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Enterococci and their interactions with the intestinal microbiome

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BACKGROUND

The Enterococcus genus is comprised of 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). 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, 4, 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 within the fecal content of insects, birds, reptiles, and mammals (2, 10). Named 'Entero' to denote their intestinal residence, Enterococcus faecalis and faecium were first isolated in the early 1900s (11, 12, 13). Based on SNPs within 16S ribosomal RNA (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 evolutionarily 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, comprising up to 1% of the adult intestinal microbiota (15, 16, 17, 18, 19).

Enterococci have recently emerged as a prevalent multidrug-resistant nosocomical pathogen. Since the late 1970s and 1980s, enterococcal species have developed increased resistance to several classes of antibiotics (20, 21, 14). Resistant Enterococci densely colonize the gut following antibiotic treatment, which can deplete the GI tract of large swaths of protective commensals (22, 23, 24). Antibiotic use has increased the spread of drug-resistant Enterococci within the hospital setting, leading to Enterococci becoming one of the most common causes of hospital-associated infections (25).

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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 Enterococci's dual life as commensals and nosocomial pathogens. To do so, we discuss the road Enterococci have traveled to become multi-drug-resistant hospital-associated infectious agents that possess diversified genomes that allows 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 chapter, we discuss the Enterococci's 1) clinical importance, 2) development of antibiotic resistance, 3) diversity in genomic composition and habitats, and 4) 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 US, Enterococci cause roughly 66,000 infections each year (27). Enterococci 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 (UTIs) (20, 26, 29). Enterococci are the second most common cause of nosocomial 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 (31, 23). 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, 33, 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 form biofilms on damaged heart valves, which grow into structures called vegetations. Prosthetic values 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 (GU) tract that gained access the bloodstream (37, 38). Over 10% of catheter-associated UTIs 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, 40, 41, 42, 43, 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 healthcare 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 healthcare 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 healthcare workers touch (49).

The GI tract represents the major site colonized by antibiotic-resistant Enterococci and thus constitutes 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 chapter. 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 use of catheters in the bloodstream or urinary tract, prior surgery, length of hospital stay, and exposure to VRE-colonized patients (51, 52, 40, 53, 47, 54).

Treatment

Severe cases of enterococcal infections, such as infections of heart valves, has 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 (IE) cases, which previously had a mortality rate of between 20% to 50%, and became standard therapy by the 1950s (38, 57). Today, for IE 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 regime 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 pencillin-binding proteins (PBPs), the enzymes involved in bacterial cell wall synthesis (60). For ampicillin- and vancomycin-resistant isolates causing IE, the majority of which are E. faecium, daptomycin or linezolid can be used, although the clinical data as to their efficacy is limited (61, 62).

There are few other examples of bactericidal synergy against Enterococci, and novel antibiotic therapies are urgently needed for multi-drug 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 to 1) acquire traits that confer antibiotic resistance through mobile genetic elements, 2) diversify over time into lineages specifically adapted to the hospital environment, and 3) colonize the GI tract at greater densities following antibiotic exposure (37). We discuss each point in the following three sections.

DEVELOPMENT OF ANTIBIOTIC RESISTANCE

Development of antibiotic resistance in E. faecium and E. faecalis

Roughly one-third of enterococcal infections in the US 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 nosocomial E. faecium strains has been attributed to the increased use of vancomycin and broad-spectrum antibiotics (20, 25, 37, 65). To date in the US, E. faecium causes nearly a third of all enterococcal nosocomial infections and constitutes over 75% of all healthcare-associated VRE strains (29, 27). The majority of E. faecium infections associated with medical equipment are vancomycin-resistant and ampicillin-resistant (80–87% and 90%, respectively) (25, 66).

