MICROBIAL BIOFILMS

Biofilm-associated infection by enterococci

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Abstract | Enterococci are ubiquitous members of the human gut microbiota and frequent causes of biofilm-associated opportunistic infections. Enterococci cause 25% of all catheter-associated urinary tract infections, are frequently isolated in wounds and are increasingly found in infective endocarditis, and all of these infections are associated with biofilms. Enterococcal biofilms are intrinsically tolerant to antimicrobials and thus are a serious impediment for treating infections. In this Review, we describe the spatiotemporal development of enterococcal biofilms and the factors that promote or inhibit biofilm formation. We discuss how the environment, including the host and other co-colonizing microorganisms, affects biofilm development. Finally, we provide an overview of current and future interventions to limit enterococcal biofilm-associated infections. Overall, enterococcal biofilms remain a pressing clinical problem, and there is an urgent need to better understand their development and persistence and to identify novel treatments.

Endocarditis
The potentially deadly inflammation of the heart valves and endocardium; infectious endocarditis is characterized by the formation of vegetations composed of platelets, fibrin and microorganisms.

Enterococci are Gram-positive lactic acid-producing members of the gut microbiota in humans and animals. These facultative anaerobes are highly tolerant to diverse environmental conditions, including extreme pH, temperature and salt concentrations. This tolerance contributes to their colonization of diverse host niches, persistence in the environment and utility as an indicator for faecal contamination. Commensal gut enterococci are not typically pathogenic in healthy hosts, and both *Enterococcus faecalis* and *Enterococcus faecium* are the predominant species readily isolated from faeces of healthy babies[1]. In susceptible hosts, however, they can cause infection and are a frequent cause of hospital-acquired infections. Infections with *Enterococcus casseliflavus*, *Enterococcus gallinarum* and *Enterococcus raffinosus* can also occur, although less frequently. The ability of enterococci to form biofilms increases their hardiness and contributes both to their persistence during infection and also their contamination of the environment and the food industry (BOX 1).

Enterococci are intrinsically resistant to antibiotics such as aminoglycosides and β-lactam-based antibiotics. Moderate resistance to aminoglycosides is due to the intrinsic low permeability of the enterococcal cell wall to the large aminoglycoside molecules[2] and is more prevalent in *E. faecium* than *E. faecalis* (49.2% and 8.9% of isolates, respectively)[3]. Intrinsic β-lactam resistance is due to the overexpression of penicillin-binding proteins with low affinity for β-lactams[4], which makes *E. faecalis* 10–100 times more resistant to penicillin than streptococci and *E. faecium* 4–16 times more resistant than *E. faecalis*[5]. Moreover, enterococci can readily acquire resistance to antimicrobials, and vancomycin-resistant enterococci (VRE) (BOX 2) are among the priority pathogens for which new antibiotics are needed[6].

Enterococcal biofilms are observed in a number of infections, including in the urinary tract, wounds, dysbiotic gastrointestinal tract and endocarditis. Biofilm-associated enterococcal infections not only are difficult to eradicate but also serve as a nidus for bacterial dissemination and as a reservoir for antibiotic resistance genes. Several in vitro, ex vivo and in vivo models exist to study enterococcal biofilms (Supplementary Table 1). Modifications to in vitro biofilm assays, such as nutrient supplementation, iron chelation and use of specific media, allow the mimicking of different niches[7–9], whereas use of continuous flow models simulates the biophysical stress and nutrient perfusion characteristics of the host environment[10]. These models have revealed some of the molecular mechanisms that underlie biofilm formation and, together with infection models, have increased our knowledge of how biofilms contribute to disease development.

In this Review, we highlight the spatiotemporal dynamics of biofilm formation and the enterococcal factors that are involved in biofilm formation by *E. faecalis* and, where data are available, *E. faecium*. We describe the cause and effect of biofilm variability and biofilm-associated pathology in different niches and infections, outline treatment strategies and suggest what is most needed to advance biofilm eradication in a post-antibiotics era.

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https://doi.org/10.1038/s41579-018-0107-z

Quorum sensing
A cell density-dependent gene regulatory response, which results in beneficial phenotypes to the population as a whole.

Catheter-associated UTI
(CAUTI). A urinary tract infection (UTI) that is associated with the use of urinary catheters, which increase the risk of infection of bladder and kidneys.

E. faecalis biofilm formation

Biofilm development generally comprises four stages: initial attachment, microcolony formation, biofilm maturation (which is in part governed by quorum sensing) and dispersal. In contrast to model biofilm-forming organisms such as *Pseudomonas aeruginosa* and *Bacillus subtilis*, in which the spatiotemporal contribution of different factors to biofilm formation is better characterized[11–14], enterococcal biofilm development is less well understood. Various factors contribute to enterococcal biofilm formation in vitro and in vivo[15,16] (FIG. 1; Supplementary Table 2), but mediators of dispersion have yet to be identified.

Initial attachment. Surface adherence is the first step in establishing a biofilm, and several surface adhesins, proteases and glycolipids have a role in this early stage. The endocarditis and biofilm-associated pilus (Ebp), which is composed of subunits A, B and C, mediates surface adherence in vitro and in vivo[17–22]. Deletion of *ebpABC* attenuated binding to platelets[19], fibrinogen and collagen[20], reduced initial attachment and consequently impaired biofilm formation in vitro[17]. The contribution of Ebp to early biofilm formation was also observed using in vivo models of urinary tract infection (UTI), catheter-associated UTI (CAUTI) and infective endocarditis, in which bacteria with deletions of pilus components were substantially attenuated[17,19,20,23]. Similarly, the absence of surface adhesins, including aggregation substance (Agg), enterococcal surface protein (Esp) and adhesin to collagen from *E. faecalis* (Ace), reduced adherence to cultured human cells and attenuated biofilm formation in vivo[24–28]. Esp-deficient bacteria showed reduced initial attachment[28] and reduced bladder colonization in an ascending UTI model[29], which is not unexpected because Esp binds fibrinogen and collagen, and these ligands are present in the bladder. Similarly, Ace is involved in binding to collagen, laminin and dentin[30–32], and deletion of Ace led to reduced colonization in rat endocarditis[33] and UTI models[34]. However, Ace deletion did not reduce the bacterial burden in a peritonitis model[33], suggesting that Ace-mediated biofilm formation is not relevant in peritoneal infection. By contrast, deletion of Agg reduced adherence to renal epithelial cells[25,26], binding to lipoteichoic acid (LTA) of other *E. faecalis* cells (and thus inter-bacterial clumping)[35] and bacterial titres recovered from endocarditis vegetations on aortic heart valves[36]. However, Agg is not involved in the colonization of the urinary tract, suggesting that Agg-mediated biofilms are not necessary for ascending UTIs[36]. Biofilm-associated glycolipid synthesis A (BgsA) also contributes to initial adherence and biofilm formation in vitro, but its contribution in vivo has yet to be determined[37]. Deletion of the extracellular secreted protein encoded by *salB* (SagA-like protein B) improved fibronectin and collagen binding but paradoxically decreased biofilm formation[24], which was suggested to be due to decreased hydrophobicity of the *salB* mutant cells. Together, these studies show that multiple factors contribute to the initial attachment and that their contribution likely depends on the surface to which the bacteria attach. Therefore, targeting of any single factor as an anti-adherence or anti-biofilm strategy is unlikely to completely abrogate the initiation of enterococcal biofilms.

