142). Early-colonizing strains of commensal enterococci have been shown to contribute to colonic homeostasis through peroxisome proliferator-activated receptor-γ1-induced interleukin-10 and transforming growth factor-beta expression in vitro and can reduce the severity of infectious diarrhea in children (143–145). Perturbations to the intestinal microbiota disrupt this symbiotic relationship established with our microbial inhabitants, with important health consequences. Susceptibility to infections is the most well-documented pathology to result from changes in the microbiota, particularly in the context of antibiotic treatment, as detailed in the following section.

**INTERACTIONS WITH THE INTESTINAL MICROBIOME**

**Colonization Resistance Mediated by the Intestinal Microbiota**

The intestinal microbiota of healthy individuals is composed of a diverse consortium of bacteria (17, 146, 147). Individuals harbor a range of bacterial compositions, consisting of hundreds of microbial strains in the colon that mainly fall into the two major phyla: Gram-negative *Bacteroidetes* and Gram-positive *Firmicutes* (17, 148, 149). In addition to variations among individuals, differences in community structure are also found across body sites that exhibit different levels of stability over time, such as between the stable lower (fecal) and variable upper (oral) regions of the alimentary canal (150).

As previously noted, administration of broad-spectrum antibiotics allows drug-resistant strains such as VRE to expand dramatically in the gut by perturbing this sensitive microbial ecosystem (22–24, 151). VRE can expand to 99% of the intestinal lumen’s microbiota in both antibiotic-treated mice and hospitalized patients (23). This overwhelming colonization is associated with translocation into the bloodstream and resulting VRE bacteremia (23, 24). In allo-HSCT patients, VRE colonization was found in over one-third of recipients, and
these dominated patients had a 9-fold greater risk for VRE bacteremia (24). This risk persists over time; ampicillin administration leaves mice susceptible to VRE colonization for up to 4 weeks posttreatment, and VRE stably persists in the cecum for at least 60 days (23). In patients, resistant enterococci can persist for years after antibiotic exposure (152).

The concept of colonization resistance refers to the microbiota’s ability to prevent the entry and growth of exogenous bacteria within its established, complex community (15, 153). Antibiotic treatment abrogates colonization resistance by depleting large swaths of intestinal commensal microorganisms, particularly anaerobic bacteria, that mediate this defense (15, 154–156).

Obligate anaerobes, such as members of the Barnesiella genus and Clostridium cluster XIVa, are highly correlated with intestinal VRE clearance following fecal microbial transplantation (156, 157). How obligate anaerobes provide a robust defense against invading VRE has not been fully elucidated. However, there are broad mechanisms that commensals can employ to exert colonization resistance and prevent infection: (i) indirect elimination that relies on stimulating innate mucosal immunity, (ii) continual maintenance of mucosal barrier integrity, and (iii) direct antagonism.

### Indirect Inhibition through Innate Immune Defense

Intestinal microbes can stimulate innate receptors on immune cells and induce the production of antimicrobial peptides in other intestinal cell types. Paneth cells and intestinal epithelial cells produce RegIIIγ, a C-type lectin driven by Toll-like receptor (TLR) signaling with bactericidal activity against Gram-positive bacteria (158–160). Secreted RegIIIγ kills bacteria by binding to peptidoglycans of the bacterial cell wall and forming pores (161). Antibiotic treatment reduces expression of RegIIIγ and, in mice, increases susceptibility to VRE colonization and bacteremia (162). Oral administration of lipopolysaccharide mimics commensal microbial signals and restores RegIIIγ production, thereby increasing resistance to VRE (162). A signaling pathway driving RegIIIγ expression was delineated by administration of the bacterial TLR5 ligand, flagellin. Flagellin administered intravenously stimulates the CD103+ CD11b+ subset of dendritic cells to produce interleukin-23, which drives the interleukin-22-mediated production of RegIIIγ by intestinal epithelial cells (163). Commensals can thus work in concert with the mucosal immune system to suppress VRE outgrowth within the intestinal ecosystem.

### Indirect Inhibition through Intestinal Barrier Maintenance

Intestinal microbes are separated from the mucosal epithelium and its distal lamina propria by mucus that coats the epithelial surface. The colonic epithelium is covered by a dense 50-μm-thick inner mucus layer composed primarily of Muc2 and a less dense outer stratum (164). Maintenance of a healthy epithelial barrier and intact gut physiology, such as gastric acid production, inhibits bacterial colonization of the GI tract (165). Goblet cells produce mucin, and secretion is stimulated by commensal bacteria in a MyD88-dependent manner (166–168). Following antibiotic treatment, the mucin layer thins; without a robust physical barrier, intestinal microbes can directly access and potentially breach the epithelium (165). Both the density and composition of the mucus layers limit bacterial invasion. RegIIIγ is associated with mucin and reduces the density of intestinal bacteria near epithelial cells (170–172).

