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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MECHANISMS OF *ENTEROCOCCUS FAECALIS*
PATHOGENESIS IN WOUND INFECTION**

CHONG KIAN LONG KELVIN
B.Sc. Biological Sciences (Hons)
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**Interdisciplinary Graduate School
HealthTech NTU (NTU institute for Health Technologies)**

A thesis submitted to the Nanyang Technological University in partial fulfillment of the
requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

29th January 2019

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Date

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Chong Kian Long Kelvin

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

29th January 2019

.....
Date

.....
Assoc/Professor Kimberly Kline

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the first and/or corresponding author.

Chapter 2 is published as Chong, K. K. L., Tay, W. H., Janela, B., Yong, A. M. H., Liew, T. H., Madden, L., Keogh, D., Barkham, T. M. S., Ginhoux, F., Becker D. L., & Kline, K. A. (2017). Enterococcus faecalis modulates immune activation and slows healing during wound infection. *The Journal of infectious diseases*, 216(12), 1644-1654.

The contributions of the co-authors are as follows:

- A/Prof Kline, Prof Becker, Dr Barkham, and I provided the initial project direction.
- Dr Tay and I prepared the manuscript drafts. The manuscript was revised by A/Prof Kline, Prof Becker, Dr Barkham, and Yong, A. M. H.
- Yong, A. M. H. and I conducted all *in vivo* co-infection experiments.
- I co-designed the study with Dr Tay and performed all the laboratory work at the Singapore Centre for Environmental Life Sciences Engineering and Lee Kong Chian School of Medicine. I also analyzed the data.
- Dr Janela performed all the flow cytometry experiments. Data was analyzed by Dr Janela, A/Prof Ginhoux, and me.
- All scanning electron microscopy was conducted by me and Dr Tay at Temasek Life Sciences Laboratory.
- All confocal images were taken by Dr Tay at the Singapore Centre for Environmental Life Sciences Engineering.
- All tiled scan images were taken by me and Madden, L at Lee Kong Chian School of Medicine. Images were analyzed by me and Prof Becker.
- Dr Keogh provided general animal procedural advise.
- Liew, T. H. generated the *mprF1/2* strain used in this study.

29th January 2019

.....
Date

.....
Chong Kian Long Kelvin

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Mechanisms of host-pathogen interactions in *Enterococcus faecalis* wound infections

Chong Kian Long Kelvin

Interdisciplinary Graduate School
HealthTech NTU (NTU institute for Health Technologies)
Nanyang Technological University

Summary

Enterococci are among the most prevalent pathogens isolated from wound infections, and *Enterococcus faecalis* is typically in the top four most commonly isolated bacterial species in diabetic wounds. Despite its prevalence, very little is known about the pathogenic mechanisms of *E. faecalis* in wound infections. Our overall aim was to identify *E. faecalis* fitness factors in wounds and to understand the host-pathogen interplay that promotes infection. To achieve this aim, we established a mouse *E. faecalis* wound infection model to study its infection dynamics. High titer *E. faecalis* inocula into wounds resulted in acute bacterial replication with a concomitant pro-inflammatory neutrophilic response, followed by a reduction in bacterial burden that can persist within wounds with limited inflammation. To better understand the bacterial factors that contribute to chronic *E. faecalis* wounds, we collected 49 *E. faecalis* isolates from a variety of infected human wounds and performed comparative genomics. We found that *E. faecalis* wound isolates were predominantly clustered in ST16/179 and CC2/6. Isolates with the greatest *in vitro* biofilm-forming capacity correlated with the presence of *ecbA*, a putative cell wall surface anchor family protein that binds to collagen type V and fibrinogen, suggesting it may be a potential indicator of high biofilm formation *in vivo*. To elucidate additional *E. faecalis* wound fitness factors, we performed transposon sequencing and also tested *E. faecalis* mutants in a hypothesis-driven manner. We found that *E. faecalis* MprF, which confers resistance to cationic peptides, and a phosphotransferase system are required for *E. faecalis* persistence in wounds. In *E. faecalis* polymicrobial wound infections with *S. aureus*, we observed that *E. faecalis* can

augment biofilm formation and titers, possibly through utilization of *S. aureus*-derived L-arginine. Together, these findings suggest that *E. faecalis* can both modulate the host immune response and tune its metabolism during monomicrobial and polymicrobial infections to mediate its persistence in wounds.

Dissertation supervisors:

Associate Professor Kimberly Kline, MPH PhD
Assistant Dean (Special Projects), College of Science
Public Health & Medical Biofilms
Singapore Centre for Environmental Life Sciences Engineering

Professor David Becker, PhD
Professor of Tissue Repair and Regeneration
Principal Investigator, Tissue Repair & Regeneration Laboratory
Lee Kong Chian School of Medicine

Adjunct Associate Professor Timothy Mark Sebastian Barkham, MBBS, MSc, FRCPath
Senior Consultant
Department of Microbiology and Immunology/Yong Loo Lin School of
Medicine/National University of Singapore
Laboratory Medicine/Tan Tock Seng Hospital

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Abbreviations

A	Adenine
<i>aac(6')-aph(2'')</i>	Bifunctional 6'-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase enzyme
C	Cytosine
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CAUTI	Catheter associated urinary tract infections
CC	Clonal complex
CCL	Chemokine ligand with two adjacent cysteines
CD	Colonization dose
CI	Competitive index
CFU	Colony forming units
CSF	Colony stimulating factor
CXCL	Chemokine ligand with two cysteines separated by one amino acid
Dpi	Days post infection
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>E. faecium</i>	<i>Enterococcus faecium</i>
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FISH	Fluorescent <i>in situ</i> hybridization
G	Guanine
GI	Gastrointestinal

H&E	Hematoxylin and eosin
Hpi	Hours post infection
ID	Infection dose
IFN-γ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MALDI	Matrix-assisted laser desorption/ionization
MBEC	Minimum biofilm elimination concentration
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
N	Biological replicate
n	Technical replicate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
P-value	Probability value
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCA	Principal component analysis

PCR	Polymerase chain reaction
Phos	Phosphate
PTS	Phosphotransferase system
RNA	Ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SEAP	Secreted embryonic alkaline phosphatase
SEM	Scanning electron microscopy
SNPs	Single nucleotide polymorphisms
Spp.	Species
SSIs	Surgical site infections
ST	Sequence types
T	Thymine
TSB	Tryptic soy broth
TSBG	Tryptic soy broth supplemented with 0.25% glucose
TNF-α	Tumor necrosis factor alpha
TLRs	Toll-like receptors
Tn-seq	Transposon sequencing
UTI	Urinary tract infections
VRE	Vancomycin-resistant <i>Enterococcus</i>
VRSA	Vancomycin-resistant <i>Staphylococcus aureus</i>

Chapter 1: Background and Significance

1.1 Motivation

Enterococci are ubiquitous in the gastrointestinal tract (GI) of almost every animal and insect, and have been so since the time of animal terrestrialization. This same innocuous commensal that is so well-adapted to life in the GI tract has now emerged as a hospital pathogen due to increased use of antibiotics, changing both the microbial landscape in the GI tract as well as selecting for antibiotic resistance (François Lebreton et al., 2017; Van Tyne & Gilmore, 2014). Moreover, Enterococci can serve as a nexus for acquiring and disseminating antibiotic resistance genes through lateral gene transfer, where Enterococci can exist in complex microbial communities and/or persist in environments enriched for antibiotic-resistance elements (Van Tyne & Gilmore, 2014; Courvalin, 1994). Hospital-adapted Enterococci strains carry varying numbers of antibiotic resistance and virulence genes, acquired through horizontal gene transfer events, to promote colonization and transmission in patients (Van Tyne & Gilmore, 2014). In particular, *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) have emerged as human pathogens in gastrointestinal (GI) tract-associated infections, wound infections, urinary tract infections (UTI), bacteremia, and endocarditis (**Figure 1.1**) (Arias & Murray, 2012; Fisher & Phillips, 2009; Flores-Mireles, Walker, Caparon, & Hultgren, 2015; François Lebreton et al., 2017; Morrison, Woodford, & Cookson, 1997; Murray, 1990).

Known *Enterococcus faecalis* infection sites

Figure 1.1. *E. faecalis* infection in known host niches. Schematic diagram showing *E. faecalis* associated infections. Figures were modified with permission. (Goh, Yong, Chong, & Kline, 2017)

Three key observations formed the motivation for investigating *Enterococcus* wound pathogenesis. First, among the *Enterococcus* genus, wounds tend to be infected mainly by *E. faecalis* followed by *E. faecium* (Dworniczek et al., 2012; Heitkamp et al., 2018; Rajkumari, Mathur, & Misra, 2014; Shettigar, Bhat, Satyamoorthy, & Murali, 2018). This observation suggests either a tropism for *E. faecalis* to infect wounds or a consequence of antibiotic use in hospitals selecting for *E. faecalis* in wounds. Second, while *E. faecalis* are commonly found in wounds, it is debated whether its presence is associated with more severe disseminated or invasive disease. Third, *E. faecalis* wound infections are mostly polymicrobial, and recent evidence suggests that *E. faecalis* can promote the virulence of other co-infecting microorganisms in wounds. Co-infecting microbes may act synergistically and/or the presence of one microbe might predispose wounds to infections by another microbe leading to infection and/or complicate treatment options. Hence, we reasoned that there is a threshold at which *E. faecalis* wound colonization can result in infection, and that predisposing factors such as co-infecting microbes, along with host health status, can lower this threshold for infection by *E. faecalis*.

The overarching goal of this thesis was to develop an *in vivo* model to study host-pathogen interactions during *E. faecalis* wound infections and use it as a platform to determine mechanisms that aid in *E. faecalis* wound pathogenesis. The model could also be used to study *E. faecalis* polymicrobial infections commonly found in hospital wounds. Furthermore, by taking a comparative genomics approach, we sought to use *E. faecalis* hospital wound isolates to identify common genetic determinants that may be required during wound infection. By undertaking these research goals, I aimed to fill the knowledge gap of how *E. faecalis* cause wound infections in monomicrobial and polymicrobial settings, with the long term goal of

developing new therapeutics and adopting more informed strategies in combating *E. faecalis* wound infections.

1.2 Enterococcus faecalis infections

1.2.1 Emergence of *Enterococcus faecalis* as an opportunistic pathogen

E. faecalis is an early commensal resident of constituting the gastrointestinal (GI) tract, where it makes up about 1% of the newborn microbiota, and which are capable of causing opportunistic infections (Malani, Kauffman, & Zervos, 2002; Sghir et al., 2000; S. Wang, Hibberd, Pettersson, & Lee, 2014; Younge, Araujo-Perez, Brandon, & Seed, 2018). *E. faecalis* belongs to group II and III in the five designated groups of Enterococci, classified by its ability to form acid in mannitol in the former but not latter group, with both groups able to hydrolyze arginine (Witte, 2003). The ability to grow in high pH, salinity and temperatures is postulated to allow *E. faecalis* to colonize a wide variety of host niches (Sherman, 1937; Wei & Jingping, 2015). In hospitals, these survival traits enable Enterococci to persist on abiotic surfaces such as medical equipment and clothing of healthcare workers, which facilitate the dissemination of Enterococcus into patients (Jackson et al., 2018; Neely & Maley, 2000). Moreover, the ability for Enterococci to acquire antibiotic resistance allows them to become enriched in microbial depleted niches. Furthermore, Enterococcus colonization has been correlated with antibiotic consumption in hospitalized patients (Ruiz-Garbajosa et al., 2012; Ubeda et al., 2010), with vancomycin-resistant *E. faecalis* enriched in the skin of patients warded in intensive care units (Jackson et al., 2018; Lee et al., 2018). These observations show that *E. faecalis* can readily colonize host niches to cause infection and have the capacity to persist in the host.

E. faecalis can colonize a variety of wound types ranging from surgical sites, diabetic wounds, pressure ulcers and venous ulcers (Dowd et al., 2008; Dworniczek et al., 2012; Fisher & Phillips, 2009; Giacometti et al., 2000; Shettigar et al., 2018;

System, 2004). Wounds remain the second most common infection site for *E. faecalis* after catheter-associated urinary tract infections (CAUTI) (Chakraborty, Pal, Sarkar, & Gupta, 2015; Salem-Bekhit et al., 2012). *E. faecalis* isolated from wounds display *in vitro* biofilm-forming capabilities but how that ability contributes to wound colonization and infection remains poorly understood (Barie et al., 1990; Dalton et al., 2011; Dworniczek et al., 2012; Hurlow, Blanz, & Gaddy, 2016). *E. faecalis* can also cause infections in diabetic wounds, based on both culture-dependent and culture-independent studies (Bowler, Duerden, & Armstrong, 2001; Dowd et al., 2008; Giacometti et al., 2000; System, 2004). Moreover, these wound infections tend to be polymicrobial, consisting of other co-isolated organisms infecting wound sites (Bowler et al., 2001). Recent efforts have been directed towards understanding these polymicrobial wound infections through development of an *in vitro* biofilm wound model (DeLeon et al., 2014; Ganesh et al., 2015; Sun, Dowd, Smith, Rhoads, & Wolcott, 2008) and via metagenomic studies (Dowd et al., 2008; Gardiner et al., 2017; Misic, Gardner, & Grice, 2014). However, the individual contributions of these wound microbes and how they contribute to infection remains poorly understood.

1.2.2 *Enterococcus faecalis* virulence during nosocomial infections

Commensal and hospital-associated *E. faecalis* strains can express different virulence factors to cause opportunistic nosocomial infections. *E. faecalis* can express microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which facilitate *E. faecalis* colonization at different host niches (Sillanpää et al., 2004). Cell-wall anchored proteins constitute a proportion of MSCRAMMs that *E. faecalis* can display on its outer surface. These cell-wall anchored proteins are recognized by sortases through a C-terminal “LPXTG” or “LPXTG-like” signal, allowing for substrate recognition and anchoring to the cell wall of *E. faecalis* (**Figure 1.2**) (Hendrickx, Willems, Bonten, & van Schaik, 2009; Kline et al., 2009;

Nielsen et al., 2013). In particular, sortase A is a housekeeping enzyme which anchors the majority of *E. faecalis* LPXTG-containing proteins to the cell-wall via its transpeptidase activity (Guiton et al., 2009; Kline et al., 2009). Understanding *E. faecalis* MSCRAMM-mediated surface attachment can inform the mechanism on how *E. faecalis* is able to colonize host niches. Here we will review only *E. faecalis* MSCRAMMs related to colonizing surfaces, and their role in biofilm formation will be reviewed in section 1.2.4.

Figure 1.2. LPXTG-anchored proteins of enterococci involved in surface attachment and biofilm formation. Schematic diagram of LPXTG-containing proteins in *E. faecalis* and *E. faecium*. Virulence factors shown are associated with their function during infection. Figure is adapted with permission from (Hendrickx et al., 2009).

E. faecalis can bind to various ECM host proteins to aid in its colonization. The *E. faecalis* genome encodes various collagen binding proteins, such as collagen binding Ace protein, which is the most extensively studied. Ace is a LPXTG containing protein capable of binding host collagen, fibrinogen, and laminin (Nallapareddy & Murray, 2008; Rich et al., 1999; Xiao, Höök, Weinstock, & Murray, 1998). Ace contains a non-repetitive segment denoted as the A-domain which facilitate binding to collagen through a proposed “collagen-hug” model. The A-domain contains two subdomains, N1 and N2, both adopting an Immunoglobulin G-like fold, enabling binding to the triple helix of collagen (Liu et al., 2007). Expression of Ace is important for *E. faecalis* colonization onto dentins in the oral cavity (Hubble et al., 2003), for heart valve colonization (Singh, Nallapareddy, Sillanpää, & Murray, 2010), and colonization during UTIs (Lebreton et al., 2009). Another LPXTG-containing protein is the *E. faecalis* pheromone-responsive aggregation substance which is encoded on several *E. faecalis* plasmids (Chow et al., 1993; Hendrickx et al., 2009). Aggregation substance allows *E. faecalis* to bind to fibronectin, type I collagen, thrombospondin, and vitronectin (Rozdzinski et al., 2001). Aggregation substance-mediated binding allows *E. faecalis* to colonize during endocarditis and directly contributes to mortality in endocarditis models (Chow et al., 1993; Hirt, Schlievert, & Dunny, 2002; Schlievert et al., 2010). Lastly, the *E. faecalis* pilus, consisting of three Ebp subunits, is able to bind host fibrinogen and collagen (Flores-Mireles, Pinkner, Caparon, & Hultgren, 2014; Nallapareddy et al., 2011). Each pilus is made up of three subunits (designated as EbpA, EbpB, and EbpC), where EbpA can bind host fibrinogen during CAUTI (Flores-Mireles et al., 2014). In sera isolated from endocarditis patients, antibodies against all three subunits of Ebp have been reported (Sillanpää et al., 2004). Moreover, an Ebp deficient mutant is attenuated in an *in vivo* model of infective endocarditis (Nallapareddy et al., 2006). Taken together, *E.*

faecalis cell-wall anchored proteins can serve as adhesins capable of binding to host surfaces and aid in colonization.