Microcolony formation. After initial attachment, bacteria typically begin to multiply and produce small quantities of biofilm matrix to form aggregates known as microcolonies[38]. However, the enterococcal factors driving microcolony formation are unclear, and no transcriptomic data are available from early-stage biofilms or microcolonies. What has been shown is the relevance of microcolonies for gut colonization. Rather than producing the largely 2D biofilm sheets (2–3 cells high) that are typically observed in biofilm models in vitro, *E. faecalis* colonization of the gut of germ-free mice resulted in discrete microcolonies covered in a fibrous sweater-like matrix within a week[39]. Although microcolonies are often thought to be a transient phase of early biofilm development, these findings suggest that microcolonies might represent a mature biofilm stage in this niche that is especially important for gut colonization. Furthermore, enterococcal microcolonies form in vitro in response to antibiotic treatment[40,41]. In contrast to the typical biofilm sheets, biofilms treated with sub-inhibitory levels of daptomycin began to extensively restructure into microcolonies as early as 8 hours after drug exposure. Deletion mutants of *epaOX*, which encodes a glycosyltransferase involved in production of cell wall-associated rhamnopolysaccharide (Epa), also formed microcolonies in vitro, even in the absence of antibiotics. Interestingly, these Δ*epaOX* microcolonies showed reduced structural integrity compared with the monolayer biofilms, as evidenced by their easy detachment during washing.

Biofilm growth and maturation. Biofilm maturation requires active growth and production of extracellular matrix components such as extracellular DNA (eDNA), polysaccharides, LTA and extracellular proteases. The best-characterized matrix component of enterococcal biofilms is eDNA: eDNA can be observed at the bacterial septum, as part of intercellular filamentous structures and also as part of the wider biofilm matrix[42], and its release from cells is dependent on autolysin AtlA[43,44]. Cells that are associated with eDNA showed no substantial cell lysis, and they had a membrane potential[42], suggesting that eDNA is released from metabolically active cells. Accordingly, treatment with DNase reduced biofilm stability and increased detachment[15,45], and the deletion of *atlA* reduced eDNA release and biofilm

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Box 1 | **Enterococcal biofilms in the environment and food**

Environment

Enterococcal biofilms have been detected in recreational beaches, with the highest levels in supratidal sands, and enterococcal numbers displayed a nonlinear relationship with the production of extracellular polymeric substances (EPS)[155]. Enterococcal levels peaked at intermediate EPS levels, suggesting that biofilms may promote survival but paradoxically inhibit proliferation. Another study showed that more beach sand isolates (62%) form biofilms than beach water isolates (30%)[156], probably because biofilm formation is more important for *Enterococcus* spp. survival in sand than in water. Biofilm-mediated colonization of plants has also been demonstrated: three tested *Enterococcus faecalis* strains (FA-2-2, OG1RF and V583) can form biofilms on and infect the roots and leaves of *Arabidopsis thaliana*[157].

Food

Biofilms are an important source of contamination in the food-processing industry, and *E. faecalis* and *Enterococcus faecium* strains isolated from food-processing facilities can form biofilms[147]. Therefore, biofilm-control strategies have increased over the past decade, including antibacterial surfaces, biofilm detachment procedures and matrix-degrading techniques[154]. Specifically, phage therapy has been used against dual-species biofilms formed by *E. faecium* and *Staphylococcus aureus* isolates derived from food-processing surfaces[154]. Peracetic acid is effective, whereas hypochlorite is ineffective, against triple-species biofilms of *E. faecalis*, *E. faecium* and *Bacillus cereus* isolated from the ricotta-producing industry[147]. As the requirements for anti-biofilm treatments in a medical context are stringent in terms of safety, tolerability and efficacy and constrained by what is physiologically compatible, novel biofilm-targeting strategies that do not meet medical criteria may be useful in industrial disinfection or antifouling applications.

However, differences in biofilms growing in different niches, for example, in humans versus the environment, may limit the repurposing of anti-biofilm drugs in this way. For example, *gelE*, *esp* and *ace* affected biofilm formation of clinical isolates in vitro, but *gelE* or *esp* had no effect in strains isolated from raw and fermented meat[158]. In another study, in vitro biofilm formation of antibiotic-resistant enterococci isolated from several other foods was similarly independent of gelatinase activity. A third study showed that 95% of *E. faecalis* isolates from different food samples formed biofilms but that *ace* and *gelE* were not associated with strong biofilm formation[159]. Together, these findings suggest that alternative mediators of biofilm formation may be important for food-associated enterococcal biofilms and that studies specifically investigating the basis of biofilm formation in the food industry are warranted.

formation[43]. Although there is no evidence that eDNA directs the spatial organization of enterococcal biofilms (as has been suggested for other bacterial species[46]), eDNA remains a potential therapeutic target.

Non-proteinaceous cell surface structures, including glycoproteins, polysaccharides and modified lipids, also contribute to biofilm development. The *dltABCD* operon is involved in production of D-alanine esters of LTA, which is an important constituent of the Gram-positive bacterial cell wall, and deletion of this operon reduced biofilm formation in vitro, decreased adherence to epithelial cells and increased susceptibility to antimicrobial peptides[47]. In addition, a putative sugar-binding transcriptional regulator, biofilm on plastic D (BopD), contributes to biofilm formation in vitro[48]. Interestingly, absence of *bopABC*, upstream of *bopD*, increased biofilm growth in glucose but reduced biofilm growth in maltose and colonization levels in the murine gut[48,49], suggesting that the ability to use maltose is necessary for biofilm growth in the gut. Furthermore, deletion of a paralogue of multiple peptide resistance factor (MprF), MprF2, also increased eDNA release and biofilm growth[50]. MprF2 mediates electrostatic repulsion of cationic antimicrobial peptides via aminoacylation of phosphatidylglycerol to reduce the net positive charge of the membrane. Whereas deletion of *mprF2* alone had no effect in a murine bacteraemia model[50], deletion of both *mprF1* and *mprF2* reduced biofilm persistence in a wound infection model[51], suggesting that cell membrane modifications and membrane charge may contribute to biofilm growth and virulence in vivo. Together, these observations support the hypothesis that cell surface glycoproteins, membrane phosphatidylglycerol and polysaccharides contribute to biofilm maturation.

Matrix modification is regulated by the quorum sensing response regulator FsrA, which upregulates the expression of *gelE*, *sprE* and *atlA*[15,44,52–54]. *gelE* and *sprE* encode proteases, and their deletion reduced biofilm formation in vitro and reduced bacterial burden in several in vivo models[55–58]. However, in a rabbit endocarditis model, loss of *gelE* alone increased the deposition of fibrinous matrix in aortic vegetations[57], leading to the hypothesis that gelatinase may be digesting the biofilm matrix to facilitate dispersion[57]. In vitro, *sprE* deletion increased autolysis and eDNA release and accelerated biofilm formation, whereas *gelE* deletion prevented eDNA release[58] and increased *ace* expression[59], which may increase surface attachment but make the biofilm susceptible to detachment.