Compared to other antibiotic-resistant pathogens such as Klebsiella pneumoniae, VRE are spatially segregated from the intestinal mucus layer and adjacent epithelium even after antibiotic treatment with its notable mucin reduction (173). Visualization of the colonic lumen reveals that VRE do not infiltrate the inner mucin layer and, despite high luminal density, very few bacteria translocate to the mesenteric lymph nodes (173). Interestingly, cocolonization of mice with VRE and K. pneumoniae, which can more deeply penetrate the mucus coating, enables VRE to gain access to the mesenteric lymph nodes, possibly by K. pneumoniae-induced alterations to the mucin composition (173). Intact mucin production, which is in part regulated by commensal microbes, likely limits the invasive potential of intestinal enterococci.

### Direct Inhibition by Anaerobic Commensals

In the first study of its kind for VRE, a defined consortium of commensals was identified as capable of restoring colonization resistance in mice (157). Antibiotic-treated mice were orally administered diluted doses of fecal microbiota from a colony of mice that had received ampicillin for over 15 years. Bacterial isolates in low-dose fractions that conferred resistance to VRE were identified, cultured, and administered in discrete combinations to mice maintained on ampicillin. Through a series of leave-one-out adoptive transfers, a minimum of four anaerobic isolates were found to successfully prevent and clear VRE from the gut: Blautia producta, Clostridium bolteae, Bacteroides sartorii, and Parabacteroides distasonis (157). Of the four-commensal mixture,
**Enterococci as Probiotics**

The benefits of using enterococci as probiotics have been controversial (182). Given the capacity of enterococcal isolates to share mobile virulence elements in the gut, there is concern about spreading antibiotic resistance if carried or obtained by probiotics. However, enterococcal strains such as *E. faecium* SF68 and *E. faecalis* Symbioflor have been marketed as probiotics for 2 decades without incident and with very few reported adverse events (182–184). Enterococcal probiotics have been shown to be effective in limiting GI infectious burden. A Cochrane meta-review of the literature found *E. faecium* SF68 to be an efficacious treatment of GI infections (184). Inoculation of *E. faecium* SF68 alone to adults and children with enteritis reduced the length of illness (182, 184–186). A probiotic mix containing *E. faecalis* as well as *Bacillus mesentericus* and *Clostridium butyricum* shortened the severity and duration of infectious diarrhea in children (145). In studies of diarrhea lasting 4 days or more, live *Lactobacillus casei* strain GG had a larger treatment effect size (0.59) than live *Enterococcus* SF68 (0.2), although the former had nearly twice as many participants enrolled in all trials (184).

**Fecal Microbiota Transplantation and Probiotics as Treatment for VRE Colonization**

Given the rise of antibiotic resistance, fecal microbiota transplantation (FMT) is an attractive alternative therapy to treat antibiotic-resistant pathogens and is an area of active research. FMT is remarkably successful at curing chronic, intractable *C. difficile* infection (187). A secondary analysis of a study involving patients with recurrent *C. difficile* infection showed that a human-derived FMT can reduce VRE colonization (188). However, the risk of unwittingly transmitting pathogenic microorganisms through FMTs is not insignificant, especially since many constituents of the microbiota have only recently been identified, if not characterized. This concern is particularly relevant to patients colonized with VRE, who are often immunocompromised. Researchers are actively exploring methods to perfect the acquisition of transferred bacteria and define critical members of FMTs that target infectious agents (189, 190).

To date, clinical trials studying the impact of probiotics on intestinal VRE carriage are limited. In a randomized study of 21 renal patients harboring VRE in their GI tract, ingestion of a yogurt supplemented with *Lactobacillus rhamnosus* GG reduced VRE density to the limit of detection in all patients receiving the

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*B. producta* was shown *ex vivo* as the member that directly inhibits VRE growth, although the exact mechanism remains unknown. One possible mechanism of inhibition is through the production of toxic substances such as bacteriocins, which are small molecules with antimicrobial activity. *Lactococcus lactis* strains engineered to express bacteriocins significantly inhibited VRE growth *in vitro* (174). Oral administration of bacteriocin-producing *L. lactis* MM19 eliminated VRE at a faster rate from the gut of mice than mock treatment (175).

**Direct Inhibition by Commensal Enterococci**

Recent studies have examined the colonization dynamics between enterococcal commensals and health care-related isolates in the GI tract. While resistant isolates outcompete sensitive enterococci in the context of antibiotic pressure, intestinal colonization in patients declines following discharge (176). In *in vivo* competition assays that compared the colonization ability of *E. faecium* strains in antibiotic-treated mice, isolates from clade B (commensal-associated) outcompeted those from subclade A1 (hospital-derived) after 2 weeks (177).