Apart from cell-wall binding proteins, *E. faecalis* can also express proteases capable of degrading host proteins for immune evasion and infection. The *E. faecalis* genome encodes gelatinase (GelE), serine protease (SprE), and cytolysin (CylA) which are able to aid in *E. faecalis* virulence. *E. faecalis* GelE is a metalloprotease capable of cleaving host endothelin, insulin β -chain, and collagen (Mäkinen, Clewell, An, & Mäkinen, 1989; Makinen & Makinen, 1994). Expression of GelE results in differential severity in rabbit endocarditis models, with increasing proteolytic activity positively correlating with mortality (Dupont, Montravers, Mohler, & Carbon, 1998; Singh, Qin, Weinstock, & Murray, 1998). The mechanism by which SprE contributes to pathogenesis and virulence is not known. However, deletion of SprE renders *E. faecalis* attenuated during *C. elegans* gut infection (Garsin et al., 2001) and rabbit endophthalmitis (Suzuki et al., 2008), but not in infective endocarditis (Thurlow et al., 2010). This finding suggests that other proteases, such as GelE, might be more crucial than SprE during infections such as endocarditis. The cytolysin CylA targets host neutrophils and macrophages for destruction (Miyazaki et al., 1993). When *E. faecalis* cytolysin is expressed together with aggregation substance, increased mortality is observed in an endocarditis model of infection (Chow et al., 1993). This increased mortality suggests that cytolysin may lower host defenses to cause more severe infections. Therefore, *E. faecalis* expresses a repertoire of virulence factors that enable colonization and infection of a wide variety of niches, while also modulating the host microenvironment for its survival and persistence. Taken together, although *E. faecalis* possess the necessary virulence machinery to cause infection, its virulence strategies in wounds remains uncharacterized.

1.2.3 Host-pathogen interactions during *E. faecalis* infections

E. faecalis must overcome host defenses in order to colonize, infect, and persist in host niches. In general, one of the ways the host immune system is able to recognize invading microbes is through recognition of pathogen-associated molecular patterns (PAMPs) (Akira, Uematsu, & Takeuchi, 2006; Molina et al., 2015). Toll-like receptors (TLRs) are PAMPs which are categorized by their varying domains of leucine-rich repeats and belong to the type I integral membrane glycoproteins. These receptors are able to bind to bacterial encoded products and trigger a downstream signaling cascade to mount an immune response against the infection. In particular, TLR2 can recognize purified *E. faecalis* lipoteichoic acids (LTA) which increase macrophage chemokine expression in murine macrophages (Park et al., 2013). The recognition of *E. faecalis* LTA triggers the MAP kinase signaling pathway for immune activation (Park et al., 2013; Zou & Shankar, 2015). Therefore, the host immune system is able to recognize *E. faecalis* products and mount an immune response against it. Hence, *E. faecalis* must possess virulence factors in order to escape clearance by the host immune system and consequently persist within the host.

E. faecalis has multiple strategies to resist host clearance. These include forming biofilms, resisting phagocytosis, and immunomodulating host cells (Baldassarri et al., 2005; Gentry-Weeks et al., 1999; Rakita et al., 1999; Zou, Baghdayan, Payne, & Shankar, 2014). Additionally, some *E. faecalis* strains can suppress the immune system by downregulating the NF- κ B response in intestinal cell lines and macrophages (S. Wang et al., 2014; S. Wang, Ng, Chow, & Lee, 2008; Zou et al., 2014). *E. faecalis* can also mask surface display of its PAMPs by producing a capsule, or by modifying its peptidoglycan by incorporating secondary wall polymers (alanylated LTA) which prevents complement-mediated opsonization and hence reduces opsonophagocytosis by neutrophils and macrophages (Geiss-Liebisch et al.,

2012; Thurlow, Thomas, Fleming, & Hancock, 2009). Besides surface masking, some strains of *E. faecalis* encode a TLR agonist (TcpF) capable of downregulating the NF- κ B response through binding to the NF- κ B adapter protein MyD88 (Zou et al., 2014). Interestingly, the ability of *E. faecalis* to modulate inflammatory processes can alleviate inflammatory skin conditions, such as atopic dermatitis, in experimental mouse models (Choi et al., 2016). Lastly, *E. faecalis* can resist phagocytosis-mediated clearance through expression of virulence factors such as aggregation substance and GelE (Baldassarri et al., 2005; Gentry-Weeks et al., 1999; Rakita et al., 1999; Süßmuth et al., 2000). This lack of *E. faecalis* clearance allows for its survival intracellularly in host cells. Taken together, *E. faecalis* is capable of modulating the immune response and resisting clearance, which aids in its persistence within host niches.

1.2.4 *Enterococcus faecalis* biofilms implicated in nosocomial infection

Enterococcal biofilm formation has been proposed to mediate pathogenesis in the urinary tract, wounds, gut, and heart valves. Enterococcal biofilms are formed through a four-stage process; planktonic cell attachment, microcolony formation, biofilm maturation, and dispersal (**Figure 1.3**).

Figure 1.3. Proposed lifecycle of *E. faecalis* biofilm formation. *E. faecalis* biofilm formation is initiated when planktonic cells attach to abiotic/biotic surfaces. *E. faecalis* then forms microcolonies, which is indicative of early biofilm formation. *E. faecalis* biofilms then undergo maturation where secretion of matrix proteins such as polysaccharides and eDNA is important in this phase. Finally, *E. faecalis* biofilms can undergo dispersal to disseminate the infection from microcolonies or matured biofilms, where the mechanisms remain poorly understood. Factors that are known to mediate *E. faecalis* biofilm formation are listed in the figure above.

These Enterococcus biofilms can serve as reservoirs for transmission of antibiotic resistance genes and dissemination to other sites. Enterococcus biofilms can form on catheters and allow for ascending infection into the bladder and kidneys during catheter associated urinary tract infections (CAUTI) (Guiton et al., 2013). In addition to forming a surface for colonization, the catheter alone also induces inflammation, which is required for CAUTI (Guiton et al., 2013). Pioneering studies revealed the importance *E. faecalis* Ebp pilus in surface adhesion (Flores-Mireles et al., 2014). The N-terminal domain of EbpA facilitates binding of *E. faecalis* to catheters and subsequent biofilm formation. Immunization of the N-terminus of EbpA

reduced *E. faecalis* catheter titers by >10000-fold. This finding is the first report of immunization against an adhesin that successfully limits *E. faecalis* CAUTI (Flores-Mireles et al., 2014). More recently, the role of host proteases has been suggested to be important during *E. faecalis* CAUTI (Xu et al., 2017). A 12-hour routine treatment with the cysteine protease inhibitor E64 reduced ascension of *E. faecalis* into the kidneys, suggesting that host cysteine proteases may play a role in *E. faecalis* CAUTI (Xu et al., 2017). Other virulence factors have also been implicated in *E. faecalis* CAUTI, including *eep* and *ahrC* (Frank et al., 2013). Taken together, *E. faecalis* biofilm formation on catheters is important for establishing an inflammation-mediated infection of the bladder and kidneys.

Enterococcus gut infections are usually caused by antibiotic resistant strains and have been extensively reviewed (Arias & Murray, 2012). Vancomycin resistant Enterococci (VRE) can colonize the gut after antibiotic treatment, growing to high numbers, which is thought to allow for dissemination of VRE to other sites (Arias & Murray, 2012). Recently, the mechanism underlying *E. faecalis* gut colonization in colitis was described. In an IL-10 deficient mouse colitis model, *E. faecalis* deficient in *epaB* and *lgt* showed reduced inflammation of the distal colon despite similar bacterial density enumerated from the organs (Ocvirk et al., 2015). Moreover, the absence of EpaB attenuated *E. faecalis* binding to intestinal mucosa *in vivo* and prevented biofilm formation on murine intestinal epithelial cells (Ocvirk et al., 2015). Therefore, the presence of inflammation and maturation of lipoproteins can facilitate *E. faecalis* infections during colitis.

During endocarditis, Enterococci can cause biofilm-like vegetations which block heart flow and may result in mortality of infected patients. Clinical isolates of *E. faecalis* isolated from endocarditis formed *in vitro* biofilms that were >2-fold higher in density compared to biofilms formed by isolates from other sites (Mohamed

et al., 2004). Moreover, deletion of *fsr*, *gelE*, *epa*, or *atn* from clinical endocarditis isolates reduced biofilm formation by >30% and adhesion by >50% (Mohamed et al., 2004). Glycosyltransferases are important for glycolipid production during biofilm formation. In a rat model of infective endocarditis, absence of either of the glycosyltransferases, *bgsA* or *bgsB*, showed attenuated vegetation formation and CFU counts by 10-fold and 10000-fold respectively (Haller et al., 2014). Other virulence factors include *eep* and *ahrC*, deletion of which reduce heart valve titers in an *in vivo* rabbit endocarditis model by >10000 fold (Frank et al., 2012; Frank et al., 2013). Disruption of the aggregation domain of aggregation substance (Asc10) mildly reduces CFU in a porcine heart valve model by 10-fold (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010). Taken together, glycolipid production and biofilm aggregation are important in the development of *E. faecalis* endocarditis.

E. faecalis rarely causes endodontic infections though their association with persistent oral infections has been reported (Zhang, Du, & Peng, 2015). In root canal infections, *E. faecalis* forms biofilms on dentin and causes persistent infections (Ferrer-Luque et al., 2014; Rosen et al., 2016; Tennert et al., 2014; Zand et al., 2016). Most of these studies have mainly focused on *in vitro* models, pre-incubating extracted teeth with *E. faecalis* before analysis. Addition of 2-2' bipyridyl, D-leucine, EDTA, or sodium hypochlorite are able to reduce biofilm CFU by >4-fold (Rosen et al., 2016). These compounds either chelate metal or increase the pH. Hence, the microenvironment of *E. faecalis* biofilms is important in causing root canal infections.

Taken together, *E. faecalis* relies on biofilms for surface attachment and resisting host environmental stressors. Moreover, specific biofilm factors can be niche-specific. However, given the clinical importance of *E. faecalis* biofilms during infection, more studies are needed to better understand and develop treatments for these infections.

1.2.5 Mechanisms of antibiotic resistance in *E. faecalis*

Enterococcus infections are a healthcare concern given their antibiotic tolerance and resistance. Enterococcus can acquire antibiotic resistance genes that enable survival during infections (Dale et al., 2015; Holmberg & Rasmussen, 2016; Komiyama et al., 2016; Shettigar et al., 2018). Moreover, the ability of *E. faecalis* to form biofilms can act as an external barrier to antibiotics (Dale et al., 2015; Dale, Nilson, Barnes, & Dunny, 2017). Overcoming these *E. faecalis* biofilms is key to treating these infections.

E. faecalis biofilms can contribute to antibiotic tolerance. In one study, *E. faecalis* isolated from urinary tract infections were able to form biofilms which conferred resistance to tigecycline but sensitivity to vancomycin treatment at its planktonic minimum concentration (MIC) (Hashem et al., 2017). In another study, biofilm-producing *E. faecalis* strains were enriched with antimicrobial resistance genes to antibiotics such as tetracycline, chloramphenicol, ciprofloxacin, and erythromycin, independent of their ability to form biofilms (Shettigar et al., 2018). This correlation suggests that *E. faecalis* can use both biofilms and carriage of antimicrobial resistance genes to resist treatment by antibiotics. However, the specific contribution of single *E. faecalis* virulence genes to biofilm-mediated resistance to specific antibiotics remains unknown.

The *E. faecalis* biofilm matrix allows encapsulation of the bacterial cells, protecting them from external antimicrobial assault (Dale et al., 2015; Hashem et al., 2017). *E. faecalis* biofilms are DNase sensitive and have decreased minimum biofilm eradication concentration (MBEC) after DNase degradation of its matrix (Torelli et al., 2017). However it is noted that the remaining *E. faecalis* biofilm cells are able to adhere strongly to the surfaces after DNase treatment, indicating that *E. faecalis* surface colonization may independent of its ability to form a biofilm matrix (Torelli et

al., 2017). *E. faecalis* biofilms mature as time progresses. In one study, *Enterococcus* biofilms grown for 120 hours showed a ≥ 2 -fold increase in MBEC for antibiotics compared to 24 hour grown biofilms *in vitro* (Holmberg & Rasmussen, 2016). The 120 hour biofilms were also recalcitrant to combinatorial treatment of multiple antibiotics compared to the 24 hours grown biofilm. This indicated that *E. faecalis* biofilms are able to mature to resist external antibiotics for persistence. Efforts have been made to try and understand the mechanisms underlying biofilm-mediated resistance of antibiotics. Transcriptomic profiling of *E. faecalis* comparing biofilm and planktonic cells *in vitro* revealed differential expression of two regulators of the arsenic resistance operon, *arsR* and *arsD*, and protease synthase, *paiA*, both of which are postulated to play a role in antibiotic resistance (Lim, Teh, & Thong, 2017; Seneviratne et al., 2017). In addition, the recently characterized glycosyltransferase Epa allows for biofilm retention on surfaces in the presence of daptomycin and gentamicin (Dale et al., 2017). Moreover, Epa-deficient *E. faecalis* biofilms resulted in decreased adhesion to Concanavalin A, although the mechanisms are not known. These observations indicate that *E. faecalis* can use biofilm formation as a strategy to confer tolerance to antibiotics.

E. faecalis in biofilms increase transfer of antibiotic resistance genes compared to planktonic cells. Several virulence factors of *E. faecalis* have been shown to be important for transfer of genetic material. The Ebp pilus and PrgABC can increase conjugation efficiency in *E. faecalis* (Bhatty et al., 2015; La Rosa, Montealegre, Singh, & Murray, 2016). In biofilm-associated infections, the transfer of vancomycin from *E. faecalis* to *S. aureus* has been observed clinically (Weigel et al., 2007). Therefore, *E. faecalis* biofilms can serve as reservoirs for transfer of antibiotic resistance genes and the emergence of resistant strains. Taken together, the ability to

form biofilms and intrinsic resistance to antibiotics serve as major healthcare barriers in the treatment of enterococcal infections.

1.3 *E. faecalis* wound infections

1.3.1 *E. faecalis* in wounds, not just a contamination

Bacterial wound infections can be clinically segregated into four stages: contamination, colonization, critical colonization, and infection (Edwards & Harding, 2004; Kirketerp-Møller, Zulkowski, & James, 2011; Siddiqui & Bernstein, 2010). Contamination represents the presence of host microbiota in wounds where the immune system can keep it in check. However, colonization of an invading bacterial species can occur. Critical colonization occurs when the local immune system fails to keep the colonizing microbes in check and represents a transition point toward infection. During critical colonization, microbes are not yet invading into the deeper layers of wounds but wound healing is inhibited. However, this concept is under debate as it is hard to define when critical colonization begins and ends (White, 2006). Finally, infection is defined by invading bacteria exceeding a threshold of microbial burden in wounds, triggering an inhibition of the wound healing response and a symptomatic inflammatory response. To date, the consequence of *E. faecalis* monoinfections in wounds remain poorly defined, and nothing is known about its mechanisms of colonization or infection.

Bacteria must be able to overcome medical interventions to allow them to persist in wounds. As discussed above, the ability to form bacterial biofilms can result in persistent infections. Current treatments to wound biofilms include debridement, topical anti-microbials, and anti-biofilm agents. Serial and regular debridement of wound biofilms can promote healing by reducing the bacterial burden (Wolcott et al., 2010). Moreover, treatment of wound sites with anti-microbials such as iodine and silver derivatives can aid in maintenance of an infection-free wound environment

(Jones & Kennedy., 2012). Furthermore, patients are able to reduce the microbial burden through coupling these anti-microbials with systemic antibiotics (Attinger & Wolcott., 2012). The shortcomings of the current treatment debridement regimens are 1) the inability to completely remove the bacteria leading to relapse, and 2) the need for regular debridement every 7 days or even more frequently. Additionally, any biofilm that is not sufficiently removed is likely to be recalcitrant to anti-microbial targeting. Hence, *E. faecalis* success as a wound pathogen is attributed to its ability to persistently colonize wound sites and to form biofilms.