VRE emerged in the mid-1980s, first in Europe among livestock and then in the US within hospitals (67, 68). In the US, glycopeptide resistance developed among hospital-adapted ampicillin-resistant isolates that were the predominant Enterococci within 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 (IV) is not correlated with the development of VRE infection (69, 22, 24). 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, 72, 73). Avoparcin was subsequently banned from use in 1996, and the prevalence of VRE in animals decreased (74, 75, 76). However, VRE has made a recent appearance in European hospitals with isolates closely related to healthcare-associated strains found in the US (77).

Enterococci harbor resistance through two means: 1) resistance that is encoded in the core genome of all enterococcal strains (*intrinsic*), and 2) resistance that is passed among isolates on mobile genetic elements by horizontal transfer (*acquired*). An overview of some of the mechanisms by which Enterococci developed resistance to ampicillin, vancomycin and daptomycin are briefly outlined.

Antibiotic resistance: Ampicillin

Beta-lactams, such as ampicillin, inhibit bacterial growth by modifying and thereby inactivating a group of enzymes called penicillin-binding proteins (PBPs). PBPs cross-link side chains of peptidoglycan peptides during cell wall synthesis. Enterococcal strains harbor

some intrinsic resistance to beta-lactams by producing penicillin-binding protein 5 (PBP5), which is chromosomally encoded (78, 79). Given their low affinity to beta-lactam drugs, PBP5s can continue peptidoglycan synthesis as other PBPs become 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, 82, 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 nosocomial E. faecium isolates developed in the late 1970s and 1980s (85, 21, 86).

Antibiotic resistance: Vancomycin

Glycopeptide antibiotics, such as vancomycin, prevent peptidoglycan cell wall synthesis by forming complexes with the D-Ala-D-Ala peptide terminus of peptidoglycan precursors, blocking enzymatic binding sites. Resistant isolates alter peptidoglycan precursors to form D-Ala-D-Lactate or D-Ala-D-Serine, with 1000-fold to 7-fold lower drug binding affinity respectively (87, 88, 65). These modifications inhibit antibiotic binding while still allowing PBP enzymes to use these substrates to build a functional cell wall. In Enterococci, 9 genes 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 on 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 multi-drug 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 in part results from alterations in the composition of its 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 two weeks of *in vitro* serial passage with increasing concentrations of daptomycin (93). The transfer of cls mutation to susceptible E. faecalis strains confers resistance to daptomycin (93). Comparative sequencing analyses were

performed on 5 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 comprise 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 vanA 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 VanB resistance operon (100, 101, 102). The gene encoding PBP5 can also be transferred between enterococcal isolates with the Tn916-family conjugative transposon Tn5386 that carries the VanB 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, there are hundreds of completed or draft genomes available (104). The GC content of enterococcal species can vary from 37% to 45%, and genome sizes can range from 2.7 Mb to 3.6 Mb (105, 106, 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 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). Multi-drug 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, 111, 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. Nosocomial strains are in fact more closely related to animal isolates than human commensals (107, 115, 116, 117). Whole genome sequencing of E. faecium isolates has revealed two major clades, one comprised 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 nosocomial 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, VanA 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 (MLST) 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 MLST found a distinct cluster of isolates that were enriched in hospitalized patients, named clonal complex 17 (CC17) (89). E. faecalis isolates derived from the hospital environment also group together by MSLT, namely into clonal complexes C2 and C9, which possess more resistance elements and pathogenicity island genes than other clusters (113, 125, 126, 127). However, clinical E. faecium isolates grouped in CC17 are not strictly clonal (111). In phylogenetic analyses that rely on the algorithm eBURST, spurious groupings can occur for species with high recombination rates like E. faecium (128). Analyses of E. faecium strains employing Bayesian models found three major hospital-associated lineages, indicating that nosocomial 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 (STs), and sequence variation was found within STs (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 for Enterococci. In animals, E. faecalis, E. faecium, E. hirae, and E. durans are the enterococcal species found most commonly 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, 132, 133). Enterococci possess the ability to adapt to extraintestinal environments, as discussed with regard to the hospital. 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 that were resistant to tetracycline (136). In water, Enterococci are used by the EPA, in addition to total coliform bacteria, as a marker of fecal contamination, after 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 were classified as impaired in the United States, 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 flora, with E. faecalis and E. faecium as the most common inhabitants (15, 16, 17, 18, 19). Average Enterococci density in the GI tract is between $10^{\wedge}4$ and $10^{\wedge}6$ 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 millenia, 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 PPARγ1-induced IL-10 and TGF-B expression *in vitro* and can reduce the severity of infectious diarrhea in children (143, 144, 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 comprised of a diverse consortium of bacteria (17, 146, 147). Individuals harbor a range of bacterial compositions, consisting of hundreds of different 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 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 (151, 22, 23, 24). 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 fact 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 four weeks post-treatment 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, 155, 156). Obligate anaerobes, such as members of the Barnsiella 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: 1) indirect elimination that relies on stimulating innate mucosal immunity, 2) continual maintenance of mucosal barrier integrity, and 3) direct antagonism.