Quorum sensing. Biofilm formation is influenced by population density-dependent signalling[60,61]. Although it is known that quorum sensing and peptide pheromone signalling coordinate gene expression and direct enterococcal biofilm development[62–64], studies on these small signalling molecules and secondary messengers in enterococci are limited. The exception is the cCF10 peptide pheromone[62,65,66], which mediates the transfer of the conjugative plasmid pCF10. This plasmid can transfer genes encoding antibiotic resistance and virulence determinants such as Agg between cells[62,65,66]. Transfer of pCF10 relies on the accumulation of cCF10, which induces proteins involved in conjugation[66]. The mechanism underlying peptide pheromone-mediated gene regulation and plasmid transfer has been well described[67] and was recently shown to mediate pCF10 transfer between *E. faecalis* cells in the gut of mice[68]. The membrane protease Eep processes the immature peptide pheromones cAD1 and cCF10 (REFS[69,70]). Eep also mediates proteolytic processing of RsiV, which is the anti-sigma factor for SigV, leading to increased environmental stress resistance[71]. Similar phenotypes were observed for a *sigV* mutant[71], consistent with the role of Eep in the regulation of SigV production. Eep, along with AhrC, an ArgR family transcriptional regulator, also contributes to biofilm formation in vitro[72], and deletion of the genes encoding either protein attenuated colonization in a rat osteomyelitis model[73] and reduced bacterial burden in UTI[72] and endocarditis models[74]. Moreover, *eep* deletion mutants form small aggregates that are not typical of wild-type biofilms[74]. Another quorum sensing system is *fsrABC*: FsrC is the membrane sensor kinase that recognizes density-dependent accumulation of the FsrB peptide and that transduces a signal to the FsrA response regulator[75]. This system regulates several biofilm-associated genes and operons (including *bopABCD*, *ebpABC*, *gelE* and *sprE*[15]), and consequently, deletion of *fsrABC* completely abolishes

Pheromone
A secreted chemical that is used to communicate between cells of the same species and triggers a population response.

Box 2 | **Vancomycin-resistant enterococci**

Since the 1990s, a second wave of hospital-acquired enterococcal infections emerged in the United States and has spread abroad, which has seen a sharp increase in *Enterococcus faecium* (*Enterococcus faecalis* was predominant in the first wave)[160]. With the use of vancomycin and broad-spectrum antibiotics, the proportion of *E. faecium* strains that are resistant to vancomycin increased from 0% before the mid-1980s to more than 80% by 2007; by comparison, ~5% of *E. faecalis* isolates are vancomycin resistant. Vancomycin-resistant enterococci (VRE) frequencies are highest in the US (35.5%), followed by Latin America (13.0%), Asia-Pacific (12.0%), Canada (6.0%) and Europe (4.0%)[161,162].

During peptidoglycan synthesis in enterococci, two D-alanine molecules are ligated and joined to UDP-*N*-acetylmuramyltripeptide, which is then incorporated into a nascent peptidoglycan. Vancomycin binds with high affinity to the D-Ala-D-Ala ends of the pentapeptide and inhibits polymerization and subsequent crosslinking. Emergence of high-level resistance to vancomycin in enterococci occurred in 1986, and the detailed mechanism of resistance has been reviewed elsewhere[163]. Eight phenotypic variants have been described (VanA, VanB, VanD, VanE, VanG, VanL, VanM and VanN) along with one type of intrinsic resistance (VanC) that is unique to *Enterococcus gallinarum* and *Enterococcus casseliflavus*[162]. Briefly, resistance entails the substitution of terminal D-Ala with either D-lactate (high-level resistance) or D-serine (low-level resistance) in peptidoglycan precursors and the inhibition or elimination of precursors ending with D-Ala-D-Ala by carboxypeptidases and dipeptidases. The D-Ala-D-Lac substitution (caused by VanA, VanB, VanD and VanM) reduces one of the five hydrogen bonds mediating vancomycin and D-Ala-D-Ala interactions, reducing binding by almost 1,000-fold, whereas substitution with D-Ser (caused by VanC, VanE, VanG, VanL and VanN) results in a sevenfold decrease[164]. VanA is the most prevalent resistance determinant in VRE and is carried primarily by *E. faecium*[162].

Although host characteristics are important risk factors, prior exposure to antibiotics (including oral and intravenous vancomycin, aminoglycosides, cephalosporins, clindamycin, metronidazole and carbapenems) is the strongest predictor of VRE colonization. Mouse models of intestinal colonization have suggested that lipopolysaccharide and flagellin of Gram-negative bacteria increase REGIIIγ secretion by Paneth cells located in the intestinal epithelium, which in turn kills Gram-positive bacteria including VRE[165]. Broad-spectrum antibiotic killing of Gram-negative bacteria therefore increases gut colonization by VRE, and the dominance of VRE in the gut precedes bloodstream infection[166]. Intestinal VRE can persist in the gut for months to years and tends to resist decolonization. Coupled with its tolerance to desiccation and disinfection (VRE can survive for up to 1 hour on hands and as long as 4 months on surfaces), dominance in the gut also facilitates VRE spread in hospitals, where transmission is associated with VRE concentration in patient stool and improper hand hygiene of health-care workers — the most consistent source of transmission[167]. The role of livestock microbiota as reservoirs of vancomycin resistance genes and the role of resistant zoonotic *E. casseliflavus* and *E. gallinarum* in human infection require further attention[168].

biofilm formation[76]. The Fsr quorum sensing system also governs the production of FsrD, which is a precursor for the cyclic peptide gelatinase biosynthesis-activating pheromone (GBAP)[77]. Finally, autoinducer 2 (AI-2), whose production depends on *S*-ribosylhomocysteine lyase (LuxS), is also implicated in *E. faecalis* biofilm formation. AI-2 supplementation increases *E. faecalis* biofilm formation in vitro[78], and deletion of *luxS* results in aberrant biofilm formation with aggregates and dense structures, in contrast to the confluent monolayers of wild-type in vitro biofilms[79].

Biofilm development in *E. faecium*

E. faecium, the second-most frequent *Enterococcus* species associated with disease, often harbours vancomycin resistance genes and is a priority pathogen for which new antibiotics are needed[6] (BOX 2). Several *E. faecium* genes are involved in biofilm development, including *atlA*, *ebpABC*, *esp*, *fsrB*, *luxS*, *spx*, *acm*, *scm*, *sgrA*, *pilA*, *pilB*, *ecbA* and *asrR*[15,80–83]. Only a few of these genes have been shown to affect biofilm-associated infection in vivo: *atlA*, *ebpABC*, *esp*, *acm* and *asrR*[15,81]. Here, we focus on these biofilm factors and the corresponding in vivo phenotypes presented in recent studies (FIG. 2).

EbpABC and Esp are involved in initial attachment preceding biofilm formation. Deletion of the pilus-encoding *ebpABC* operon reduced biofilm formation and surface attachment in vitro and virulence in UTI and infective endocarditis models[84,85]. The cell surface adhesin Esp is also involved in surface attachment. Similarly to *E. faecalis*, deletion of *esp* in *E. faecium* reduced biofilm formation in vitro[86]. Expression of *esp* is regulated by enterococcal biofilm regulator B (EbrB) (a putative AraC family transcriptional regulator), and accordingly, *ebrB* deletion reduced *esp* expression and biofilm formation and attenuated intestinal colonization in mice[87]. However, another study showed that *esp* expression is not essential for intestinal colonization[88] and that *ebrB* may instead affect intestinal colonization by regulating other factors, such as a putative NADH oxidase, muramidase, a hypothetical phage protein and a putative drug resistance transporter[87].