Predisposing factors that may promote *E. faecalis* colonization of wounds include comorbidities such as diabetes, an impaired immune system, as well as consumption of antibiotics. Diabetes is a risk factor for wound infection by organisms including *E. faecalis* (Williams, Hilton, & Harding, 2004). Co-morbidities associated with diabetes include poor perfusion to wounds, an impaired immune response, and alteration in wound metabolic homeostasis (Pozzilli & Leslie, 1994). Elevated blood glucose levels (hyperglycemia) have been linked to reduced chemotactic activity but increased spontaneous attachment, and inflammation by neutrophils and macrophages (Delamaire et al., 1997; Kanter et al., 2012). Moreover, in diabetic conditions, neutrophils are more likely to undergo NETosis, where the lack of clearance of neutrophil extracellular traps (NETs) prevents the wound from closing (Flach & Czech, 2015; Wong et al., 2015). In diabetics, macrophages are primed towards a pro-inflammatory state and thus prevent tissue remodeling and wound healing (Kanter et al., 2012; Pozzilli & Leslie, 1994; Williams et al., 2004). Therefore, the failure of the host to recover, coupled with the inability of the immune system to properly maintain wound homeostasis, serve as risk factors which *E. faecalis* can take advantage of in order to infect wounds.

Another possible risk factor of developing *E. faecalis* wound infection can arise from the consumption of antibiotics. One study reported that intensive care patients had the same strain of VRE present in both their guts and on the skin surface, where patients suffering from fecal incontinence had a higher risk of VRE skin colonization (Beezhold et al., 1997). Moreover, a subset of these patients contracted VRE-mediated bacteremia, although the causative source of infection was unclear. Hence, cross-contamination and cross-infection of *E. faecalis* from adjacent niches can occur and possibly serve as a reservoir for skin colonization. Moreover, VRE can be present on the skin of hospitalized patients, and serves as a risk factor for spreading into wounds (Jackson et al., 2018). Thus, antibiotic stewardship and proper hygiene is necessary for preventing VRE spread into wounds.

1.3.2 A step towards understanding *Enterococcus faecalis* wound infections

Recent advancements in modeling wound infections can help in studying *E. faecalis* wound infections. An extensive review of model systems used to study bacterial biofilms has been reported (Goh et al., 2017; Lebeaux, Chauhan, Rendueles, & Beloin, 2013; Seaton, Hocking, & Gibran, 2015; J. F. Wang et al., 2001). A main goal of this thesis was to develop a model where we can measure the pathogenic potential of *E. faecalis* wound infections. The pathogenic potential of an organism is defined as a proportion of symptomatic individuals over the total population of infected people at a given initial inoculum (Casadevall, 2017). Defining the pathogenic potential allows us to draw relationships between symptoms as a consequence of infection with the bacterial burden, which overcomes limitations associated with defining bacterial “classical virulence” through relative comparisons to other infecting microbes or host niches (Casadevall, 2017). Moreover, we hypothesized that the majority of *E. faecalis* wound infections stem from colonization by a mixture of environmental and hospital-derived isolates, where the presence of

specific virulence factors is not known in either group. Using the pathogenic potential framework, bacterial wound infections can be defined symptomatically through the use of wound assessment measurements (Harries, Bosanquet, & Harding, 2016; Leaper et al., 2012). Moreover, our ability to control the inoculating load allows us to assess *E. faecalis* virulence in terms of colonization and infection.

The ability of *E. faecalis* to inhibit the wound healing process can determine its pathogenic potential during wound infection. The wound healing process typically consists of four phases: hemostasis, inflammation, proliferation, and maturation (Enoch, Grey, & Harding, 2006; Harries et al., 2016; Singer & Clark, 1999). The first stage, hemostasis, involves recruitment of clotting factors to the site of epithelial breach in order to seal the opening. Following hemostasis, the inflammatory phase occurs shortly after where there is recruitment of immune cells to the site of infection, which aids in clearance of foreign materials entering the wound site. A proliferative phase typically begins one day after wounding where collagen deposition, keratinocyte re-epithelialization, and angiogenesis occurs. Finally, a remodeling phase occurs when the wound is completely healed, and loosely packed ECM are replaced with more densely packed collagen to prevent abnormal scarring and strengthen the skin layer after closure (Xue & Jackson, 2015). The ability of animal models to closely mimic human wound healing processes needs to be considered before choosing the model to study host-pathogen interactions. The most recognized animal model for studying wound infections is the use of porcine wound excisional model due to their similarity with human skin structure (Sullivan, Eaglstein, Davis, & Mertz, 2001). By contrast, the rodent wound healing response is different from humans, mainly with respect to wound contraction due to the loose skin structure in rodents, and where the muscle layer beneath and adjacent the wound contract to promote healing (Chen et al., 2015; Davidson, Yu, & Opalenik, 2013). However, wound

closure by reepithelization can still be assessed through measuring the degree of epithelial regrowth (Chen et al., 2015). Given the ease of use and the ability to assess microbial burden during wound infections, we decided to develop an *in vivo* mouse excisional wound model to study *E. faecalis* infections which will be covered in **Chapter 2**.

Our overall aim of this dissertation was to characterize *E. faecalis* pathogenic potential during wound infections in both monomicrobial and polymicrobial settings. Moreover, the fitness factors identified in these studies will feed forward to future studies in understanding *E. faecalis* pathogenesis, and later, designing of specific diagnosis and therapeutics particularly for wound infections. Considering our overarching goals, we wanted to develop a mouse wound infection model to study *E. faecalis* wound infections and as a platform for understanding *E. faecalis* virulence factors in wounds. The specific outlines and aims of this thesis will be covered in the following section.

1.4 Thesis outline and aims

This dissertation is broken up into four specific aims:

Aim 1: To establish and characterize a mouse wound infection model to study *E. faecalis* wound infections. We will characterize the effects of different *E. faecalis* inoculating doses into wounds and determine the threshold for both colonization and infection. We will characterize the infection dynamics of *E. faecalis* temporally and employ fluorescence *in situ* hybridization (FISH) along with scanning electron microscopy to view its localization *in vivo*. To characterize the immune response to *E. faecalis* wound infections, we will utilize multiplex ELISAs and flow cytometry. Lastly, we will utilize histology to evaluate the effect of *E. faecalis* on wound healing. This portion of the work is mainly described in **Chapter 2**.

Aim 2: To perform comparative genomics on *E. faecalis* clinical wound isolates from human. In collaboration with Tan Tock Seng Hospital (Singapore), we prospectively obtained 49 *E. faecalis* strains isolated from wounds. To phenotypically characterize their pathogenic potential, we will perform antimicrobial susceptibility testing and assess their ability to form biofilms *in vitro*. To perform comparative genomics on these *E. faecalis* wound isolates, we will extract genomic DNA and perform whole genome sequencing. Furthermore, we will attempt to correlate our phenotypic observations of these clinical isolates with our genome-wide association analysis. This portion of the work is mainly described in **Chapter 3**.

Aim 3: To determine *E. faecalis* fitness factors that contribute to wound pathogenicity. We will identify virulence factors that are responsible for *E. faecalis* wound infection through two different genetic-based strategies. The first strategy entails a targeted approach utilizing the wound model we set up in **Chapter 2** and testing for mutants involved in adhesion, biofilm formation, and host immunity resistance as previously characterized in other *E. faecalis* infection models. In the second strategy, we will use an unbiased transposon sequencing approach to globally identify *E. faecalis* virulence factors in wounds. Identification of mutants will be described in **Chapter 4**.

Aim 4: To characterize the interactions between *S. aureus* and *E. faecalis* during wound infections. We will utilize macrocolony and 96-well biofilm assays to assess the interaction of both organisms *in vitro*. Furthermore, we will utilize our wound model established in **Chapter 2**, to look at mixed species interactions *in vivo* and assess their impact on the immune response by measuring cytokines, chemokines, and growth factors. To gain insight into the molecular mechanisms underlying *E. faecalis* and *S. aureus* mixed species interactions, we will perform transcriptomic analyses to identify pathways that are influenced during co-culture. These

observations will be described in the first half of **Chapter 5**, and the remaining half of the chapter will describe work performed in collaboration to understand the mechanisms underlying *E. faecalis* and *S. aureus* using a transposon library screen, together with MALDI-based biochemical analysis. These findings will conclude **Chapter 5** of this thesis.

Overall, the research undertaken in this thesis will serve as a platform to understand *E. faecalis* wound infections in mono-microbial and poly-microbial contexts. This work and model systems has many future applications, including use in testing antimicrobial compounds against *E. faecalis* wound infections in a physiologically relevant host environment of, as summarized in **Chapter 6**. Taken together, understanding virulence mechanisms of *E. faecalis* wound infections will allow for better diagnosis, and the information here can feed forward toward developing therapeutics against wound pathogens.

1.6 References

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Chapter 2: *E. faecalis* modulates immune activation and slows healing during wound infection

2.1 Introduction

Wound infections affect between 7-15% of hospitalized people globally (WHO, 2016). *Enterococcus faecalis* is frequently isolated from many types of wounds, including diabetic foot ulcers, burns, and surgical sites (Bowler, Duerden, & Armstrong, 2001; Dowd et al., 2008; Giacometti et al., 2000). In surgical site infections (SSIs), *E. faecalis* is the third most commonly isolated organism (Gjødtsbøl et al., 2006; System, 2004). Similarly in diabetic wounds, *E. faecalis* is among the top five most commonly isolated bacterial species (Citron et al., 2007; Mottola et al., 2016). *E. faecalis* infections are increasingly difficult to treat due to their intrinsic and acquired resistance to a range of antibiotics (Hollenbeck & Rice, 2012). Despite the high frequency of *E. faecalis* in wound infections, little is known about its pathogenic strategies in this niche.

Microbes present within wounds can inhibit the wound healing processes, and contribute to the development of chronic wounds (Edwards & Harding, 2004; Flora, 2002; White, Cooper, & Kingsley, 2001). Bacterial wound titers of $<10^6$ CFU/mL in exudates correlate with better healing in chronic wounds (Bendy, 1964; Levine, Lindberg, Mason, & Pruitt, 1976). However, the reliability of using swabs and tissue biopsies to quantify bacterial titers in wounds is poor, and is confounded by the complexity and number of microorganisms present at the wound site and site of sampling (Bowler et al., 2001; Kallstrom, 2014). Predisposing factors that associated with a higher risk of wound infection include an impaired immune system or other comorbidities such as diabetes (Citron et al., 2007; Thomsen et al., 2010). Overall, the interplay between the health of the host and the virulence of the bacteria can determine the outcome of wound healing.

Bacterial biofilms, which are often polymicrobial in nature, are a major factor in wound healing (Hurlow et al., 2015; Metcalf & Bowler, 2015; Tay, Chong, & Kline, 2016). Biofilm-associated wound infections are associated with a poorer healing prognosis (Hurlow et al., 2015; Metcalf & Bowler, 2015). Moreover, biofilm formation promotes survival and persistence of infecting microbial species because it facilitates defense against the host immune response (Hall-Stoodley & Stoodley, 2009). *E. faecalis* wound isolates can readily form biofilms *in vitro*, suggesting that biofilm formation may contribute to their persistence during wound infection (Hurlow et al., 2015; Metcalf & Bowler, 2015). Bacteria can exist as aggregates in wounds that are encapsulated within a biofilm matrix (Hurlow, Blanz, & Gaddy, 2016; Zhao et al., 2010). However, there are no clear *in vivo* markers of *E. faecalis* biofilm formation in wounds. Instead, biofilms in wounds are presently defined by the presence of bacteria forming microcolonies based on fluorescent microscopy techniques (Kirketerp-Møller et al., 2008; Thomsen et al., 2010) or electron microscopy (Ashrafi et al., 2018; Hurlow et al., 2016; Zhao et al., 2010). These biofilms form complex structures that act as a physical barrier to protect embedded bacterial cells against immune cell penetration (Seth et al., 2012; Zhao et al., 2010). *E. faecalis* biofilm or microcolony formation has never been described or reported within human wounds (Dalton et al., 2011; Hurlow et al., 2016). Host factors can also contribute directly to biofilm formation. During CAUTI, *E. faecalis* can directly bind host fibrinogen that is deposited on urinary catheters, as a substrate for biofilm formation (Flores-Mireles, Pinkner, Caparon, & Hultgren, 2014; Flores-Mireles et al., 2016).

We sought to optimize an *in vivo* model of *E. faecalis* wound infection in excisional wounds, meant to mimic the surgical wounds which *E. faecalis* is commonly found (Dowd et al., 2008; Giacometti et al., 2000; WHO, 2016). Previously, the mouse wound excisional model was primarily used to study wound

healing processes (Cross, Naylor, Coleman, & Teo, 1995; Kirchner et al., 2003; Stiernberg et al., 2000). This wound excisional model is sometimes modified to include a splint, which promotes wound healing by re-epithelialization and granulation tissue formation similar to human skin wound healing, as compared to healing by wound contraction which occurs in the absence of the splint and which is what occurs in mice (Brandenburg et al., 2015; Schierle, De la Garza, Mustoe, & Galiano, 2009; Wang, Ge, Tredget, & Wu, 2013). However, we opted for the non-splinted model for several reasons: 1) Our goal was to utilize this wound model to probe for bacterial virulence factors implicated in wound infection and since the non-splinted wound model has previously been used to examine bacterial factors required for wound infection by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus* (DeLeon et al., 2014; Thompson et al., 2014; Turner, Everett, Trivedi, Rumbaugh, & Whiteley, 2014; Watters et al., 2013), we opted for the non-splinted model as well. 2) The non-splinted model still allows for the measurement of wound healing parameters, relying on histological analysis for assessment (DeLeon et al., 2014; Thompson et al., 2014; Turner et al., 2014; Watters et al., 2013). 3) The use of the non-splinted model being less time consuming and able to identify bacterial mutants of interest. Hence, we decided to utilize the wound excisional model to look at *E. faecalis* virulence *in vivo*.

In this chapter, we characterized the dynamics of *E. faecalis* infection in a wound excisional model, looking at both non-diabetic mice and diabetic mice. We have established the minimal doses of *E. faecalis* required for colonization and infection of wounds and describe its infection dynamics *in vivo*. We applied both fluorescence *in situ* hybridization techniques and scanning electron microscopy to look at the spatial distribution of *E. faecalis* during *in vivo* wound infection. Finally, we show that modulation of early inflammatory responses and delayed wound healing

are coupled with persistence of *E. faecalis* within wounds, with diabetic wounds having a greater inflammatory response than non-diabetic wounds. Overall, this is the first thorough characterization of *E. faecalis* wound infection using a mouse model of surgical wounds.

2.2 Results

2.2.1 The minimum colonization dose for *E. faecalis* in wounds is 10 CFU

To investigate the colonization and infection dynamics of *E. faecalis* OG1RF in wounds, we first determined the minimum colonization dose required to colonize $\geq 80\%$ of excisional wounds in C57BL/6 mice (CD_{80}). We examined a range of infection inocula, from 10^1 to 10^6 CFU, at 24 hours post inoculation (hpi) and determined the CD_{80} to be 5.8×10^1 CFU, which resulted in 80% of the mice displaying recoverable CFU (**Fig 2.1A**). The CD_{100} was 6.2×10^2 CFU. In general, we observed that the median recoverable CFU for all inocula at 24 hpi was similar to the initial inoculum. At inocula of 10^2 , we observed no visible, macroscopic signs of inflammation (**Fig 2.1B**). By contrast, at 24 hpi, inocula of 10^6 resulted in visible, macroscopic inflammation with redness and accompanied by presence of serous exudates at the wound site of all infected mice (**Fig 2.1B**). Henceforth, we defined 10^6 as the infectious dose (ID_{90}). These findings suggest that the initial bacterial inoculum can result in wounds of two different states: colonization or infection.

Figure 2.1. The CD_{50} of *E. faecalis* wound infection is 10^1 CFU. (A) Male C57BL/6 mice were wounded and infected with *E. faecalis* OG1RF with an inocula of 5.8×10^1 , 6.2×10^2 , 6.6×10^3 or 2.8×10^6 colony forming units (CFU). Wounds were harvested at 24 hpi and the recovered bacteria enumerated. Each dot represents one mouse, and the solid horizontal lines indicate the median. The horizontal dashed line indicates the limit of detection, $n=5$. (B) Male C57BL/6 mice were wounded and infected with *E. faecalis* OG1RF with an inoculum of 10^2 or 10^6 CFU or mock-infected with PBS. Pictures were taken at 24 hours post infection. Images shown are representative of three independent experiments.