Indirect inhibition through innate immune defense

Intestinal microbes can stimulate innate receptors on immune cells and induce the production of antimicrobial peptides (AMPs) in other intestinal cell types. Paneth cells and intestinal epithelial cells produce RegIIIγ, a C-type lectin driven by TLR signaling with bactericidal activity against gram-positive bacteria (158, 159, 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 LPS 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 IL-23, which drives the IL-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-

um thick inner mucin 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, 167, 168). Following antibiotic treatment, the mucin layer thins; without a robust physical barrier, intestinal microbes can directly access and potentially breach the epithelium (169). Both the density and composition of the mucus layers limits bacterial invasion. RegIIIγ is associated with mucin and reduces the density of intestinal bacteria near epithelial cells (170, 171, 172).

Compared to other antibiotic-resistant pathogens such as Klebsiella pneumoniae (KP), VRE is 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 does not infiltrate the inner mucin layer and, despite high luminal density, very few bacteria translocate to the mesenteric lymph nodes (MLN) (173). Interestingly, co-colonization of mice with VRE and KP, which can more deeply penetrate the mucus coating, enables VRE to gain access to the MLNs, possibly by KP-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 administrated diluted doses of fecal microbiota from a colony of mice that had received ampicillin for over fifteen years. Bacterial isolates in low-dose fractions that conferred resistance to VRE were identified, cultured, and administrated 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, Blautia producta was shown *ex vivo* as the member that directly inhibit 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 Lactococcus 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 nosocomial 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 two 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 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 multi-drug resistant E. faecalis isolate V583 harbors a plasmid pTEF2 that renders it susceptible to a killing mechanism induced by commensal-derived pheromone cOB1 (179). Bacteriophages 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, intraperintoneal 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).

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 of spreading antibiotic resistance if carried or obtained by probiotics. However, enterococcal strains such as E. faecium SF68 and E. faecalis Symbio-flor have been marketed as probiotics for two decades without incidence and with very few reported adverse events (182, 183, 184). Enterococcal probiotics have been shown to be effective in limiting gastrointestinal infectious burden. A Cochrane meta-review of the literature found E. faecium SF68 to be an efficacious treatment of GI infections (184). Inoculation of the E. faecium SF68 alone to adults and children with enteritis reduces the length of illness (182, 184, 185, 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 on 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 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. The field is 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 on the impact of probiotics on the 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 probiotic (191). VRE burden decreased during a three-week oral supplementation with L. rhamnosus GG in a randomized clinical trial of 61 children (192). This effect was not seen with five-week administration of L. rhamnosus Lcr35 in a randomized study of nine patients (193). A two-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 in 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 Blautia producta in ampicillin-treated mice required the adoptive transfer of three additional commensals. Bacteroides sartorii and Parabacteroides distasonis inactivate ampicillin through the production of β-lactamase, which was critical for ampicillin-sensitive isolates' survival in the GI tract, while Clostridium bolteae supported Blautia 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 for 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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