Biofilm growth and maturation in *E. faecium* involve similar factors as in *E. faecalis*. For example, AtlA-dependent release of eDNA is important for biofilm formation in both species. Deletion of *atlA* in *E. faecium* reduced eDNA release, biofilm formation and binding to collagen in vitro[89]; however, there are no reports on whether AtlA contributes to *E. faecium* biofilm formation in vivo. In a transcriptomic analysis, 177 genes were upregulated and 599 downregulated in 1-day-old biofilms compared with planktonic, exponentially growing *E. faecium* cells[80]. Upregulated genes included genes involved in plasmid replication, conjugative transfer and surface adhesion such as *ebpABC*, whereas other biofilm factors such as *fsrB*, *luxS* and *spx* were downregulated[80], which might suggest that LuxS and the *fsr* locus negatively regulate biofilm development in some *E. faecium* strains. Spx is involved in stress responses in *E. faecalis*[90] and *Staphylococcus aureus*[91]. In *S. aureus*, deletion of *spx* increased biofilm formation[91]. Thus, downregulation of *spx* in *E. faecium* might similarly promote biofilm maturation and stress resistance; however, this has not been experimentally tested in *E. faecium*. In addition, deletion of *asrR*, which encodes antibiotic and stress response regulator (AsrR), increased biofilm formation and persistence in the gut of *Galleria mellonella* and intraperitoneally in mice[92], suggesting that AsrR may also regulate genes involved in biofilm-associated infections; however, the identity of these genes is unknown. There are no studies yet on biofilm formation in other enterococcal species.

Biofilm persistence and antibiotics

Low penetration of antibiotics through the biofilm matrix and the presence of persister cells contribute to antibiotic tolerance of biofilms, leading to persistent infections.

Persister cells. Although very little is known about persister cell formation in enterococci, *E. faecalis* requires the alarmone guanosine tetraphosphate (ppGpp) to tolerate cell wall-disrupting antibiotics[93]. Ubiquitous in almost all bacteria, ppGpp has been suggested to have a central

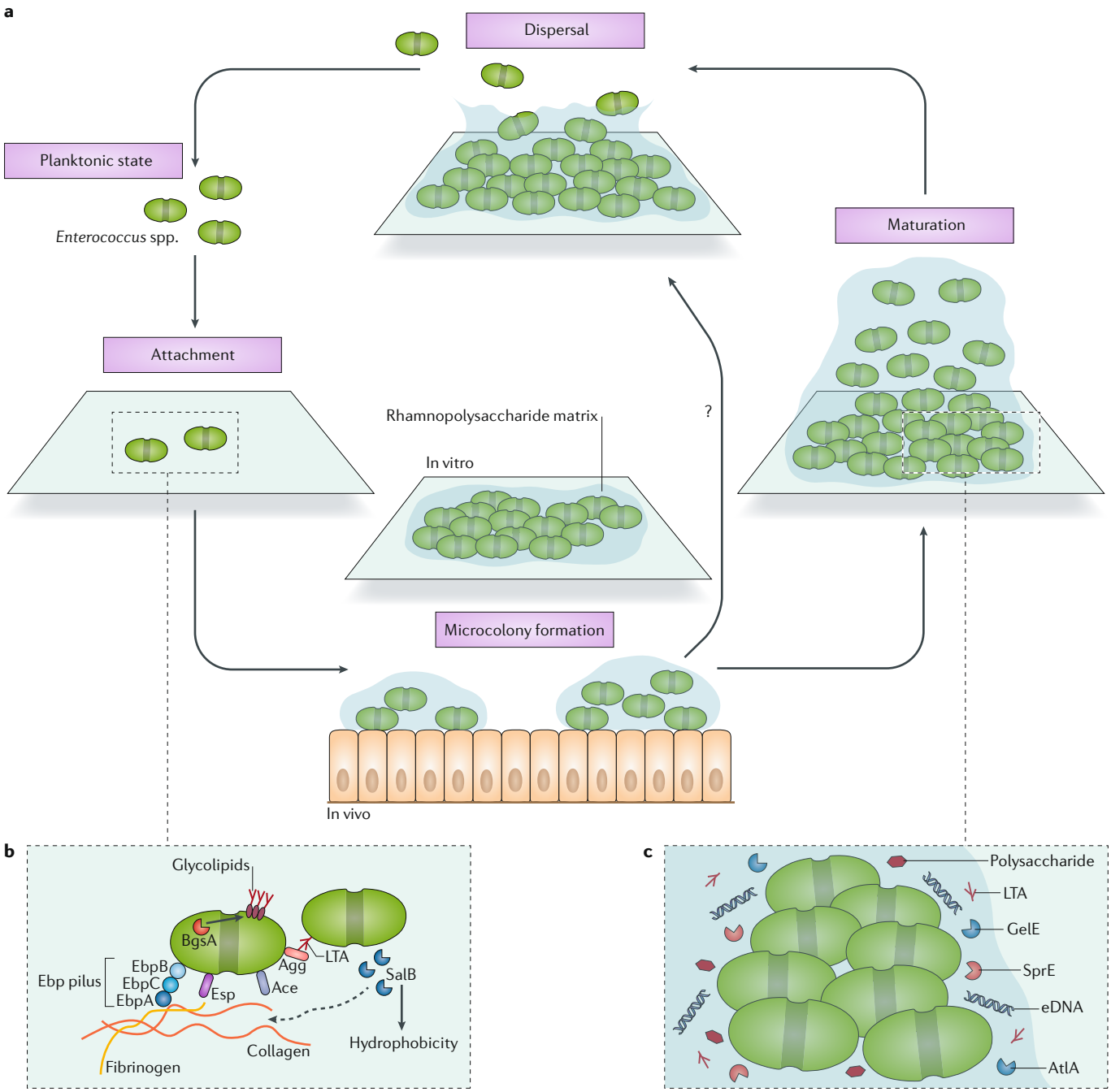

Fig. 1 | **Stages of biofilm development in enterococci. a,b** | *Enterococcus faecalis* biofilm formation begins with planktonic cells that attach to a surface in a process involving adhesins, including the endocarditis and biofilm-associated pilus (Ebp), aggregation substance (Agg), enterococcal surface protein (Esp), adhesin to collagen from *E. faecalis* (Ace), proteases and glycolipids. Following attachment, microcolonies form, and rhamnopolysaccharide is produced (in vitro, *E. faecalis* typically forms biofilm sheets). Some microcolonies may be readily dispersed, but others may further develop into a mature biofilm with a thicker and more complex matrix. **c** | Mature enterococcal biofilms are characterized by the accumulation of extracellular DNA (eDNA), polysaccharides, extracellular proteases, including autolysin AtlA, gelatinase (GelE) and serine protease (SprE), and lipoteichoic acid (LTA) in the matrix. Factors driving progression from mature biofilms to the dispersal stage are unknown, and it is unclear whether microcolonies can disperse planktonic cells. BgsA, biofilm-associated glycolipid synthesis A; SalB, SagA-like protein B.