2.2.2 *E. faecalis* infection is associated with high titer persistence in wounds

To further investigate the differences between low-inoculum colonization and high-inoculum infection dynamics, we inoculated mice with the CD_{80} colonization dose of 10^2 CFU, or the ID_{90} infection dose of 10^6 CFU, and monitored the mice for 7 days post inoculation (dpi). We observed that, regardless of the initial inoculum, viable bacteria were recovered at all time points (24 hpi to 7 dpi). However, after a 10^2 CFU inoculation, *E. faecalis* persisted at 10^2 CFU and only decreased at 7 dpi where we could not recover *E. faecalis* CFU from 5 out of 8 of the mice at the limit of detection (**Fig 2.2A**). By contrast, when mice were inoculated with 10^6 CFU, we

observed a rapid increase to 10^8 CFU by 8 hpi, followed by a decrease at 3 dpi to 10^5 CFU which was maintained throughout the course of the experiment (**Fig 2.2B**). Consistent with this, at inocula of 10^6 , we observed visible inflammation and wound exudates only at 8 and 24 hpi, which resolved after 2 dpi (**Fig 2.2B**).

E. faecalis wound infection dynamics were not strain-specific because the clinical blood isolate *E. faecalis* V583 (Bourgogne et al., 2008) displayed similar infection kinetics to that of strain OG1RF (**Fig 2.2C,D**). In addition, clinical *E. faecalis* wound isolates inoculated at the infection dose resulted in similar high titer wound infections at 8 hpi (**Fig 2.3**). Together, these results demonstrate and confirm that the initial inoculum can result in the following states: colonization in the absence of increased *E. faecalis* titers or overt inflammation, or infection associated with acute bacterial replication and overt inflammation.

Figure 2.2. Colonization and infection dynamics of *E. faecalis* in wounds. Wounds were harvested at the indicated time points post-inoculation and the colony forming units (CFU) enumerated. Mice were inoculated with (A) 10^2 CFU of OG1RF, (B) 10^6 CFU of OG1RF, (C) 10^2 CFU of V583, or (D) 10^6 CFU of V583. Each dot represents one mouse, and the solid horizontal lines indicate the median, N=2-3, n=5-15. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$.

Figure 2.3. *E. faecalis* infection of wounds is not strain specific. Male C57BL/6 mice were wounded and infected with 10⁶ CFU of 4 different *E. faecalis* clinical wound isolates. Wounds were harvested at 8 hpi and CFU were enumerated. Each dot represents one mouse, and the solid horizontal lines indicate the median. N=1, n=5.

2.2.3 *E. faecalis* forms microcolonies on the wound surface

Since we observed marked changes in the CFU recovered from infected wounds over time; with macroscopic inflammation present at 8 hpi but absent at 3dpi, we hypothesized that the spatial distribution of *E. faecalis* may also vary across time during infection. To address this question, we performed scanning electron microscopy (SEM) at 8 hpi and 3 dpi which represent the peak of infection and the onset of stable persistence, respectively. At 8 hpi, we observed *E. faecalis* microcolonies on infected wounds that appeared to be encased within a matrix, indicative of early biofilm development, which are absent in our mock-infected group (**Fig 2.4A,B**). This is consistent with findings observed of microcolonies clusters observed in cocci biofilm formers observed clinically (Ashrafi et al., 2018; Hurlow et

al., 2016). By contrast, at 3 dpi we were unable to detect *E. faecalis* on the surface of the wounds neither at the wound edge nor the wound bed (**Fig 2.4C**). Since the CFU burden was still high at 3 dpi, we reasoned that *E. faecalis* may instead be embedded within host tissue. To determine the spatial localization of sub-superficial *E. faecalis* in 3 dpi wounds, we performed fluorescence *in-situ* hybridization (FISH) on 3 dpi wound samples. Using FISH probes specific for *E. faecalis* and 16S ribosomal RNA, we observed *E. faecalis* microcolonies at the wound edge (**Fig 2.5A,C**), and in the wound bed (**Fig 2.5B,C**). These results suggest that *E. faecalis* form biofilm-like microcolonies on wound surfaces and can be encapsulated or internalized within the host tissues. We postulate that both properties may contribute to protection from the host immune response and persistence within wounds.

Figure 2.4. *E. faecalis* forms microcolonies in acutely infected wounds. Mice were wounded and infected with 10⁶ CFU *E. faecalis* OG1RF or mock-infected with PBS. Wounds were harvested at the indicated post-infection time points for scanning electron microscopy. *E. faecalis* microcolonies encapsulated by fibrous material were visible at 8 hpi (white arrows, **A**), but not in mock-infected wounds (**B**) or in infected wounds at 3 dpi (**C**). Bar represents 5 μ m. Images shown are representative of three independent experiments.

Figure 2.5. *E. faecalis* is present at the wound edge and in the wound bed. Male C57BL/6 mice were wounded and infected with 10^6 CFU of *E. faecalis* OG1RF. Wounds were harvested at 3 days post-infection, cryosectioned, and subjected to (A,B) FISH or (C) H&E staining. (A) *E. faecalis* specific probe or probe specific for the domain bacteria (Eub) were used for FISH. The brightfield channel is represented in greyscale. Red and blue arrows (C) correspond to the red and blue boxes (A,B) and represent the wound edge and wound bed, respectively. (A,B) Bar represents 2 μ m. Images shown are representative of three independent experiments.

2.2.4 High titer *E. faecalis* infection alters wound healing and delays wound closure

Infection of wounds by *P. aeruginosa* and *S. aureus* correlate with delayed re-epithelization and wound healing (Goldufsky et al., 2015; Schierle et al., 2009). To determine whether *E. faecalis* similarly affects wound healing, we performed histology on skin tissue obtained from wounds of infected mice at 7 dpi. Hemotoxylin and eosin (H&E) staining revealed a hyper-thickened epidermis, indicative of non-progressive wound healing, with delayed closure in the infected tissues which was not seen in the wounded, mock-infected controls (**Fig 2.6A,B**). Moreover, we also observed large numbers of polymorphonuclear leukocytes in H&E stained infected samples as late as 7 dpi as compared to mock-infected controls (**Fig 2.6A**). In addition, granulation tissue, which is indicative of dermal healing, was not properly formed in infected wounds, whereas healing was visible in mock-infected controls (**Fig 2.6A,B**). These observations show that high titer *E. faecalis* infection negatively affects the wound healing process and delays the onset of wound closure.

Figure 2.6. *E. faecalis* infection alters wound healing dynamics. Wounds were harvested at 7 dpi and subjected to H&E staining. **(A,B)** Red and blue boxes represent the wound edge for the infected wounds and mock-infected controls, respectively. **(B)** Higher magnification images of the boxes depicted in A and dashed line indicate the wound edge. Clusters of polymorphonuclear leukocytes are present at the 7 dpi wound (white arrows) but absent from the mock-infected wound. Bar represents 20 μ m. Images are representative observations from three independent samples examined.

2.2.5 *E. faecalis* can persist within wounds while escaping immune clearance

We next hypothesized that *E. faecalis* might escape host detection during wound infection, contributing to its ability to persist and delay wound healing. Therefore, to examine the host immune response to *E. faecalis* infection, we first performed cytokine, growth factor and chemokine analysis on supernatants from wound homogenates inoculated with either 10^2 or 10^6 CFU, or PBS. At both 8 hpi and 3 dpi, wounds inoculated with 10^2 *E. faecalis* CFU had cytokine and growth factor levels similar to the mock-infected controls (**Fig 2.7A,B**). By contrast, wounds infected with 10^6 CFU displayed significantly higher levels of the inflammatory cytokine IL-1b, as well as growth factors/chemokines CSF3, CXCL1, CCL2, CCL3 and CCL4 compared to the mock-infected controls at 8 hpi (**Fig 2.7A, Appendix Fig 2.1A**), when macroscopic inflammation was observed. At 3 dpi, when *E. faecalis* wound titers resolved to 10^5 CFU (**Fig 2.2B**), we observed significantly lower levels of IL-2, IL-5, IL-10, IL12-p70, CCL11, IFN- γ and CSF2 compared to both 10^2 CFU-inoculated and mock-infected controls (**Fig 2.7B, Appendix Fig 2.1B**). Reduced cytokine and chemokine levels during steady state infection suggest that *E. faecalis* can modulate the host immune response in wounds to promote persistence.

To gain further insight into the spectrum of soluble factors that were most associated with *E. faecalis* immune modulation during infection, we performed principal component analysis (PCA) (**Appendix Fig 2.1C**). The PCA profiles of wounds infected with 10^6 CFU at 8 hpi and 3 dpi were distinct and clustered separately, confirming that high inoculum infection results in a temporally distinct inflammatory profile (**Appendix Fig 2.1C**). Differences in IL-1 β , IL-2, IL-12p70 and CCL11 specifically explained the variation between the PCA profiles and best represented differences between all sample groups. Among these, IL-2, IL-12p70 and CCL11 were significantly decreased in the 10^6 CFU infected group when compared to

the mock-infected controls, suggesting that downregulation of these cytokines in particular may be associated with an attenuated immune response (**Appendix Fig 2.1B**).

To complement the analysis of soluble immune effectors, we performed flow cytometry to quantify the immune cell types present during infection (**Appendix Fig 2.2**). Nearly all immune cell types examined were significantly increased in the infected wounds compared to the healthy skin at 1 and 3 dpi (**Appendix Fig 2.2**); and neutrophil infiltration correlated with neutrophil-related chemokine expression in the infected wound compared to the mock-infected controls (**Fig 2.7A,C**). Notably, at 3 dpi, there were significantly more MHCII- macrophages in the infected wounds compared to mock-infected controls (**Fig 2.7D**). Despite the presence of significant immune infiltrates at 3 dpi, the *E. faecalis* bacterial burden in the wounds persisted at $>10^5$ CFU.

Taken together, these data demonstrate that high titer inoculation into healthy mice results in high titer wound infection, is associated with an acute inflammatory response concomitant with the peak of infection. The resolution of acute high titer infection to a lower steady state infection at 3 dpi corresponds both a suppression of cytokine and chemokine levels yet the presence of immune cellular infiltrate, suggests a complex immunomodulatory program that is insufficient to resolve acute *E. faecalis* wound infection.

Figure 2.7. *E. faecalis* modulates the soluble and cellular host response at the wound site. Mice were wounded and infected with 10² CFU or 10⁶ CFU of *E. faecalis*, or mock-infected with PBS. At the indicated times, wounds were processed into single cell suspensions and subjected to (A-B) cytokine analysis, shown in pg/mL. Total number of (C) neutrophils (CD45⁺ MHCII⁻ CD11b⁺ Ly6G⁺) and (D) macrophages (CD45⁺ MHCII⁻ CD11b⁺ Ly6C⁻ CD64⁺) infiltrating into and accumulating in the skin analyzed by flow cytometry. N=2, n=5. Error bars represent the standard error of mean. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-hoc test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

2.2.6 *E. faecalis* displayed sustained pro-inflammatory response in diabetic wounds compared to non-diabetic wounds

Since *E. faecalis* is commonly isolated from diabetic wounds, we hypothesized that diabetic mice might be more susceptible to *E. faecalis* wound infection, which would correlate with reduced CD₈₀ or ID₉₀ as compared to healthy mice, and would result in higher titer infection and persistence states due to the impaired immune system and delayed host response in diabetic mice. To test this, we first inoculated 10⁶ and 10² *E. faecalis* CFU into excisional wounds in *db/db* homozygous mice, which are a common model for type 2 diabetes (Michaels et al., 2007; Zhao et al., 2010). The *db/db* mice possess mutations in its leptin receptors, resulting in development of diabetic symptoms of obesity-induced hyperglycemia and hyperinsulinemia. Surprisingly, *db/db* mice were not more susceptible to *E. faecalis* wound colonization or infection. At the 10² colonization dose, we could recover only *E. faecalis* CFU from 83% of the mice at 1 dpi, and from 66% of the mice at 3 dpi (**Fig 2.8A**), as compared to normal mice where we recovered 100% and 80%, respectively. Moreover, the CD₈₀ for *E. faecalis* in diabetic wounds was 2.84 x 10² CFU, higher than in non-diabetic mice (**Fig 2.1A, 2.8A**), together suggesting that *E. faecalis* is less fit during low titer persistence in diabetic wounds compared to non-diabetic wounds. In addition, at an inocula of 10⁶ CFU, we observed no difference in recovered *E. faecalis* CFU titers between diabetic and non-diabetic mice at all time points (**Fig 2.2B, 2.8B**).

Figure 2.8. Colonization and infection dynamics of *E. faecalis* in diabetic wounds. Wounds were harvested at the indicated time points post-inoculation and the colony forming units (CFU) enumerated. Mice were inoculated with (A) 10^2 CFU of OG1RF, or (B) 10^6 CFU of OG1RF. Each dot represents one mouse, and the solid horizontal lines indicate the median, N=2, n=3. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$.

To determine whether the soluble host immune response to *E. faecalis* infection differed between diabetic and non-diabetic wounds, we measured cytokine, growth factor and chemokine from diabetic wound inoculated with either 10^2 or 10^6 CFU, or PBS. At both 8 hpi and 3 dpi, we observed sustained increased levels of cytokines IL-1 β , IL-3, IL-4, IL-10, CSF3, CSF2, CXCL1, TNF- α , CCL3, and IL-17a (Fig 2.9A,B, Appendix Fig 2.3). This is consistent with the prediction that diabetic wounds tend to have a sustained pro-inflammatory response associated with improper wound healing, accompanied by an increase influx of pro-inflammatory cells in wounds (Koh & DiPietro, 2011; Wong et al., 2015). By contrast, we observed lower levels of IL-5 and IFN- γ in diabetic wounds infected at 10^6 CFU at both 8 hpi and 3 dpi compared to mock-infected mice (Fig 2.9A,B, Appendix Fig 2.3). IL-5 and IFN- γ are the only two cytokines measured that were both lower in both diabetic and non-diabetic wounds as compared to mock-infected controls, suggesting that *E. faecalis* might modulate the host response through these two cytokines or that downregulation

of these cytokines might be a direct consequence of *E. faecalis* infection in wounds (**Fig 2.9**). PCA analysis of the global soluble response to *E. faecalis* in diabetic versus non-diabetic wounds could be separated by protein expression relating to TNF- α , CCL4, and CCL5 expression between the different genotypes (**Fig 2.9E**). Taken together, *E. faecalis* contributes to a persistent and longer pro-inflammatory response which inhibits the wound healing and may promote its persistence in non-diabetic mice. Furthermore, these data suggest that biomarkers differentiating *E. faecalis* diabetic wounds and non-diabetic wounds could serve as potential predictors for *E. faecalis* persistence in wounds.

Figure 2.9. *E. faecalis* modulates the soluble and cellular host response at the wound site. Mice were wounded and infected with 10^2 CFU or 10^6 CFU of *E. faecalis*, or mock-infected with PBS in (A-B) diabetic mice, or (C-D) non-diabetic

mice. At the indicated times, wounds were processed into single cell suspensions and subjected to cytokine analysis, shown in pg/mL. Luminex data sets for normal mice are the same as **Figure 2.7**, shown again here for ease of comparison with diabetic data. **(E)** Principal component analysis was done for all analytes measured at all time points. Principal component 1 (PCA1) and principal component 2 (PCA2) represent 30.7% and 26.4% of the variations of all analytes respectively. Each dot represents one mouse and are clustered plotted according to their positions in PCA1 and PCA2. Clustering was performed to group analyte profiles measured at 3 dpi and represented as follows: Non-diabetic mice 10^6 CFU; red circle, diabetic mice 10^6 CFU; yellow triangle, non-diabetic mice 10^2 CFU; green square, diabetic mice 10^2 CFU; cross, non-diabetic mock-infected mice; box with cross, diabetic mock-infected mice; star. Arrows in the biplot represent analytes that separates the different individuals in the plot. Ellipses represent the standard error of variation by each group.

2.3 Discussion and conclusion

SSIs and diabetic wounds are prevalent and can extend the average hospitalization time by 5 to 17 days (WHO, 2016). These open wounds have an increased tendency to become infected, leading to poorer prognoses and poorer wound healing. Despite the prevalence and clinical importance of *E. faecalis* wound infection, we know nothing of its pathogenic mechanisms in this infection setting. Here, we established a modified mouse wound excisional model using diabetic and non-diabetic mice to study the infection dynamics of *E. faecalis* in a model for surgical site infections and diabetic wounds respectively.

We show that acute high titer *E. faecalis* wound infection at $\geq 10^6$ CFU is associated with a robust cellular host immune response and visible signs of inflammation in both diabetic and non-diabetic mice, whereas inflammation is suppressed or absent in lower titer infections. Our observations are consistent with reports showing that bacterial counts of $\geq 10^6$ perturb healing in human wounds (Bendy Jr et al., 1963; Robson & Heggers, 1970). Surprisingly, we did not observe a change in infection dynamics of *E. faecalis* between diabetic and non-diabetic wounds at 8 hpi or 3 dpi, suggesting that the immune system in both diabetic and non-diabetic mice can limit the initial infection by *E. faecalis* in wounds, but cannot prevent long term persistence. This finding suggests that the clearance threshold of *E. faecalis* by the host immune system is similar in both diabetic and non-diabetic wounds. However, despite an early robust inflammatory response with accompanying neutrophilic response (at least in non-diabetic mice), *E. faecalis* can persist in the local wound site regardless of the inoculum load, at levels similar to the original inoculating dose. Furthermore, we observed *E. faecalis* in both the wound bed and at the epidermal wound edge at 3 dpi in non-diabetic mice, suggesting that *E. faecalis* reservoirs within host cells may promote persistence in this niche in non-diabetic mice.