Commensal enterococci have developed sophisticated defense mechanisms to eliminate exogenous enterococcal competitors from the gut. Bacteriocin-coding genes are commonly harbored on plasmids in enterococci. Commensal *E. faecalis* strains that express a pheromone-responsive conjugative plasmid encoding bacteriocin bac-21 outcompeted VRE lacking it (178). This plasmid, pPD1, is also quickly transferred to naive intestinal commensals by conjugation (178). Pheromones are secreted short lipoprotein signal peptide fragments that act as chemical messengers between bacteria and can mediate cell death. The multidrug-resistant *E. faecalis* isolate V583 harbors a plasmid called pTEF2 that renders it susceptible to a killing mechanism induced by commensal-derived pheromone cOB1 (179). Bacteriophages, or phages, are viruses that selectively infect and kill microbes. Given their selective killing, phages could be used therapeutically as a narrow-spectrum antimicrobial. *E. faecalis* strains that contain the bacteriophage φV1/7 in their genetic repertoire possess a growth advantage over related bacteria that lack it through phage-mediated lysis of competitors (180). In a mouse model of VRE bacteremia, intraperitoneal injection of ENB6 phage protected all mice when administered shortly after lethal VRE challenge and half of the mice when administered after the mice were moribund (181).
probiotic (191). VRE burden decreased during a 3-week oral supplementation with *L. rhamnosus* GG in a randomized clinical trial of 61 children (192). This effect was not seen with 5-week administration of *L. rhamnosus* Lcr35 in a randomized study of nine patients (193). A 2-week course of *L. rhamnosus* GG administration in 11 patients with comorbidities also did not affect VRE colonization (194). Studies of enterococcal probiotics have failed to demonstrate their potential to limit drug-resistant enterococci colonization. In a prospective cohort study with over 500 hospitalized patients, a 10-strain mixture that contained *E. faecium* and numerous *Lactobacillus* isolates did not prevent ampicillin-resistant *E. faecium* acquisition (195).

The optimal design of probiotic consortia utilizes preclinical mouse models for candidate screening and follow-up mechanistic studies. Microbiome research relies on deep 16S rRNA gene and shotgun sequencing to profile bacterial communities of the gut and to predict candidate commensals that confer colonization resistance in time-series microbiota-reconstitution experiments. Ecological modeling of the microbiota using 16S sequencing data accurately predicted fluctuations in the composition of the microbiota following clindamycin administration and *C. difficile* colonization, and proposed the anaerobe *Coprobacillus* as a commensal capable of inhibiting enterococcal growth (196). *In vivo* adoptive transfer experiments allow investigators to further elucidate the mechanisms of colonization resistance provided by reconstituted commensals. In a mouse model of *C. difficile* infection, *Clostridium scindens* protected antibiotic-treated mice from *C. difficile* colonization by restoring secondary bile salt levels that inhibit the pathogen’s growth (190). How these findings are best translated to treating at-risk patients is yet to be determined. In a promising phase 1b trial, orally administered capsules of 50 human-derived live *Firmicutes* spores prevented recurrent *C. difficile* infection, while the phase II clinical study found no efficacy (197, 198). A key question facing the translation of optimal bacterial combinations into patient therapy is what is required for a high transplantation efficacy. The study that defined a minimal consortium for VRE in mice highlights this challenge (157). Successful colonization of *B. producta* in ampicillin-treated mice required the adoptive transfer of three additional commensals. *B. sartorii* and *P. distasonis* inactivate ampicillin through the production of beta-lactamase, which was critical for ampicillin-sensitive isolates’ survival in the GI tract, while *C. bolteae* supported *B. producta*’s engraftment through an unknown mechanism (157). Modulating the local gut environment through drug inactivation with probiotics is of particular importance for preventing VRE colonization in patients currently receiving antibiotics (199). Probiotic commensals can limit pathogen colonization in the gut by mitigating the disruptive effects of antibiotics to begin with. A *Bacteroides thetaiotaomicron* strain that produces a cephalosporinase has been shown to prevent intestinal VRE outgrowth by inactivating ceftriaxone and thus mitigating any significant changes to the microbiota (200).

Another open question is whether a protective microbial consortium should be tailored to individual patients, and if so, how to scale such a design. Given the falling costs of deep sequencing, profiling patients’ microbiota may occur regularly in clinical practice. In the context of VRE, patients with different degrees of immune system impairment and treatment histories may benefit from personalized alterations to the minimally defined protective consortium. For example, patients who recently received antibiotics may be deficient in nutrients that resistance-mediating bacteria require to survive in the gut, necessitating additional isolates to support successful engraftment. Mouse models would not be a scalable approach to test these individual modifications. In this era of deep sequencing, we can potentially integrate diet, treatment regimens, and gut microbiome data to build machine-learning algorithms that can assess a patient’s risk of VRE colonization and optimize probiotic combinations. Incorporating information on microbiome composition and function improved predictions of individuals’ glycemic response following a meal and helped design dietary interventions for better glycemic control (201). Such data-driven approaches may help tailor preclinical findings to individual patients at scale to successfully mitigate their susceptibility to VRE colonization.

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**REFERENCES**

3. MacCallum WG, Hastings TW. 1899. A case of acute endocarditis caused by *Micrococcus zymogenes* (nov. spec.), with a description of the


Enterococci and the Intestinal Microbiome

44. Howden BP, Holt KE, Lam MM, Scennan T, Ballard S, Coombs GW, Tong SY, Grayson ML, Johnson PD, Stinear TP. 2013. Genomic insights to control the emergence of vancomycin-resistant enterococci. mBio 4:e00412-13
Dubin and Pamer


Enterococci and the Intestinal Microbiome


154. Dubin and Pamer


Enterococci and the Intestinal Microbiome