role in environmentally induced persister cell formation through the stringent response[94], slowing growth by differentially regulating ~500 genes in *Escherichia coli*[95]. In a clinically isolated VRE strain with a single *relA* missense mutation, a constitutively activated stringent response increased ppGpp levels, leading to antibiotic tolerance and delayed eradication[96]. However, the role of ppGpp in persister cell formation seems to be drug-specific and is dependent on culture conditions[97]. Therefore, further work is required to determine the

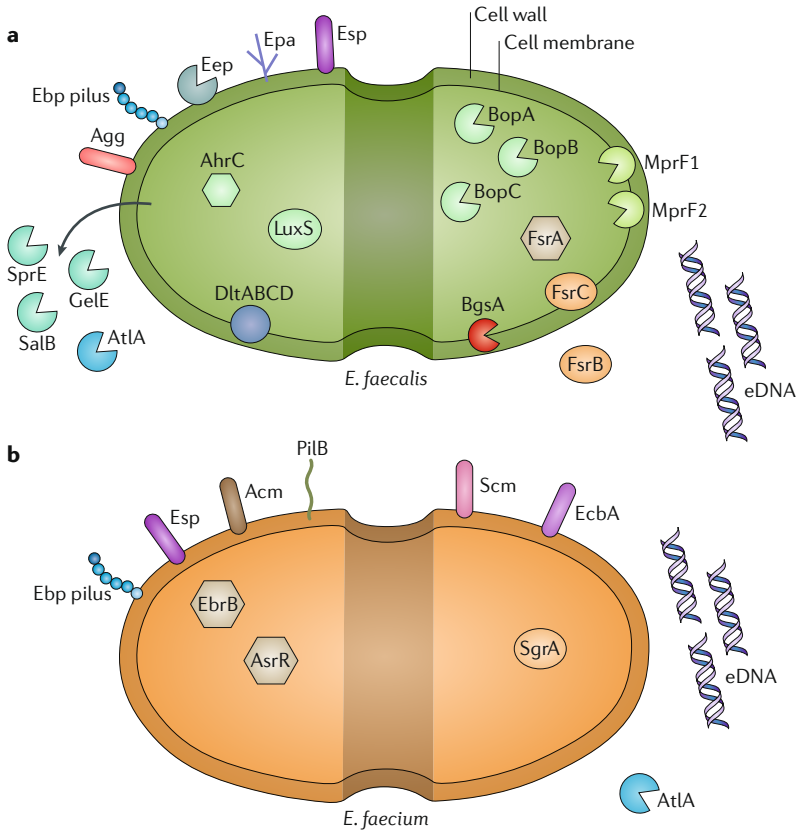

Fig. 2 | **Differences and similarities between factors involved in *E. faecalis* and *E. faecium* biofilm formation. a** | Several genes and proteins (circles) have been experimentally validated to be important for *Enterococcus faecalis* biofilm formation including the endocarditis and biofilm-associated pilus (Ebp), surface adhesins such as aggregation substance (Agg) and enterococcal surface protein (Esp), transcriptional regulators (hexagon) such as AhrC and FsrA, enzymes, such as the secreted proteases gelatinase (GelE), autolysin AtlA, SagA-like protein B (SalB) and serine protease (SprE), and polysaccharides such as rhamnopolysaccharide (processed by the glycosyltrasnferases EpaI and EpaOX), as well as extracellular DNA (eDNA). Signalling molecules, such as the quorum sensing regulators, *S*-ribosylhomocysteine lyase (LuxS) and FsrB, are important for the coordination of biofilm formation. **b** | Biofilm formation in *Enterococcus faecium* is less well described but has been shown to involve overlapping factors such as Ebp, AtlA and eDNA but also *E. faecium*-specific factors such as PilB pilin. Acm, adhesion to collagen from *E. faecium*; AsrR, antibiotic and stress response regulator; BgsA, biofilm-associated glycolipid synthesis A; Bop, biofilm on plastic; *dltABCD*, operon for D-alanylation of lipoteichoic acid; EbrB, enterococcal biofilm regulator B; EcbA, *E. faecium* collagen-binding protein A; Eep, enhanced expression of pheromone; MprF, multiple peptide resistance factor; Scm, second collagen adhesin of *E. faecium*; SgrA, nidogen-binding LPXTG surface adhesin.

precise mechanisms by which enterococcal persister cells form and to define their role in biofilm persistence.

Biofilm tolerance. Addressing enterococcal tolerance and resistance to antibiotics is a global health priority[6]: *E. faecium* is intrinsically more resistant to antibiotics than *E. faecalis*[98], whereas *E. faecalis* forms thicker biofilms that enable tolerance to antibiotics[99,100]. Compared with the minimum inhibitory concentrations (MICs) of planktonic cells, biofilms of clinical *E. faecalis* isolates had increased tolerance to tigecycline and vancomycin[101,102]. Furthermore, older biofilms had increased tolerance to antibiotics, with day 3 and day 5 biofilms of clinical *E. faecalis* and *E. faecium* isolates showing a higher minimum biofilm eradication concentration (MBEC) of vancomycin than day 1 biofilms[103]. Additionally, there was a synergistic effect on MBEC if rifampicin was combined with other antibiotics in 1-day-old biofilms, but this effect was absent in mature biofilms[103]. The underlying mechanism was not determined but could be due to high levels of matrix production in mature biofilms and thus low antibiotic penetration or an increase of antibiotic-tolerant cells, as is reported for other bacterial species[104,105].

The quantity and composition of the biofilm matrix contribute to the virulence and persistence of many bacterial species, but this has not been examined for enterococci, likely owing to the limited understanding of the enterococcal matrix composition. However, it is known that the matrix composition is dynamic and dependent on external cues. For example, cell density regulates the production of biofilm components that are enriched in the matrix through signals, such as AI-2 (REF.[106]) and the *fsr* quorum sensing system[76]. Moreover, *E. faecalis* biofilms can incorporate material contributed by the host and neighbouring microorganisms. For example, fibrin, platelets and immune cells shield *E. faecalis* from antibodies in endocarditis-associated biofilms[107].

Antibiotic resistance genes in biofilms. Planktonic and biofilm-embedded enterococci respond to antibiotics differently. In vitro, the transcriptome of vancomycin-treated *E. faecalis* biofilm cells revealed 101 differentially regulated genes compared with planktonic cells[108]. Genes encoding ATP-binding cassette (ABC) transporters and penicillin-binding 1 A family proteins were the most highly upregulated, suggesting increased antibiotic efflux and resistance against β-lactams in the biofilm-associated bacteria compared with their planktonic counterparts[108]. By contrast, untreated *E. faecium* biofilms had 776 differentially expressed genes in biofilm cells compared with planktonic cells[80]. Upregulation of the antibiotic resistance gene *tetS* and genes associated with mobile genetic elements suggests that *E. faecium* biofilms may resist tetracycline by increasing *tetS* expression and promoting genetic exchange between biofilm cells[80].