Consistent with reports that most wound infections involve biofilms and that microcolony formation precedes biofilm formation in wounds (Hurlow et al., 2016; Hurlow et al., 2015; Metcalf & Bowler, 2015), we observed the presence of microcolonies at the surface of *E. faecalis* infected wounds at 8 hpi. These findings suggest that *E. faecalis* are able to cluster within host wounds and lead to biofilm formation which may contribute to antimicrobial and immune tolerance, leading to persistence in wounds. At 1 dpi, we observed a strong pro-inflammatory cytokine and chemokine response, as well as a 2-log increase in neutrophils at the site of infection compared to mock-infected wounds, which began to dissipate by 3 dpi. We also observed significantly more MHCII- macrophages in infected wounds compared to mock-infected wounds and we only observed this at 3dpi, when bacterial titers stabilized at 10^5 CFU/wound. It is tempting to speculate that *E. faecalis* can modulate macrophages *in vivo* leading to its persistence in wounds, possibly by either inhibiting macrophages chemotaxis or response. Consistent with this, we and others have shown that *E. faecalis* can survive within and suppress immune activation of macrophages through the NF- κ B pathway (Tien et al., 2017; Jun Zou & Shankar, 2014; J. Zou & Shankar, 2016).

Importantly, we show that *E. faecalis* wound infection results in immunomodulation. At 3 dpi, IL-2, IL-5, IL-10, IL-12p70, CCL11, IFN- γ , and CSF2 levels were significantly lower in infected wounds compared to mock-infected wounds, suggesting active immune suppression at the cytokine and chemokine level (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008; Leoni, Neumann, Sumagin, Denning, & Nusrat, 2015). However, this differs for *E. faecalis* infection in diabetic wounds where we observed sustained inflammation at 3 dpi with induction of IL-1 β , IL-3, IL-4, IL-10, CSF3, CSF2, CXCL1, TNF- α , CCL3, and IL-17a. Interestingly, IL-5 and IFN- γ are lower in infected animals at both 8 hpi and 3 dpi.

We speculate that *E. faecalis* modulation of the host response occurs in both diabetic and non-diabetic wounds, converging at the expression of IL-5 and IFN- γ . However in non-diabetic wounds, we still observed significant immune cell infiltrate at 3 dpi, indicating that immune modulation may be insufficient to limit a full inflammatory response. Additionally, principal component analysis of the soluble factors in the different infection contexts revealed that increased levels of TNF- α , CCL4, and CCL5 can serve as indicators to differentiate between diabetic mice infected with *E. faecalis* at the infection dose compared to non-infected and/or at the colonization dose of *E. faecalis* in diabetic wounds. Moreover, we observed that these three cytokines were lower in infected non-diabetic mice compared to the rest of the groups profiled, further supporting that *E. faecalis* is able to modulate the host response in these mice. Nevertheless, the pro-inflammatory cellular infiltrate was not able to clear *E. faecalis* from the wounds. Thus, these data suggest that *E. faecalis* wound infection includes an active immune evasion or immune suppression component, which contributes to high titer infection and long-term persistence, and may lead to the development of a chronic, non-healing wound. While in diabetic wounds, sustained inflammation does not aid in clearance of *E. faecalis* titers which may be due to the anergic activity of immune cells due to hyperglycemia in diabetic wounds (Fadini et al., 2016; Hesketh, Sahin, West, & Murray, 2017; Wilgus, Roy, & McDaniel, 2013; Williams, Hilton, & Harding, 2004). Further, even modest *E. faecalis* mediated immune suppression may provide an advantage for co-infecting organisms commonly found with *E. faecalis* in polymicrobial wound infections (Keogh et al., 2016; Tay et al., 2016). Given the widespread prevalence of Enterococcal wound infections, further studies into factors that promote *E. faecalis* pathogenesis in wounds and its consequences on wound healing are critical.

2.4 Materials and Methods

2.4.1 Bacterial strains and growth conditions

Strains used are shown in **Appendix Table 2.1**. For mouse infections, *E. faecalis* was grown statically at 37 °C for 15-18 hours in Brain Heart Infusion medium (Neogen, Lansing, USA) without antibiotics. Clinical strains isolated from patient wounds were provided by Tan Tock Seng Hospital, Singapore.

2.4.2 Mouse wound excisional model

Mouse wound infections were modified from a previous study (Keogh et al., 2016). Male C57BL/6 mice (7-8 weeks old, 22 to 25 g; InVivos, Singapore) or Male *db/db* (BKS.Cg-*Dock7^m* *+/+* *Lepr^{db}*/J) mice (7-8 weeks old, The Jackson Laboratory, USA) were anesthetized with 3% isoflurane and the dorsal hair trimmed. Following trimming, Nair™ cream (Church and Dwight Co, Charles Ewing Boulevard, USA) was applied and the fine hair removed via shaving with a scalpel. This two-step shaving method ensured the wound dressing remains for >5 days without detachment. The skin was then disinfected with 70% ethanol. A 6-mm biopsy punch (Integra Miltex, New York, USA) was used to create a full-thickness wound and 10 µL of the respective bacteria inoculum applied. The wound site was then sealed with a transparent dressing (Tegaderm™ 3M, St Paul Minnesota, USA). At indicated time points, mice were euthanized and a 1x1 cm piece of skin surrounding the wound site was excised into sterile PBS. Excised wounds were homogenized and viable bacteria enumerated by plating onto BHI agar with (**Appendix Table 2.1**) and without antibiotics to check for contamination. Mice with contaminants were excluded from our datasets and subsequent analysis. For coinfection experiments, competitive index (CI) was determined with the following formula: $CI = (OG1RF (output)/OG1X (output))/(OG1RF (input)/OG1X (input))$.

2.4.3 Histology

Wound tissues were excised as described above and fixed in 4% paraformaldehyde in 1x PBS (pH 7.2) for 24 hours at 4 °C. Samples were then submerged in 15% and 30% sucrose gradient for 24 hours each, embedded in Optimal Cutting Temperature (OCT) embedding media (Sakura, California, USA), and frozen in liquid nitrogen. 10 µm thin sections were then obtained with a Leica CM1860 UV cryostat (Leica Biosystems, Ernst-Leitz Strasse, Germany) and stained with hemotoxylin and eosin (H&E). Images of H&E sections were acquired using an Axio Scan.Z1 slide scanner (Carl Zeiss, Göttingen, Germany) fitted with a 20×/0.8 Apochrome objective.

2.4.4 Gene probe and Fluorescence *in-situ* Hybridization (FISH)

E. faecalis was detected with the oligonucleotide probe 5'-GGTGTTGTTAGCATTTTCG/Cy3/-3' (IDT Technologies, Iowa, United States). Probe 5'-GCTGCCTCCCGTAGGAGT/AlexaFluor®488/-3' (IDT Technologies, Iowa, United States) targets the 16S rRNA of organisms in the domain of *Bacteria* (Kempf, Trebesius, & Autenrieth, 2000). Cryo-sectioned tissue sections were dehydrated in a graded ethanol series (70% and 80%) for 3 mins each. Tissue sections were then immersed in a 0.2% Sudan Black solution (prepared in 96% ethanol) for 20 minutes and washed thrice with a 0.02% Tween solution (prepared in 1X PBS). A total of 25 µL of 25% formamide hybridization buffer (20 mM Tris-HCl [pH 8.0], 5M NaCl, 0.1% sodium dodecyl sulfate, and 25% formamide) containing 100 pmol of the labelled probe (50 µg/mL stock) was added to the sections and incubated overnight at 48 °C. Slides were then immersed in 50 mL of wash buffer (0.5M EDTA and 5M NaCl, 20mM Tris-HCl [pH 8.0]) for 30 mins in a 46 °C water bath. After washing, slides were plunged into ice cold water for 5 seconds and left to dry. Hybridized samples were mounted using Citifluor™ (Citifluor Ltd, Enfield Cloisters, London)

and imaged using an Elyra PS.1 LSM780 inverted laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany) fitted with a 63×/1.4 Plan-Apochromat oil immersion objective using the Zeiss Zen Black 2012 SP2 software suite. Laser power and gain were kept constant between experiments.

2.4.5 Scanning Electron Microscopy

Excised skin samples were fixed using 2.5% glutaraldehyde (prepared in 0.1M PBS pH 7.4) for 48 hours at 4 °C and then washed three times with 0.1M PBS. Fixed samples were then dehydrated with a graded ethanol series (once with 30%, 50%, 70%, 80%, 90% and twice with 100% for 15 minutes at each step) together with gentle agitation. Samples were then subjected to amyl acetate immersion for 30 minutes. Samples were next critical point dried with the Bal-Tec CPD-030 Critical Point Dryer (Bal-Tec AG, Balzers, Liechtenstein) overnight and deposited onto SEM specimen stubs using NEM Tape (Nisshin Em. Co. Ltd, Tokyo, Japan). Samples were then sputter coated with gold using a Bal-Tec SCD 005 sputter coater (Bal-Tec AG, Balzers, Liechtenstein). Samples were viewed using a JSM-6360LV (JEOL, Tokyo, Japan) scanning electron microscope.

2.4.6 Cytokine Luminex® xMAP® analysis

Luminex MAP analysis was performed using the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, California, USA) as previously described (Rousseau et al., 2016).

2.4.7 Flow cytometry

Skin was cut into pieces and incubated in RPMI containing 10% serum, 0.2 mg/mL Collagenase IV (Roche, Basel, Switzerland) and 20000U/mL of DNase I (Roche, Basel, Switzerland) for 1 hour at 37 °C. Cells were then passed through a 19 G syringe and filtered through a 100 µm cell strainer (BD Biosciences, New Jersey,

USA) to obtain a homogenous cell suspension which was stained with the fluorochrome or biotin-conjugated monoclonal antibodies listed in **Appendix Table 2.2**. Multi-parameter analyses of cell suspensions were performed on a LSR II (BD Biosciences, San Jose, USA). Data were analyzed with FlowJo software (TreeStar, Oregon, USA).

2.4.8 Statistical Analyses

Statistical analyses were performed with GraphPad Prism software (Version 6.05 for Windows, California, United States) and are described in the respective figure legends. Principal component analysis performed done in R (Version 3.3.2) with the packages factoextra (Version 1.0.4) and FactoMineR (Version 1.34).

2.4.9 Ethics statement

All procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee (IACUC) in Nanyang Technological University (ARF SBS/NIEA0198Z).

2.5 Authors contribution

I initiated the study of this project. Tay Wei Hong, Adeline Yong Mei Hui, and I optimized the wound infection model. Tay Wei Hong and I performed the experiments in determining *E. faecalis* colonization and infection doses in this dissertation. I analyzed the *in vivo* data. Tay Wei Hong and I performed, designed, optimized, and analyzed the scanning electron microscopy and FISH experiments. Tay Wei Hong imaged the FISH sections. I performed and imaged the histological sectioning and analyzed the data, under guidance of Professor David Becker, Leigh Madden, and Milton Kwek Sheng Yi. I performed the diabetic infection wound model, assisted by Eliza Soh Ye Chen, Adeline Yong Mei Hui, Tay Wei Hong, and Sharon Goh Hwee Mian. Sharon Goh Ye Chen and I performed the Luminex

experiments. Baptiste Janela performed the flow cytometry experiments. I analyzed the flow cytometry data together with Baptiste Janela and Florent Ginhoux. Tay Wei Hong and I wrote and edited the paper for this study.

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Chapter 3: Comparative genomics and characterization of *Enterococcus faecalis* wound isolates

3.1 Introduction

Enterococcus faecalis (*E. faecalis*) wound infections are highly prevalent in surgical and chronic wounds (Dowd et al., 2008; Giacometti et al., 2000; Hurlow et al., 2015; Percival, Vuotto, Donelli, & Lipsky, 2015). *E. faecalis* is an opportunistic pathogen capable of colonizing and causing infection in wounds, particularly in chronic wounds where healing is delayed and immune defenses are lacking. Mechanisms of *E. faecalis* virulence in wounds remain poorly understood and represent a knowledge gap in this prevalent infection.

Besides our mono-infection *in vivo* studies of *E. faecalis* described in **Chapter 2**, mechanisms of *E. faecalis* biofilm formation in wounds are limited to polymicrobial infections (Dalton et al., 2011; Stacy et al., 2016; Tay, Chong, & Kline, 2016). One approach to better understand *E. faecalis* mono-infection is to identify genes enriched in *Enterococcus* clinical isolates. The presence of such *E. faecalis* genes encoding adhesins (*esp*, *agg*, *efaA*), proteases (*gelE*, *cylA*), and biofilm factors (*fsrABC*), can then be associated to its virulence. In one study, comparing 510 isolates derived from urine, wounds, blood, and the genital tract, invasive *Enterococcus* isolates were reported to have a higher percentage of *ace* whereas *esp*, *agg*, and *efaA* were enriched in non-invasive strains (Strateva et al., 2016). In contrast, another study of 197 urinary tract clinical isolates showed that the presence of *gelE* and *agg* were predictors of stronger *in vitro* biofilm formation (Hashem et al., 2017; Kristich, Li, Cvitkovitch, & Dunny, 2004; Soares et al., 2014). However, in *E. faecalis*, most clinical isolates are able to form biofilms regardless of their source of isolation, with less than half of the strains being *esp* or *gelE* positive (Rosa et al., 2006). More recently, in a study of 90 *E. faecalis* urinary tract isolates, *gelE* alone was not

predictive for virulence unless *fsrB* is also present, since *fsrB* is important in determining gelatinase and biofilm activity (Hashem et al., 2017). These data suggest that examining single “prototypic” biofilm-associated factors, such as Esp and/or Gelatinase, are often not predictive of virulence or biofilm forming capacity. Interestingly, the presence of the cytolysin, encoded by *cylA*, correlated with weak biofilm formation compared to isolates with *agg*, as well as *esp* presence, which formed better biofilms (Zheng et al., 2017; Zheng et al., 2017). Given that the conditions in which *E. faecalis* forms biofilms may vary greatly across host colonization and infection sites, understanding *E. faecalis* biofilm formation in a niche specific manner may help us to identify specific virulence factors that are important in wounds.

Different clonal complexes (CC) and sequence types (STs), as determined by multilocus sequence typing (MLST), have been correlated with *E. faecalis* antibiotic resistance and biofilm formation. MLST is a nucleotide-based typing tool to characterize a bacterial isolate with a ST (Jolley & Maiden, 2010). Correlation of these different STs to virulence has led to identification of “high-risk” strains of *E. faecalis*. CC and ST do not segregate with *E. faecalis* clinical isolates versus environmental isolates. Antibiotic resistance strains also cluster by MLST (Kuch et al., 2011; Nallapareddy, Duh, Singh, & Murray, 2002; Nallapareddy, Wenxiang, Weinstock, & Murray, 2005; Ruiz-Garbajosa et al., 2006). *E. faecalis* CC2 contains strains that are enriched for antibiotic genes in USA and Europe (Kuch et al., 2011; Nallapareddy et al., 2002; Nallapareddy et al., 2005). This lineage was originally designated as a risk group containing *E. faecalis* strains that are vancomycin resistant or have β -lactamase activity (Nallapareddy et al., 2002; Nallapareddy et al., 2005). Moreover, these strains are clonally related and enriched among hospital isolates. Hence, CC2 represents a high-risk group of *E. faecalis* isolates where antibiotic

resistance strains could emerge. In Asia, antibiotic resistant *E. faecalis* strains were also associated with CC2, as well as CC87, ST4 and ST470/471 groups (Getachew, Hassan, Zakaria, & Aziz, 2013; Ruiz-Garbajosa et al., 2006; Sun et al., 2012; Yang et al., 2015). Similarly, MLST analysis of 113 *E. faecalis* catheter-associated urinary tract infections (CAUTI) isolates from China demonstrated an enrichment of ST16 and ST179, where ST16 isolates were better to form strong biofilms *in vitro* (Zheng et al., 2017). By contrast, association of *E. faecalis* CAUTI isolates from Europe/USA to specific ST lineages is not well defined. Taken together, identification of geographically relevant “risk-groups” of *E. faecalis* can help inform healthcare associates when making decisions about treatment.

Comparative genomics has been successful in identifying strains that are associated with specific infection niches. For example, in *Staphylococcus aureus* (*S. aureus*), comparative genomic clustering of methicillin-resistant *S. aureus* (MRSA) strains by their metabolic potential enabled the prediction of infection severity and infection site (Bosi et al., 2016). In addition, comparative genomics allows for the identification of virulence genes enriched in a subset of clinical isolates. For example, the *S. aureus* clone EMRSA-16 is a dominant Oxacillin-resistant clone in the United Kingdom (Moore & Lindsay, 2002). Comparative genomics of 10 osteomyelitis isolates can differentiate laboratory strains of *S. aureus* from closely related EMRSA-16 clones, based on the presence of collagen binding adhesin (*cna*) and differential expression of quorum sensing genes in the EMRSA-16 clones (Cassat et al., 2005). Collectively, these types of studies show that comparative genomics can provide a platform in looking at isolates to provide predictive insights into bacterial pathogenic potential in a particular host niche.

In this chapter, we collected, surveyed, and correlated the phenotypic and genotypic characteristics of *E. faecalis* clinical wound isolates. We identified two

sequence lineages that were enriched in the clinical isolates. A subset of the isolates was able to form strong biofilms, which correlated with the presence of *ecbA*. Overall, we report the first comparative genomic analysis of *E. faecalis* clinical wound isolates and suggest that the presence of *ecbA* in *E. faecalis* isolates may be predictive of biofilm formation in wounds.

3.2 Results

3.2.1 *Enterococcus* species identification through amplification of *sodA*

In order to investigate the genomic diversity of *E. faecalis* isolated from human wounds, we obtained 49 strains (de-identified) from the laboratory medicine department at Tan Tock Seng Hospital (Singapore). The only clinical information we were able to obtain about these strains was: 1) the isolation site, and 2) whether they came from mixed or single species wound infections (**Appendix Table 3.1**). These strains were initially determined to be *E. faecalis* in the hospital laboratory using mass spectrometry. To reconfirm the species identification of *Enterococcus* wound isolates, we adopted a PCR based on amplification of the superoxide dismutase gene, *sodA*, which can discriminate between genus and species of Enterococci while having low false positives rates (Jackson, Fedorka-Cray, & Barrett, 2004). A 360 bp *sodA* product is discriminatory for identifying *E. faecalis*, because no other species of *Enterococcus* have the same sized *sodA* amplicon. Using this approach, we validated that all 49 Enterococcal wound isolates were *E. faecalis* (**Appendix Fig 3.1, representative isolates labeled EF_001 to EF_1010**). Negative controls included an *E. faecium* clinical wound isolate collected from the same hospital which did not have a 360 bp product, along with the negative controls *Escherichia coli* and *Staphylococcus aureus*.

3.2.2 Population structure of *E. faecalis* clinical wound isolates

Comparative genomics can be a useful approach in identifying commonalities between different genomes and correlating them to phenotypic traits, as well as tracking their evolutionary behavior (Edwards & Holt, 2013; Howden et al., 2011). We therefore sequenced the genomes of all 49 *E. faecalis* clinical wound isolates, and the reads were assembled and annotated. For phylogenetic analyses, we included 16 strains of *E. faecalis* which had been previously reported to represent the deepest diversity in the MLST phylogeny (Palmer et al., 2012). We used the fully sequenced *E. faecalis* strain V583 (Paulsen et al., 2003) as reference for construction of the phylogenetic tree. We detected 66% of V583 genes were also present in all 65 isolates, indicating that those 66% were core genes present in all isolates. The core genes were then used to construct a maximum-likelihood tree, which showed that the genome clustered into five distinct groups (**Fig 3.1**). When we instead used *Lactococcus lactis* as an outgroup, we found that *E. faecalis* does not form distinct clades, similar to previously reported results (data not shown) (Palmer et al., 2012). To determine if single allele differences correlate with the phylogenetic clusters we observed, we performed MLST analysis using seven housekeeping genes previously reported to have low diversification in *E. faecalis* (*yqiL*, *aroE*, *xpt*, *gki*, *pstS*, *gdh*, and *gyd*) (Ruiz-Garbajosa et al., 2006). We observed that 21/49 isolates were assigned to ST16/179, while 9/49 isolates were assigned to the CC2/6 (**Fig 3.2**). *E. faecalis* isolates present in the ST16/179 group have been reported to be more likely to form biofilms, while *E. faecalis* in the CC2/6 group are considered to be “high-risk” *E. faecalis* lineages due to their high carriage of antibiotic resistance genes (Leavis, Bonten, & Willems, 2006; McBride et al., 2007; Zheng, Bai, et al., 2017; Zhen et al., 2017). Furthermore, we observed that all of the MLST clusters identified separated

into distinct nodes in the phylogenetic tree (**Fig 3.1**). Taken together, our data suggest an enrichment of specific *E. faecalis* lineages isolated from wounds.

Figure 3.1. *E. faecalis* wound isolates clustered phylogenetically. Single nucleotide polymorphisms (SNPs) of genes present in all strains were used to resolve phylogenetic relationships between the 49 *E. faecalis* wound isolates (red) and 20 reference *E. faecalis* isolates (blue) chosen to represent the greatest diversity of *E. faecalis* phylogeny. The fully sequenced V583 genome is used as the reference for

maximum-likelihood tree generation using the harvest suite. Node values are indicated in black.

Figure 3.2. Majority of clinical *E. faecalis* wounds isolates are clustered in CC2/6 and ST16/179. The *E. faecalis* MLST scheme was downloaded from pubMLST. Sequence type (ST) definitions and sequencing reads were used as input for MLST determination using SRST2. Black numbers represent other ST designations in the clonal group. Pink represents the ST designation of *E. faecalis* wound isolates and new STs identified in our wound isolates are indicated in green.

3.3.3 Biofilm forming strains of *E. faecalis* correlate with ST groups

Microorganisms in wounds can form biofilms and inhibit the wound healing process (Hurlow et al., 2015; Johani et al., 2017; Percival et al., 2015; Rahim et al., 2017). Our previous findings revealed that *E. faecalis* can form microcolonies on the wound bed, indicative of biofilm formation *in vivo* (**Chapter 2, Fig 2.4A**). Since we observed a majority of *E. faecalis* clinical wound isolates in ST16/179, we wanted to assess the biofilm forming capabilities of those isolates *in vitro*. Therefore, we performed a 96-well crystal violet biofilm assay on all 49 clinical isolates in TSB medium. Under these conditions, we observed that 8 of the clinical isolates were able to form biofilms of biomass greater than the biofilm-positive lab strain OG1RF (hereafter called “high biofilm formers”) (**Fig 3.3A**). Because *E. faecalis* wound infection is highly associated with diabetic ulcers (Dowd et al., 2008; Shettigar, Bhat, Satyamoorthy, & Murali, 2018; Xavier, Sukumaran, Varma, Kumar, & Chellan, 2014), we hypothesized that the presence of glucose might further promote biofilm formation in these clinical isolates. In TSB medium supplemented with 0.25% glucose, we observed 27 of the clinical isolates (inclusive of the 8 high biofilm formers in TSB) were now able to form biofilms of biomass greater than our OG1RF positive control strain (**Fig 3.3B**). Importantly, the differences in planktonic growth rate did not correlate with the ability of the *E. faecalis* clinical isolates to form biofilms, regardless of glucose supplementation (**Fig 3.3, Appendix Fig 3.2**). Among the 21 ST16/179 strains, 18 isolates showed biofilm augmentation in the presence of glucose (40_EF, EF_1010, 14_EF were not augmented), suggesting that ST16/179 isolates can use glucose to augment biofilm.

Figure 3.3. Biofilm formation is augmented in clinical *E. faecalis* wound isolates with glucose supplementation. Forty-nine clinical *E. faecalis* wound isolates were grown in TSB media or TSB media supplemented with 0.25% glucose (TSBG), and biofilm biomass was determined after 2 days. *E. faecalis* OG1RF is a biofilm-positive laboratory strain and used as a positive control. *E. faecalis* Δ srtA Δ ebpABC is a biofilm-negative strain and used as a negative control. Mean biofilm formation is indicated for each isolate in the heat map. N=3, n=6.

To identify virulence factors that may be responsible for *in vitro* biofilm formation, we queried our sequencing reads using the VFDB database (Chen et al., 2005; Inouye et al., 2014), which is a curated database of previously described *E. faecalis* virulence factors. Among the 8 *E. faecalis* isolates that form better biofilms than our positive control in TSB media, we detected the presence of EF2347 in 6 of the 8 isolates (**Fig 3.3A, Fig 3.4**). EF2347, also called *ecbA*, codes for a putative collagen-binding and sortase substrate, previously shown to facilitate binding to collagen type V and fibrinogen (Hendrickx et al., 2009). Previously, studies have suggested that the presence of either *gelE* and *fsr* together, or *agg* or *esp* alone is predictive of biofilm forming ability (Hashem et al., 2017). However, none of these genes were enriched among the high biofilm formers grown in TSB or TSBG (Hashem et al., 2017; Kristich et al., 2004; Soares et al., 2014; Tendolkar, Baghdayan, Gilmore, & Shankar, 2004). Together our results suggest that under low glucose conditions, several *E. faecalis* wound isolates are able to form biofilms independent of the “prototypic” *E. faecalis* biofilm factors *esp*, *gelE*, *agg* or *fsr*.

To investigate the relationship between antibiotic resistance genes and their resistance profiles, we determined minimum inhibitory concentrations (MICs) for the 49 clinical isolates and queried their genomes for antibiotic resistance genes using the ResFinder database (Kleinheinz, Joensen, & Larsen, 2014). We observed that all 49 *E. faecalis* clinical wound isolates were sensitive to amoxicillin, daptomycin, and vancomycin (**Appendix Table 3.2**). However, we observed that 34/49 strains were resistant to gentamicin (**Fig 3.5**). In 19 of the 34 gentamicin-resistant isolates, we observed the presence of *aac(6')-aph(2'')*, which encodes the bifunctional 6'-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase enzyme, conferring high-level of gentamicin resistance (**Fig 3.5**) (Chow, 2000). In the remaining 15 gentamicin-resistant isolates, we could not identify any other genes encoding aminoglycoside-modifying enzymes that could be responsible for the high aminoglycoside resistance. It is therefore tempting to speculate that *E. faecalis* aminoglycoside resistance might be due to polymorphisms within its genome, or that they possess novel aminoglycoside resistant genes that confer resistance.

Taken together, *E. faecalis* isolates in the ST16/179 lineage appear to have an enhanced ability to form biofilms in the presence of glucose *in vitro*, and therefore may be more likely to infect wounds rich in glucose sources such as diabetic wounds. In addition, we propose that *ecbA* expression in *E. faecalis* may promote biofilm formation in a glucose-independent manner.

Figure 3.5. High-level aminoglycoside resistance is present in several clinical *E. faecalis* wound isolates. Antimicrobial susceptibility was assayed for 49 clinical *E. faecalis* wound isolates (right y-axis). The presence of antibiotic resistance genes (x-axis) using the ResFinder database with SRST2. The corresponding gentamicin MIC values are indicated in brackets beside the strain name. Two-dimensional hierarchical clustering of isolates is indicated by the dendrogram (left y-axis). The data were plotted in binary where gene presence is indicated in blue and absence is indicated in red.

3.3.4 Several clinical *E. faecalis* isolates can suppress *in vitro* macrophage activity

In general, the pathogenic potential of bacterial species is defined by the contribution of bacterial virulence and host susceptibility (Casadevall, 2017). In addition to expressing factors that contribute to biofilm formation in some niches, *E. faecalis* is also capable of immunomodulation which promotes infection (Tien et al., 2017; Zou, Baghdayan, Payne, & Shankar, 2014). To determine if *E. faecalis* wound isolates are enriched for immunomodulatory functions, we infected macrophages with *E. faecalis* in the presence of lipopolysaccharide (LPS), and assessed their NF- κ B activity. We observed 16/49 strains had similar immunosuppressive capability as our positive control strain OG1RF (**Fig 3.6A**). Of the remaining strains, we observed 9 strains that were hyperinflammatory (**Fig 3.6B**), and the remaining 24 strains displayed an intermediate phenotype (data not shown). We did not observe any correlation between these immune modulatory phenotypes and the presence or absence of known virulence factors (data not shown).

Figure 3.6. Clinical *E. faecalis* wound isolates differentially affect macrophage activation. RAW-BLUE (RAW267.4) macrophages were infected with clinical *E. faecalis* wound isolates concurrently in the presence of LPS at a multiplicity of infection (MOI) of 100:1 for 6 hours prior to measurement of NF-κB driven secreted embryonic alkaline phosphatase (SEAP) reporter activity. **(A)** NF-κB-driven SEAP activity of *E. faecalis* wound isolates that can suppress the NF-κB response of macrophages (red bars). **(B)** NF-κB-driven SEAP activity of *E. faecalis* wound isolates that fail to suppress (black bars) or additively activate (blue bars) the NF-κB response of macrophages in the presence of LPS. Blank represents media alone for

background NF- κ B reporter activity. *E. faecalis* OG1RF is used as a positive control for immunomodulation in this assay. LPS is used as a positive control for immune activation of RAW-BLUE macrophages. N=3, n=3. Statistical analysis was performed using one-way ANOVA with post-hoc Tukey's test to correct for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$ represents statistical significance when compared to LPS. # = $p < 0.05$, ## = $p < 0.01$, ### = $p < 0.001$, and #### = $p < 0.0001$ represents statistical significance when compared to *E. faecalis* OG1RF.

3.3 Discussion and conclusion

Given the prevalence of *E. faecalis* in human wound infections, identification of potential *E. faecalis* virulence traits in this niche is an important first step for the diagnosis of high risk groups of *E. faecalis*. To this end, we wanted to investigate whether *E. faecalis* biofilms contribute to wound infection. To complement our *in vivo* studies (**Chapter 2**) and others' *in vitro* studies of *E. faecalis* biofilm factors, here we performed the first comparative genomics analysis of *E. faecalis* wound isolates in an attempt to correlate genotypic and phenotypic parameters of *E. faecalis* wound virulence.

Phylogenetic analyses revealed that majority of *E. faecalis* strains isolated from human wounds clustered in 2 different ST groups, the CC2/6 and ST16/179. We observed that a subset of *E. faecalis* clinical isolates were able to form biofilms without glucose supplementation and were enriched for the adhesin gene *ecbA* in the CC2/6 group, but not the ST16/179 group. By contrast, CC2/6 isolates did not form thicker biofilms with glucose supplementation, whereas majority of isolates in the ST16/179 isolates did. Surprisingly, none of the factors typically associated with *in vitro* biofilm such as *esp*, *agg*, *gelE* and *fsr* were enriched among the high biofilm formers. However, we have not yet determined which biofilm factors mediate biofilm formation among these clinical isolates. We are particularly intrigued by the enrichment of *ecbA* among high biofilm formers grown in TSB. This collagen-

binding protein is reminiscent of *S. aureus* where there is enrichment of the collagen binding protein *cna* in clinical isolates (Booth, Pence, Mahasreshti, Callegan, & Gilmore, 2001; Cassat et al., 2005; Papakyriacou, Vaz, Simor, Louie, & McGavin, 2000). One limitation of the comparative genomics approach to virulence factor identification is that it, by itself, does not account for differential gene expression or the role of antigenic variation of *E. faecalis* adhesins. However, it is tempting to speculate that *ecbA* expression may contribute to wound virulence independent of glucose availability. Our current analysis is also limited to correlating the presence and absence of *E. faecalis* virulence factors in the VFDB database to biofilm formation. The main limitation of using the VFDB database is that only “characterized” virulence factors are reported. However, it is widely accepted that these “classical” virulence factors are not the sole determinants contributing to bacterial fitness during pathogenesis (Turner et al., 2014). As an alternative approach to identify other biofilm factors that may contribute to wound infection in the absence of *ecbA*, we have performed transposon sequencing experiments that will be described in **Chapter 4**.

E. faecalis can immunomodulate the host, suppressing normal inflammatory responses, which is suggested to aid in its colonization and infection (Tien et al., 2017; Zou et al., 2014). We observed that 16 out of 49 clinical wound isolates were able to suppress NF- κ B activity in macrophages and the 16 strains were not phylogenetically clustered or enriched for any ST groups. In addition, we observed 9 *E. faecalis* strains that have increased NF- κ B activation. These 9 strains did not phylogenetically cluster either. Taken together, our results suggest that the interaction between *E. faecalis* and the immune system cannot be defined by MLST or comparative genomics alone. Ongoing work must assess the contribution of

individual genes and gene polymorphisms in the accessory genomes to immune modulation. In addition, differential regulatory networks should be considered.