Enterococci have higher rates of horizontal gene transfer of antibiotic resistance genes in biofilms than in planktonic cells[41,98,109], and transfer is facilitated by Ebp, Epa, pCF10 and PrgABC[41,110,111]. Ebp promotes cell aggregation and biofilm formation, which facilitates conjugation[110]. EpaOX and EpaI mediate polysaccharide production and maturation, respectively, in response to daptomycin, and the resulting polysaccharide layer sequesters daptomycin away from its cell membrane target[41]. Moreover, EpaI promotes efficient formation of mating pairs and thus can increase pCF10 conjugation[41]. Furthermore, pCF10 promotes its own transfer by encoding PrgB, which enables eDNA-dependent plasmid conjugation[111]. Although the exact role of eDNA is unclear, both cell aggregation and eDNA are important for conjugative transfer of plasmids within biofilms. Perhaps the most compelling evidence for biofilm-mediated horizontal gene transfer is the clinical observation that the vancomycin resistance gene, *vanA*,

Minimum biofilm eradication concentration (MBEC). The minimum concentration of an antimicrobial compound required to kill all cells in a preformed biofilm; the test is typically performed in vitro using biofilm grown on a Calgary biofilm device.

Horizontal gene transfer. The transfer of genetic material between organisms that are not parent-offspring, also known as lateral gene transfer.

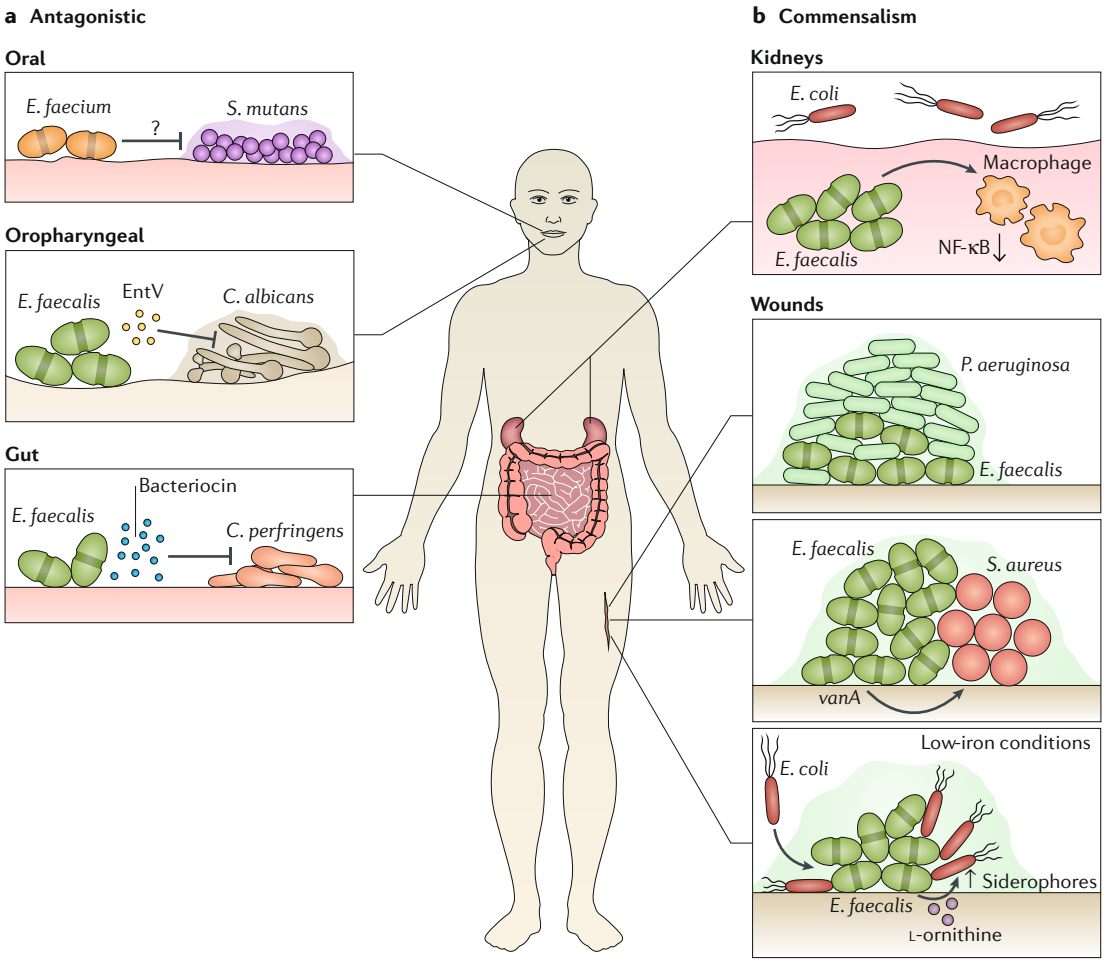

Fig. 3 | **Interactions of enterococci with other species in polymicrobial biofilms. a** | Antagonistic polymicrobial interactions can occur at different infection sites. *Enterococcus faecium* antagonizes *Streptococcus mutans* in the oral cavity by unknown mechanisms. In the oropharynx, *Enterococcus faecalis* produces enterocin EntV to inhibit *Candida albicans*. *E. faecalis* inhibits *Clostridium perfringens* in the gut by producing bacteriocin. **b** | Commensal polymicrobial interactions with enterococci occur during urinary tract infections (UTIs) and wound infections. *E. faecalis* can increase the virulence of *Escherichia coli* in UTIs by modulating the host immune response, which leads to higher *E. coli* titres in the kidneys. Furthermore, *E. coli* can migrate towards autoinducer 2 (AI-2) produced by *E. faecalis*, and the local secretion of L-ornithine by *E. faecalis* promotes *E. coli* virulence in wounds. *E. faecalis* can also co-infect with other wound microorganisms such as *Pseudomonas aeruginosa*, where it increases secretion of biofilm matrix components by *P. aeruginosa*. In wounds, vancomycin-resistant *E. faecalis* can transfer resistance to *Staphylococcus aureus* by disseminating antibiotic resistance genes. Spatial organization depicted for wounds does not necessarily reflect the in vivo situation.

was transmitted from *E. faecalis* to *S. aureus* in a polymicrobial wound biofilm[112,113] (FIG. 3). Together, these data suggest that enterococcal biofilms can serve as gene reservoirs for antibiotic resistance transmission within and between species.

Polymicrobial biofilms and infections

Most *E. faecalis* biofilm-associated infections are polymicrobial, with two or more species present at the infection site. Next-generation sequencing can be used to identify species, and especially in CAUTI and wound infections, enterococci can constitute a substantial proportion of the population[114–120].

Several uropathogens are frequently co-isolated with *E. faecalis*. For example, *Proteus mirabilis* is found in almost 40% of *E. faecalis* biofilms[117]. *E. coli* and *Klebsiella pneumoniae* have been co-isolated on catheters[118], and *P. aeruginosa* and *Candida albicans* also co-occur in UTIs[119]. By comparison, enterococci in diabetic ulcers, burns and surgical wounds are commonly co-isolated with *Staphylococcus* spp., *P. aeruginosa*, *Corynebacterium* spp. and *E. coli*[115,121,122]. Given the frequency with which these organisms co-occur with enterococci and their close interactions in multispecies biofilms (as described below), understanding interspecies relationships could be pivotal in combating complex infections.

Antagonism between enterococci and other species. Antagonistic relationships give insight into which species compete with enterococci (FIG. 3). *E. faecalis* strains isolated from the gut of farm animals suppressed *Clostridium perfringens* growth through bacteriocin production[123], whereas *E. faecalis* and *E. faecium* can inhibit botulinum neurotoxin production in *Clostridium*

botulinum[124]. Additionally, *E. faecium* can inhibit biofilm formation by the oral pathogen *Streptococcus mutans*[125]. Although these aforementioned studies were only performed in vitro, antagonistic interactions could be key in shaping the gut microbiota and additional studies should address this. In a more comprehensive study, there was inter-kingdom antagonism between *E. faecalis* and *C. albicans*: the enterocin EntV inhibited *C. albicans* hyphal morphogenesis and biofilm formation[126,127]. EntV and the resulting suppression of *C. albicans* protected murine macrophages from hyphal-dependent cytotoxicity and reduced pathogenicity in a murine model of biofilm-associated oropharyngeal candidiasis[127].

Commensalisms between enterococci and other species. In contrast to antagonistic interactions, commensal relationships might increase the virulence of enterococcal co-infections (FIG. 3). Frequently co-isolated in wounds and UTIs, *E. coli* and *E. faecalis* show evidence of such an interaction. Under iron-limiting conditions, *E. faecalis* secretes L-ornithine that triggers siderophore synthesis in *E. coli*, increasing *E. coli* growth and virulence during polymicrobial wound infections[8,128]. This commensal relationship was also demonstrated in CAUTI but through a different mechanism. *E. faecalis* suppressed NF-κB-dependent chemokine and cytokine production in macrophages, giving rise to a less inflammatory microenvironment during co-infection[129]. This immune suppression increased the virulence of co-infecting *E. coli* in CAUTI. In addition, *E. coli* can migrate towards AI-2-producing *E. faecalis* during the early stages of biofilm formation, resulting in co-aggregation and increased biofilm formation by *E. coli*[130].

E. faecalis and *P. aeruginosa* are often co-isolated in diabetic foot ulcers, and they have a synergistic effect on biofilm matrix production. In vitro, the combination of *E. faecalis* and *P. aeruginosa* produced the highest biofilm biomass compared with combinations of other co-isolated species[131]. This increase was due to the production of Psl and Pel matrix polysaccharides by *P. aeruginosa*, as deletion of *pelA* and *psl* abolished this synergy[132]. Confocal laser scanning microscopy of the dual-species biofilm revealed that *E. faecalis* were largely confined to the base of the biofilm and that the structured top layers were dominated by *P. aeruginosa*. However, the relevance of increased matrix production for pathogenesis and infection, as well as for cell growth or virulence of either species, is unclear.

Interestingly, no species has been identified that confers a benefit to enterococcal growth or biofilm formation. The identification of such species would be of particular interest because they could increase the pathogenicity of enterococci and may serve as anti-virulence targets for polymicrobial infections.

Although mechanistic studies of polymicrobial biofilms have thus far been confined to dual-species or triple-species models for simplicity, the compounded heterogeneity and resilience of complex multispecies biofilms must not be underestimated[120,133,134]. The simplification of polymicrobial interactions for the sake of experimentation may inform molecular underpinnings of community behaviours but runs the risk of generating results that are irrelevant to complex multi-dimensional infections like in chronic wounds. Models of complex multispecies in vivo biofilms (such as the grafting of multispecies Lubbock chronic wound biofilms onto porcine wounds, Supplementary Table 1) will be useful to evaluate novel anti-biofilm compounds intended for persistent polymicrobial infections.

Novel treatments

Multiple properties of biofilms, including phenotypic antibiotic tolerance and synergistic protective attributes of mixed-species communities, render biofilms recalcitrant to standard antibacterial therapies. As such, the prevention of biofilm formation, wherever possible, should be prioritized.

Surface coatings. Anti-adhesive or antibacterial surface coatings and materials can be used to prevent biofilm formation on the surface of catheters and implants. Effective coatings for preventing or reducing enterococcal biofilm formation include non-leachable cationic film coatings (applied to silicon catheters, these reduced biofilm titres of vancomycin-resistant *E. faecalis* by 97% in vitro and by 95% in a murine CAUTI model)[135], graphene coatings (applied to titanium, these reduced *E. faecalis* biofilm formation and cell growth in vitro)[136], surfactant coatings (glycerol monolaurate and lauric acid applied to sutures reduced *E. faecalis* biofilm formation and cell growth in vitro)[137] and sulfonation of bone-like material (sulfonated poly(ether-ether-ketone) reduced *E. faecalis* biofilm formation and cell growth in vitro)[138]. Of these coatings, the coating of urinary catheters promises the greatest medical benefit owing to the high number of *Enterococcus* spp.-associated CAUTIs, but further validation with other laboratory strains and clinical isolates, as well as preclinical studies, is needed.

Vaccination. Another approach to prevent biofilm formation is the use of EbpA as a vaccine against CAUTI. Located at the pilus tip, EbpA mediates the interaction of *E. faecalis* with fibrinogen (deposited on urinary catheters by the host) and has a major role in biofilm formation. Mice vaccinated with EbpA produced antibodies after 2 weeks, which conferred protection against CAUTI with a 4 log reduction in both bladder and kidney colony-forming units[139]. A subsequent study of ten *E. faecalis* and *E. faecium* strains, representing the diversity of the fibrinogen-binding amino-terminal domain of EbpA, demonstrated that antibodies were universally protective[140]. Although the possibility of developing a vaccine is appealing, vaccination must be carried out well ahead of catheterization, and immunocompromised individuals may not respond adequately to vaccination. To overcome these limitations, the use of antibody therapy (passive immunity) may be considered. Administration of anti-EbpC pilin monoclonal antibodies only 1 hour before infection significantly reduced mortality of mice in an endocarditis model[141]. Although this work is still preliminary, future studies should examine the utility of this therapy for CAUTI biofilms and could also investigate whether antibody therapy will remove pre-existing enterococcal biofilms.

Improving antibiotic effect. Because prevention is not always possible, the removal of pre-existing enterococcal biofilms remains a necessity. Even when biofilm-associated infection is suspected, the use of antibiotics as first-line treatment is commonplace. Instead of clearing biofilms, sub-inhibitory levels of antibiotics can trigger microcolony formation in *E. faecalis*[40]. Combination antibiotic therapy has been attempted but without much success. In one study, the use of combinations of ampicillin, gentamicin, ceftaroline and ceftriaxone all failed to show synergistic effects against 1-day-old *E. faecalis* biofilms compared with monotherapy[142]. Neither biofilm thickness nor titres were affected even at concentrations of up to 1,000 times the MIC. By contrast, another study showed that rifampicin improved the effect of antibiotics against *E. faecalis*[143] and *E. faecium*[144] biofilms. However, this effect was limited to 1-day-old biofilms and not older biofilms[103]. Instead of antibiotic combinations, digestion of the extracellular polymeric substance has also been suggested to render *E. faecalis* sensitive to antibiotics. As eDNA is an integral component of the early biofilm matrix of E. *faecalis*, it presents such a potential target[42]. In a small study on the synergistic effect of matrix digestion on vancomycin killing, biofilms of clinical *E. faecalis* isolates were sensitized by DNase and *E. faecium* biofilms were sensitized by alginate lyase[101]. However, the concentration of vancomycin required to completely eradicate all biofilm cells was unchanged after enzymatic digestion of the biofilm matrix. Together, these findings reiterate the inadequacy of antibiotics to completely clear biofilms.