Comparative genomics of *E. faecalis* wound isolates has given us insights into the spectrum of biofilm forming abilities it can display. We have identified possible new wound biofilm-associated factors (*ecbA*), determined that prototypical biofilm genes are not enriched in wound isolates, and that glucose may augment biofilm formation among clinical wound isolates. Moreover, because we observe enrichment ST16/179 and CC2/6 groups among wound isolates, we postulate that certain lineages of *E. faecalis* are selected for in wounds. Outstanding questions include the contribution of specific gene pathways towards biofilm formation, as opposed to single gene contributions, in mediating/promoting biofilm formation. We would also like to know whether *E. faecalis* in wounds could seed disseminated infections such as bacteremia, and whether similar ST groups are enriched in both niches. Further insights on these biofilm and virulence traits of *E. faecalis* wound isolates can provide us with improved diagnostics of *E. faecalis* wound infection.

3.4 Materials and Methods

3.4.1 *E. faecalis* clinical wound isolates

Forty-nine hospital isolates (**Appendix Table 3.1**) were prospectively isolated from patients' wounds in Tan Tock Seng Hospital, Singapore, under the coordination of Dr Timothy Mark Barkham Sebastian (Laboratory Medicine). Clinical isolates were identified by mass spectrometry, collected in individual vials.

Confirmation of *E. faecalis* was done via streaking on differential CHROMagar™ Orientation plates (CHROMagar™, France), identifying for turquoise blue colonies after incubation for 16 hours at 37 °C. Single positively identified colonies were isolated, grown in Brain-heart infusion (BHI) media (Acumedia®,

USA) overnight statically at 37 °C, and stored at -80 °C as 25% glycerol frozen stocks prior to sequencing and phenotypic assays.

3.4.2 Sequencing of *E. faecalis* clinical wound isolates

Genomic DNA was extracted from the 49 clinical wound isolates using DNeasy blood and tissue kit (Qiagen, Germany). Extracted DNA was verified on a 1% agarose gel for the presence of intact DNA and quantified using Qubit® 2.0 fluorometer (Thermo Fisher Scientific, USA).

Extracted DNA was subjected to polymerase chain reaction (PCR)-based identification to validate Enterococcal species prior to sequencing. Primers were chosen based on benchmarking studies looking at differentiating *Enterococcus* species as described (Jackson et al., 2004). The amplification of the superoxide dismutase (*sodA*) gene can be used identify *E. faecalis* from a PCR product of 30 bases using primers FL1 and FL2 (**Supplemental Table 3.4**). After verification of species identity, sequencing was performed on a MiSeq V3 (300 bp x 2), with an average coverage of 18x per genome.

3.4.3 Growth kinetics of *E. faecalis* clinical wound isolates

Clinical isolates were inoculated into BHI broth and grown statically overnight at 37 °C. Overnight bacterial suspensions were washed twice in phosphate buffered saline (PBS) centrifuged at 5,000 x g for 4 mins. Optical density of 600 nm (O.D_{600nm}) of bacterial suspensions were adjusted to 0.7, which corresponds to 2 x 10⁸ CFU/mL, and diluted 1:25 into 96-well polystyrene microtiter plates (Thermo Fisher Scientific, USA) containing a final volume of 200 µL of Tryptic Soy Broth (TSB) (Oxoid, USA) supplemented with 0.25% glucose when indicated. Growth kinetics were measured at O.D_{600nm} using an Infinite® 200 PRO plate reader (Tecan Trading AG, Switzerland) for 18 hours statically at 37 °C at 15 mins interval.

3.4.4 Microtiter 96-well plate biofilm assay

E. faecalis clinical wound isolates were assessed for their ability to form *in vitro* biofilms with a modified crystal violet assay. Bacterial suspensions were normalized to an O.D_{600nm} of 0.7 as described in the previous section, and diluted 1:25 into 96-well polystyrene microtiter plates (Thermo Fisher Scientific, USA) containing 200 µL of final volume of TSB or TSB supplemented with 0.25% glucose. Plates were incubated statically at 37 °C for 44 hours for biofilms to form. After incubation, wells were decanted to get rid of supernatants and wells were washed twice with PBS. Microtiter plates were blotted dry on absorbent hand towels to remove excess liquid. Fixation was performed with 100% methanol (Sigma Aldrich, USA) for 5 mins at room temperature and methanol was discarded, with wells dried at 55 °C for 5 mins. Biofilms were stained with 0.1% crystal violet (Sigma Aldrich, USA) for 5 mins at room temperature, excess crystal violet was removed by washing in 18.2Ω milliQ water and drying at 55 °C for 10 mins. Solubilization of crystal violet was done using 30% acetic acid for 1 hour at room temperature and absorbance at O.D_{595nm} was measured with the Infinite® 200 PRO (Tecan Trading AG, Switzerland).

3.4.5 Minimum inhibitory concentration (MIC) determination of *E. faecalis* clinical wound isolates

Bacterial colonies were inoculated into Mueller-Hinton II broth (MHB) (Becton Dickinson, USA) and grown statically overnight at 37 °C. Bacterial suspensions were washed twice in PBS centrifuged at 5,000 g for 4 mins and normalized to a McFarland standard 0.5 according to manufacturer's instructions and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. A lawn of each normalized bacterial suspensions was made using a sterile cotton

swab over a 4 mm high Mueller-Hinton II agar (Becton Dickinson, USA), air-dried, and Etest® strips containing the indicated antibiotics were applied (bioMérieux, USA). *E. faecalis* ATCC 29212 was used as a positive control and quality control for MIC determination.

3.4.6 Comparative genomics and phylogenetic analysis of *E. faecalis* clinical wound isolates

Draft genomes of 49 *E. faecalis* clinical isolates were constructed from sequencing data obtained from MiSeq V3 (300 bp x 2) and *de novo* assembled using SPAdes version 3.10.1 (Bankevich et al., 2012). The contigs were then ordered using Mauve (version 2.4.0) (Darling, Mau, Blattner, & Perna, 2004). *De novo* assembled contig were used to compare gene synteny in Mauve and phylogenetic analysis using Harvest suite (version 1.1.2) (Treangen, Ondov, Koren, & Phillippy, 2014) using DNA maximal unique matches indexes (MUMi) for building the core genome and used for phylogenetic analysis (Treangen et al., 2014). Raw reads were subjected to short read sequence typing (SRST2 version 0.2.0) (Inouye et al., 2014) to perform multilocus sequence typing (MLST) from PubMLST database, determine known virulence factors from VFDB, find known plasmids using PlasmidFinder, and antibiotic resistance genes from Resfinder.

3.4.7 RAW-Blue macrophage culture

RAW-Blue cells, derived from RAW264.7 macrophages (Invivogen, USA), contain a plasmid encoding a secreted embryonic alkaline phosphatase (SEAP) reporter under transcriptional control of an NF- κ B-inducible promoter, were cultivated in Dulbecco modified Eagle medium (4,500 mg/liter glucose, 4.0 mM L-glutamine) (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA) and 200 μ g/mL Zeocin at 37 °C in 5% CO₂ to maintain the SEAP reporter gene.

3.4.8 RAW-Blue infection NF- κ B reporter assay

RAW-Blue cells were seeded in a 96-well plate at 100,000 cells/well in 200 μ L of antibiotic-free cell culture medium. Following overnight incubation, the cells were washed once with PBS, and fresh medium was added. Cells were stimulated using EB ultrapure LPS purified from *E. coli* O111:B4 (200 ng/mL) (Invivogen, USA) and media alone serve as positive and negative controls for reporter activity respectively. RAW-Blue cells were infected with *E. faecalis* clinical isolates at MOI of 100:1 for 6 hours with LPS stimulation. Overnight bacterial cultures were grown in cell culture medium and diluted 1:10 in fresh cell culture medium. Normalized cultures were then used to infect RAW-Blue macrophages. Post infection, 20 μ L of supernatant was added to 180 μ L of Quanti-Blue reagent (Invivogen, USA) and incubated for 3 hours at 37°C. SEAP levels were determined by using O.D_{640nm} using an Infinite® 200 PRO plate reader (Tecan Trading AG, Switzerland).

3.4.9 Statistical analysis

Statistics indicated in the figure legends were performed using GraphPad Prism software (Version 6.0f for Mac OS X, California). Heatmap was produced using R package, pheatmap (version 1.0.10).

3.5 Authors contribution

I designed the study of this project, performed, and analyzed the *in vitro* experiments listed in this dissertation, assisted by student Kong Ze Qing. Human *E. faecalis* clinical isolates were obtained from Timothy Mark Barkham Sebastian's team at Tan Tock Seng Hospital (Singapore). I performed the bioinformatics analysis of the clinical isolates.

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Chapter 4: Virulence factors important for *E. faecalis* wound infection

4.1 Introduction

Enterococcus faecalis is an opportunistic bacterium capable of colonizing open wounds and developing chronic infections. Bacterial wound infection typically begins with colonization on the subdermal or dermal layers of the skin, and develops into an infection when left untreated. Moreover, the ability to develop biofilms at the wound site allows the bacteria within the biofilm protection against the host immune system (Metcalf & Bowler, 2015; Tay, Chong, & Kline, 2016; Zhao et al., 2013). To persist in wounds, *E. faecalis* has to tolerate the initial low pH of the wound and resist killing by reactive oxygen species in the wound microenvironment. Similarly, *E. faecalis* must be able to acquire nutrients present at wound sites for survival. Here, we will investigate putative *E. faecalis* virulence factors important for wound infection.

The first stage of wound infection by *E. faecalis* involves colonization of the wound. *E. faecalis* can bind a variety of host extracellular matrix (ECM) to promote colonization (Fisher & Phillips, 2009; Jett, Huycke, & Gilmore, 1994; Sava, Heikens, & Huebner, 2010). The wound bed consists of ECM proteins including collagen type III, fibronectin, vimentin, and fibrinogen, whereas healthy adjacent skin predominantly contains collagen type I (Frantz, Stewart, & Weaver, 2010; Olczyk, Mencner, & Komosinska-Vassev, 2014; Schultz & Wysocki, 2009; Xue & Jackson, 2015). The surface *E. faecalis* protein Ace can bind collagen type I, collagen IV and laminin (Liu et al., 2007; Nallapareddy et al., 2000). In addition to Ace, some *E. faecalis* strains carry plasmids that encode the pheromone-inducible aggregation substance, AS. *E. faecalis* AS is a collagen binding adhesin that facilitates *E. faecalis* attachment to fibronectin, thrombospondin, vitronectin, and collagen type I (Rozdzinski, Marre, Susa, Wirth, & Muscholl-Silberhorn, 2001). During wounding, host hemostasis (clotting) occurs immediately, in which the coagulation cascade is

activated resulting in recruitment of clotting factors to the wound site to seal the breach in the epithelial barrier. Hemostasis results in the deposition of fibrinogen, among other hemostasis proteins, onto the wound surface (Laurens, Koolwijk, & De Maat, 2006; Velnar, Bailey, & Smrkolj, 2009). The *E. faecalis* pilus, Ebp, can adhere to fibrinogen-coated catheters during catheter-associated urinary tract infections (CAUTI) and has been proposed to utilize fibrinogen as an energy source (Flores-Mireles, Pinkner, Caparon, & Hultgren, 2014; Xu et al., 2017). Moreover, *E. faecalis* encodes multiple fibrinogen (Fn) binding proteins that target different domains of Fn. It has been postulated that the different Fn binding proteins are functionally redundant and/or that they are differentially expressed in different tissues, enabling tissue tropism (Maadani, Fox, Mylonakis, & Garsin, 2007). Lastly, *E. faecalis* encodes EfbA, a PavA-like adhesin that binds host fibronectin. With this repertoire of adhesins, *E. faecalis* has the ability to bind to wound surfaces to initiate colonization and infection, but the involvement of individual adhesins at the wound site has yet to be demonstrated.

In addition to initial adhesion and colonization, *E. faecalis* must also overcome host defenses to establish infection. *E. faecalis* can form biofilms and modulate the immune response to promote its survival (Park et al., 2008; Thurlow, Thomas, Fleming, & Hancock, 2009; Varahan, Iyer, Moore, & Hancock, 2013; Zou & Shankar, 2014). *E. faecalis* encodes several factors that contribute to biofilm formation, including two sortase enzymes, SrtC and SrtA, that polymerize and attach endocarditis- and biofilm-associated pili (Ebp) to the cell wall, respectively (Kline et al., 2009; Nallapareddy et al., 2006; Nielsen et al., 2013). Ebp pili aid in the attachment of *E. faecalis* to surfaces, which is required in the initial stages of biofilm formation *in vitro* and *in vivo* during CAUTI (Flores-Mireles et al., 2014; Nielsen et al., 2012). Other biofilm-associated factors that are attached to the cell wall by SrtA

include Ace, aggregation substance, and Esp (Dunny, Leonard, & Hedberg, 1995; Nallapareddy et al., 2000; Shankar et al., 1999; Süßmuth et al., 2000). Biofilm formation, along with expression of the SrtA substrate aggregation substance, can promote *E. faecalis* survival within macrophages and polymorphonuclear leukocytes (Baldassarri et al., 2005; Rakita et al., 1999). The multiple peptide resistance factor (MprF) protein is lysyltransferase and a flippase involved in modifying the anionic phosphatidylglycerol with a lysine group, conferring a net positive charge on this lipid (Ernst et al., 2009). *E. faecalis* MprF-mediated lipid modifications confer resistance to antimicrobial peptides via electrostatic repulsion (Bao et al., 2012; Kandaswamy et al., 2013), and is important for resisting both neutrophil-mediated clearance and surviving within epithelial cells and macrophages in a variety of Gram-positive bacteria (Camejo et al., 2009; Peschel et al., 2001; Thedieck et al., 2006). Taken together, *E. faecalis* can rely on biofilms for surface attachment and immune evasion strategies to resist host responses.

To date, the pathogenic mechanisms of *E. faecalis* in wounds remain poorly understood. To elucidate *E. faecalis* factors that are important for wound infection and persistence, we used the infection model that we developed in **Chapter 2** to identify mutants attenuated for *E. faecalis* infection and persistence. We approached this problem using both forward and reverse genetics. In the reverse genetics approach, we selected *E. faecalis* mutants based on their virulence properties in other niches, particularly relating to adhesin expression, biofilm formation, and immune defense. In the forward genetics approach, we used an *E. faecalis* transposon library of ~15,000 mutants (Kristich et al., 2008) and optimized an *in vivo* transposon sequencing approach using these *E. faecalis* mutants to look for wound infection fitness factors. Fitness factors are defined through failure to recover transposon mutants in the factor after *in vivo* infection (Van Opijnen & Camilli, 2013).

Transposon sequencing has been used to elucidate factors that are important for wound infection by *P. aeruginosa* and *S. aureus* (Turner et al., 2014; Valentino et al., 2014). Similarly, we developed an unbiased transposon sequencing approach to identify *E. faecalis* fitness factors during wound infection

In this chapter, we identified *E. faecalis* mutants that are deficient during wound persistence and discuss the potential role of these mutants in infection. We show that resistance to cationic antimicrobial peptides is likely important for *E. faecalis* persistence in wounds. We also established a transposon sequencing and analysis pipeline, and identified mutants in carbohydrate uptake that are deficient in wound persistence. Overall, this is the first study identifying *E. faecalis* mutants important for wound infection.

4.2 Results

4.2.1 MprF contributes to *E. faecalis* fitness during wound infection

To determine *E. faecalis* factors involved in wound colonization and infection, we examined the fitness of previously described biofilm factors as well as factors involved in immune defense. We chose to look at timepoints at 8 hpi and 3 dpi at the infection dose of 10^6 CFU (**Chapter 2, Fig 2.2B**). We employed a competitive infection model to increase the sensitivity to detect less fit mutants. In competitive infections with OG1X (a closely related strain to OG1RF, both derived from parent OG1 (Dunny, Brown, & Clewell, 1978; Ike et al., 1983)), we found that an $\Delta ebpABC$ mutant was as fit as wild type OG1RF at 8 hpi and 3 dpi, indicating that biofilm-associated Ebp are not important for replication and wound persistence (**Fig 4.1A,B**). Similarly, a $\Delta srtA\Delta srtC$ double mutant strain defective in biofilm formation *in vitro*, was not attenuated in fitness during coinfection with OG1X at either 8 hpi or 3 dpi (**Fig 4.1C,D**). We also tested $\Delta ebpABC$ and $\Delta srtA\Delta srtC$ mutants in single (non-competitive) infections at the colonization dose, and recovered CFU at levels similar

to the wild type strain at 24 hpi (data not shown). Together these observations indicated that the inability to attach LPXTG-containing adhesins to the *E. faecalis* cell wall is dispensable for replication and persistence in wounds. We hypothesized that mutants defective in binding to host extracellular matrix proteins might be attenuated in wound infection, and so performed competitive infection at the colonization dose with OG1X with mutants of collagen-binding adhesins, *ace* and *cna*, along with the fibronectin-binding protein, *pavA*, and did not see any difference in competitive indices from the wild type strain (**Appendix Fig 4.1**).