Disinfection. Although disinfection is effective against planktonic cells, biofilms are resistant to disinfection[145] and this is of particular relevance to recurring endodontic infection. Sodium hypochlorite, which is commonly used to disinfect root canal surfaces, failed to eradicate single-species *E. faecalis* biofilms grown on dentin slabs[146] and triple-species biofilms (formed by *E. faecalis*, *E. faecium* and *Bacillus cereus* isolated from a food-processing facility) grown on stainless steel coupons[147]. Nanoparticles could be added to potentiate the effect of hypochlorite and other non-discriminating oxidizing agents. In *E. faecalis* biofilms that had grown for 3 weeks in root canals, iron oxide nanoparticles bound to biofilm-covered surfaces quickly, and their peroxidase-like activity increased the effectiveness of peroxide in killing biofilm cells at all depths of dentinal tubules[148]. It has yet to be determined whether this approach could be adapted to other accessible sites such as wounds and catheters.

Targeting dispersal. The release of planktonic bacteria during biofilm dispersal renders them susceptible to antibiotics and presents an opportunity for carefully considered adjuvant therapy. In addition to the earlier mentioned biofilm restructuring by antibiotics[40], non-conventional D-amino acids can disperse *E. faecalis* biofilms grown on dentin slabs without inhibiting planktonic growth, although the underlying mechanism has yet to be elucidated[146]. The herbal extract berberine hydrochloride disperses biofilms of *E. faecalis* UTI isolates by downregulation of sortase A and *esp*[149]. Phage therapy has also been considered to promote biofilm dispersal[106]. Interestingly, an *E. faecalis* and *E. faecium* phage, EFDG1, isolated from sewage effluent was effective against ten clinical isolates and demonstrated promising anti-biofilm potential[150]. In vitro, EFDG1 almost completely eradicated a vancomycin-resistant biofilm of 100 µm in thickness and decreased *E. faecalis* titres by 4 logs. Furthermore, EFDG1 also prevented bacterial outgrowth in an ex vivo root canal infection model and decreased *E. faecalis* titres by 8 logs. Although phage therapies must be carefully evaluated (for efficacy, safety, stability during storage, immunogenicity, collateral damage to indigenous flora, resistance, and so on[151,152].), the efficacy of EFDG1 in other enterococcal biofilm disease models should be investigated further. That being said, the approach to use dispersal as a means of biofilm eradication must be approached with caution. It has been reported that dispersed *P. aeruginosa* cells have increased expression of virulence genes, a reduced propensity to be engulfed by macrophages and an increased ability to kill *C. elegans* compared with planktonic cells[153]. Dispersed enterococcal biofilms have not been similarly studied, and it is unclear so far whether enterococcal dispersal will lead to a clinically desirable outcome.

Treating multispecies biofilms. Although developing treatments for enterococcal biofilms is challenging, the task of developing treatments against clinically prevalent multispecies biofilms is likely to be more so. Consideration of multispecies biofilms must begin at detection, and molecular methods should be used to detect fastidious organisms and organisms present at low levels that may influence infection outcomes. A recent study on the dynamics of chronic wounds showed that the species composition of biofilms changes with treatment and that species that become dominant after treatment are already detectable at an earlier stage but at low abundance[133]. With the ubiquity and resilience of enterococci to antimicrobials, their presence in multispecies biofilms, even at low levels, should be flagged as a cause for concern.

To our knowledge, the only study showing limited success in treating enterococcal multispecies biofilms used dual-species biofilms of *E. faecium* and *S. aureus* isolated from a food-processing plant[154] (BOX 1). Treatment of 1-day-old biofilms with the staphylococcal mycophage phiPLA-RODI resulted in flatter and less organized biofilms, a biomass decrease of 63% and a reduction in viable *S. aureus* titres by 0.6 log units but no decrease in *E. faecalis* titres. These results convey some promise but need to be validated in biofilms of clinical isolates. Although it is not suitable for medical use, peracetic acid is effective against triple-species biofilms (*E. faecalis*, *E. faecium* and *B. cereus*) in the food industry[147] (BOX 1) and potentially for the disinfection of hospital devices. Given the limited number of in vivo studies and even fewer studies addressing treatment of multispecies biofilms, many challenges but also possibilities lie ahead.

Conclusions

Enterococcal biofilm-associated infections cause substantial morbidity in humans. Enterococci growing as biofilms on urinary catheters and wounds are the most common cause of infections, and their prevalence will

Endodontic infection
A bacterial infection of the dental pulp located within the teeth and root canals.

only increase as the global human population continues to age, hospital stays increase and comorbidities become more prevalent. Compounding the phenotypic antibiotic tolerance that is common in biofilms is the intrinsic antibiotic resistance and the increasingly frequent horizontally acquired resistance in enterococcal strains. At this time, a thorough understanding of the mechanisms of antibiotic resistance and tolerance in enterococcal biofilms is lacking. There are several outstanding questions. Does resistance evolve differently in biofilms than in planktonic cells? Are there biofilm-specific responses to antibiotic exposure? Do persister cells form, and by what mechanism do they form? What are the relative contributions of transcriptional, post-transcriptional and even epigenetic responses to antibiotic tolerance in biofilms?

To prevent and combat enterococcal biofilm-associated infections, we must first understand the mechanisms underlying biofilm formation. Major challenges lie in a full spatiotemporal characterization of the enterococcal factors that mediate each stage of biofilm development. Strikingly, insight into the mechanisms of enterococcal biofilm dispersal is completely absent at this point in time. Given that biofilm dispersal is a promising therapeutic approach, addressing this knowledge gap is particularly important. The challenge of describing and understanding enterococcal biofilm development is heightened by our increasing appreciation that the biofilm substrate and local environment, including the host immune system and co-infecting microorganisms, can substantially change the biofilm properties and composition in different niches. However, by combining technologies that couple optical detection with meta-omics and systems level analyses, along with animal models of CAUTI and wound infections, we are poised to tackle these challenges and design new and effective therapeutics against enterococcal biofilm infections.

Published online: 18 October 2018

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Acknowledgements
Work in the Kline laboratory related to this article is supported by the Ministry of Education Singapore under its Research Centre of Excellence Program and by the Ministry of Education Singapore under its Tier 2 programme (MOE2014-T2-2-124) and the National Institutes of Health (NIH; 1 R21 AI126023-01).

Author contributions
J.-H.C., K.K.L.C., L.N.L. and J.J.W. researched data for the article. All authors made substantial contributions to discussions of the content, wrote the article and reviewed and edited the manuscript before submission.

Competing interests
The authors declare no competing interests.

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Reviewer information
Nature Reviews Microbiology thanks D. Garsin, M. Gilmore and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41579-018-0107-z.