Figure 4.1. Ebp pilus and sortases are dispensable for *E. faecalis* replication and persistence in wounds. Wounds were infected with a 1:1 ratio of *E. faecalis* strains OG1X:wild type OG1RF with (A,B) OG1X:OG1RF $\Delta ebpABC$, or (C,D) OG1X:OG1RF $\Delta srtAsrtC$ at 10^6 CFU per inoculum. The resulting CFU was determined at (A,C) 8 hpi, and (B,D) 3 dpi. Each dot represents one mouse and the solid horizontal lines indicate the median. N=1-4, n=4-5. Statistical analysis was performed using Mann-Whitney U test, no significance was observed.

Because we observed overt inflammation after high dose *E. faecalis* infection in wounds (**Chapter 2, Fig 2.1**), we predicted that resistance to host immune killing may be important for *E. faecalis* survival. *E. faecalis* encodes two paralogues of MprF (Bao et al., 2012; Kandaswamy et al., 2013). To address the contribution of MprF to fitness in wounds, we co-infected wild type *E. faecalis* OG1X with the $\Delta mprF1\Delta mprF2$ double mutant at the infection dose. We observed that the $\Delta mprF1/2$ mutant was outcompeted during co-infection at 3 dpi, but not at 8 hpi (**Fig 4.2**). However, this difference was not observed in the colonization dose (data not shown). Together, these results suggest that biofilm-associated factors described for other niches may be less important for *E. faecalis* wound persistence than its ability to resist immune defense mechanisms.

Figure 4.2. Multiple peptide resistance factor (MprF) contributes to fitness during *E. faecalis* wound persistence. Wounds were infected with a 1:1 ratio of *E. faecalis* strains OG1X:wild type OG1RF or OG1X:OG1RF $\Delta mprF1/2$, at 10^6 CFU per inoculum, harvested at **(A)** 3 dpi, and **(B)** 8 hpi, and the recovered bacteria enumerated on selective media for each strain. Each dot represents one mouse, and the solid horizontal lines indicate the median, N=3, n=4. Statistical analysis was performed using Mann-Whitney U test. **** = $p < 0.0001$.

4.2.2 Identifying *E. faecalis* wound fitness factors using *in vivo* transposon sequencing

To identify additional fitness factors involved in *E. faecalis* wound infection, we developed and optimized an unbiased transposon sequencing approach. An *E. faecalis* transposon mutant library was obtained from the laboratory of Gary Dunny and was previously sequenced (Kristich et al., 2008). The transposon library contains ~15,000 mutants inserted randomly throughout the genome in non-essential regions, giving us about ~5.5x coverage in the genome with 2,701 predicted ORFs (Bourgogne et al., 2008). While the coverage of this transposon library is not as extensive as other transposon libraries generated in other systems, our knowledge of annotation of our transposon library can help in reducing type I error (false positives) in our analysis (Goodall et al., 2018; Langridge et al., 2009; Valentino et al., 2014). The mariner-based transposon consists of a GFP gene and a chloramphenicol cassette flanked by the two terminal inverted repeats, and has been verified by sequencing in our laboratory (**Fig 4.3**). This library contains locus tag information tied to the respective insertion sites and thus in theory, we can trace the mutants in each pool. After shearing recovered genomic DNA, we used primers that annealed to the unique terminal inverted repeats to enrich for transposon mutants (**Fig 4.3**).

Figure 4.3. Schematic design of amplifying specific transposon sequences for sequencing. Genomic DNA harvested from *E. faecalis* infected wounds, was subjected to library preparation to enrich sequences which contain the transposon

insertion. **(A)** *E. faecalis* transposon mutants containing the insertion of the mariner transposon sequence. The transposon element contains a Chloramphenicol resistance gene and a GFP encoding gene, which is flanked by the two transposon terminal inverted repeats. **(B)** Genomic DNA is sheared *via* sonication and adapters with splinkerette sequences are ligated onto the end of each sheared DNA fragment. PCR primers targeting the terminal inverted repeats and the adapter is used to amplify transposon-containing sequences for downstream library preparation and sequencing.

4.2.2A. Bottlenecks in designing transposon sequencing *in vivo*

To design a successful transposon sequencing experiment tailored to our experimental system, we had to consider common issues of *in vivo* biological bottlenecks and the specificity of our custom-primers to enable unbiased recovery of transposon-inserted regions for sequencing. An extensive review of transposon sequencing design has been published (Van Opijnen & Camilli, 2013), but here we specifically consider bottlenecks in our experimental infection model. Biological bottlenecks are arguably the biggest problem in designing transposon sequencing experiments, because mutants that are lost by chance (false positives) must be differentiated from mutants lost due to their fitness defect *in vivo* (true positives). To test for possible biological bottlenecks, we co-infected wild type OG1RF strain with OG1X at 8 hpi and 3 dpi, at the colonization dose and infection dose. We hypothesize that the ratio of OG1RF and OG1X recovered should be similar in the absence of biological bottleneck. We observed similar ratios of OG1RF and OG1X obtained irrespective of inocula used (**Fig 4.4**). Hence, our system does not seem to exhibit any significant biological bottlenecks when designing our transposon sequencing method. Based on these preliminary studies, library pools containing inocula of 10^2 (the colonization dose) of each Tn mutant should be sufficient for equal recovery without a bottleneck problem. Since we deliver a total of 10^6 CFU/wound in a 10 μ L

inoculum and subsequently recovered 10^5 CFU/wound, we could create Tn mutant pools of up to 10^3 different mutants, each at 10^2 CFU.

Figure 4.4. Biological bottleneck is not present at the *E. faecalis* colonization dose. Wounds were infected with a 1:1 ratio of *E. faecalis* strains OG1X:wild type OG1RF at 10^2 CFU or 10^6 CFU per inoculum, harvested at 8 hpi, and the recovered bacteria enumerated on selective media for each strain. Each dot represents one mouse, and the solid horizontal lines indicate the median, $N=1$, $n=5$. Statistical analysis was performed using Mann-Whitney U test, no significance was observed from a competitive index of 1.

4.2.2B. Identification of transposon insertion sites

Library preparation and sequencing design is important for specific identification of transposons within genes of interest. The main challenge in the field is to reduce type I error where the majority of efforts have focused on accurate prediction of identifying transposon insertion sites (Barquist et al., 2016; Goodall et al., 2018; Langridge et al., 2009; Zomer et al., 2012). Transposon Directed Insertion Sequencing (TraDIS) is one type of transposon sequencing that can be used for assessing gene essentiality *in vivo*. Recently, a “splinkerette” design was introduced into the adapter ligation step for enriching transposon mutants (Barquist et al., 2016). This design has the advantage of increasing the odds of enriching transposon sequences with 3' genomic DNA sequence and reducing bias in amplifying the 5' region during PCR amplification in typical Illumina-based designs (Barquist et al., 2016). The splinkerette adapters also provide a binding site on the 3' end of the genomic DNA for PCR amplification (**Fig 4.3**). We then designed primers using an amplicon-based sequencing approach to enrich for transposon mutants, where we report successful amplification post-PCR enrichment of the correct size (~300bp) consistent with size of sheared genomic DNA in this assay (**Fig 4.5**). “Dark cycle” sequencing is one of the methods used to overcome the lack of complexity during transposon sequencing. This is achieved by not imaging the initial transposon sequence, which is identical for all sequences in the library, allowing for imaging only the genomic DNA sequence. Initially we faced difficulties in implementing “Dark cycle” sequencing, possible due to the custom primer design (Barquist et al., 2016). However, we adopted an amplicon based sequencing approach where transposase target sequences were added to the 5' end of the transposon sequence primer (**Fig 4.3**) which serves as a binding site for PCR addition of flow cell binding sites with index sequences added by PCR during library preparation. Using this approach, we were

successful in optimizing our sequencing run of transposon pools isolated from *E. faecalis* infected wounds. We analyzed transposon inserted sequences obtained from *E. faecalis* infected wounds and compared them to our input reference pool. To date, we have only been able to run and analyze data recovered from one MiSeq run. We were not able to achieve enough sequencing depth from this. Hence in order to gain more sequencing depth, HiSeq runs have been performed, analysis is underway, but time limitations preclude incorporate of the full data set into this thesis submission.

Figure 4.5. Genes containing transposon sequences are enriched by PCR. Genomic DNA harvested from *E. faecalis* infected wounds was subjected to shearing and adapter ligation. PCR enrichment of transposon mutants using primers targeting the end of the transposon and the splinkerette adapters showed one peak at ~327 bp, indicative of specific enrichment. Lower and upper refer the DNA ladder standards. Image shown are representative of all samples.

4.2.3 Carbohydrate utilization is important for *E. faecalis* persistence during wound infection

Based on our initial MiSeq dataset, multiple genes in the same operon were most significantly decreased in our post-infection transposon pools: OG1RF_10020 and OG1RF_10021 which encode PTS mannose/fructose/sorbose transporter subunits, being essential for *E. faecalis* persistence at 3 dpi (**Fig 4.6**). To validate our transposon sequencing hits in the PTS transporter subunits, we used individual transposon mutants in each PTS transporter subunit gene and performed competitive infection with OG1X at 3 dpi. In accordance with our transposon sequencing results, strains with mutations in each of the PTS transporter subunits had significantly reduced fitness compared to wild type OG1RF at 3 dpi (**Fig 4.6**). This result suggests that *E. faecalis* needs to uptake sugar as a carbon source in wounds, thereby promoting persistence. Taken together, we have successfully set up an unbiased platform for looking at *E. faecalis* fitness factors that are important during wound infections.

Figure 4.6. A phosphotransferase (PTS) system contributes to fitness during *E. faecalis* wound persistence. Wounds were infected with a 1:1 ratio of *E. faecalis* strains OG1X:wild type OG1RF, OG1X:OG1RF_10020::tn or OG1X:OG1RF_10021::tn, at 10^6 CFU per inoculum, harvested at 3 dpi, and the recovered bacteria enumerated on selective media for each strain. Each dot represents one mouse, and the solid horizontal lines indicate the median, N=1, n=5. Statistical analysis was performed using Mann-Whitney U test. * = $p < 0.05$ and ** = $p < 0.01$.

4.3 Discussion and conclusion

E. faecalis is commonly isolated from wounds such as surgical site infections and diabetic wounds (Dowd et al., 2008; Giacometti et al., 2000). Given its widespread prevalence and its clinical importance, especially in the context of antibiotic resistance, understanding *E. faecalis* virulence factors that contribute to colonization, infection and persistence is of great interest. To date, there are no studies reported on any virulence factors of *E. faecalis* in wounds. Here, we used *E. faecalis* mutants using both targeted and unbiased methods to look at virulence factors important for *E. faecalis* wound infection. We used the modified mouse wound excisional model described in **Chapter 2** as a platform to probe for *E. faecalis* factors that are essential for wound infection.

Presence of microcolonies is an indicator of early biofilm formation in wounds (Ashrafi et al., 2018; Hurlow, Blanz, & Gaddy, 2016). Consistent with these reports, we previously observed presence of *E. faecalis* microcolonies in wounds (**Chapter 2, Fig 2.4A**). However, a sortase null mutant, deficient in the surface display of a variety of biofilm-associated factors, was not attenuated in wounds. Furthermore, *E. faecalis* mutants disrupted for collagen-binding adhesins (*ace* and *cna*) and fibronectin binding (*pavA*), were also not attenuated in colonization when wounds were infected at low doses. Together, these findings suggest that *E. faecalis* wound-associated microcolonies or biofilms require other bacterial or host factors for their development, and that currently understood biofilm factors are less crucial in this niche. Instead, we found that a factor involved in resistance to host immune defenses is important for *E. faecalis* survival in wounds. We observed that an MprF null strain was not attenuated at 8 hpi when acute bacterial replication is occurring, but displayed a fitness defect at 3 dpi when the bacterial burden resolves to a steady state of 10^5 CFU (**Chapter 2, Fig 2.2B**). Interestingly, we previously found that MHCII- macrophages were

significantly greater in infected wounds compared to mock-infected wounds at 3 dpi (**Chapter 2, Fig 2.7D**). Based on these findings, it is tempting to speculate that MprF may contribute to *E. faecalis* survival in the face of macrophage infiltration. Consistent with this, we and others have shown that *E. faecalis* can survive within and suppress immune activation of macrophages through the NF- κ B signaling pathway (Tien et al., 2017; Zou & Shankar, 2014, 2016). Taken together, we showed through this reverse genetic approach that resistance to host cationic antimicrobial peptide may be important for *E. faecalis* persistence in wounds through the disruption of MprF.

Transposon sequencing gives us an opportunity to map fitness factors in a particular infection setting (Kristich et al., 2008; Langridge et al., 2009; Van Opijnen & Camilli, 2013). Two main caveats of transposon sequencing are complementation of gene function by the sharing of public goods or lack of phenotype by gene redundancy (Goodall et al., 2018; Turner et al., 2014; Valentino et al., 2014; West, Griffin, Gardner, & Diggle, 2006). However, transposon sequencing can be used as a sensitive tool to study gene interactions and networks. We optimized and validated a transposon sequencing method using an *E. faecalis* Mariner transposon library. We observed that disruption of two adjacent PTS transporter subunit genes, putatively important for mannose/fructose/sorbose uptake, resulted in *E. faecalis* attenuation in wounds at 3 dpi. Complementation assays are important for validating transposon sequencing results due to their high levels of false positive hits, and are underway for these PTS subunit encoding genes (Langridge et al., 2009; Turner et al., 2014; Valentino et al., 2014; Van Opijnen & Camilli, 2013). Taken together, this finding suggests that *E. faecalis* may uptake and utilize mannose/fructose/sorbose as a carbon source for ATP generation during persistence in wounds. Studies are underway to definitely determine the carbohydrate transported by this PTS system.

In summary, we have identified two factors that are important for *E. faecalis* persistence in wounds: MprF and a putative PTS mannose transporter. Importantly, the ability to utilize an *in vivo* model Tn-seq system to probe for *E. faecalis* fitness factors provides us a valuable insight into the discovery of new genes contributing to *E. faecalis* wound infection. Finally, our data suggests that *E. faecalis* is active during its infection and persistence in wounds, both in terms of nutrient acquisition and immune subversion. Given the high prevalence of enterococcal wound infections, future studies in understanding the mechanistic contribution of these virulence factors are important, especially now that we have the ability and the insight to uncover its pathogenic mechanisms in wounds.

4.4 Materials and Methods

4.4.1 Bacterial strains and growth conditions

E. faecalis strains are shown in **Appendix Table 4.1**. For mouse infections, *E. faecalis* strains were grown statically at 37 °C for 15-18 hours in Brain Heart Infusion (BHI) medium (Neogen, Lansing, USA) without antibiotics.

4.4.2 Genetic manipulation

Construction of *E. faecalis* OG1RF $\Delta mprF1$ and $\Delta mprF2$ were previously described (Kandaswamy et al., 2013). OG1RF $\Delta mprF1/2$ was made by subcloning the $\Delta mprF1$ deletion construct from pJRS213- $\Delta mprF1$ into pGCP213 to create pGCP213- $\Delta mprF1$ and transforming the plasmid into OG1RF $\Delta mprF2$. Chromosomal deletions were constructed as described previously.

Targeted screening of *E. faecalis* mutants from Mariner Transposon library

The *E. faecalis* transposon library containing ~15,000 mutants was constructed and kindly provided to us by Gary Dunny (Kristich et al., 2008). Transposon mutants were arrayed into 384 wells with the transposon sequence of

each mutant being known. Mutants of interest were streaked out from the 384 microtiter plate, with inoculation of single colonies into BHI medium (Neogen, Lansing, USA) for overnight static growth at 37 °C for 15-18 hours and normalized for *in vivo* wound infection with OG1X at a ratio of 1:1 as stated in **Chapter 2** described under the **Mouse wound excisional model** section.

4.4.3 Preparation of transposon pools for wound infection

Initial pools consisting of 100 transposon mutants per pool were made and glycerol stocked. Fifty transposon pools of 100 were then combined to achieve a final pool size of 5000 to be used for subsequent transposon sequencing, with a total of 3 pools of 5000 covering the whole transposon library. Glycerol stocks of the 5000 mutants were grown overnight in BHI medium (Neogen, Lansing, USA) for 15-18 hours at 37 °C. Overnight cultures were washed twice with PBS after pelleting at 5,000 rpm for 4 minutes and normalized to O.D_{600nm} of 0.35 corresponding to 2×10^8 CFU/mL. Wounds were made on the dorsal back of the mice as described under the **Mouse wound excisional model** section in **Chapter 2** and 10 µL of the normalized inocula was inoculated into wounds to achieve an infection CFU of 2×10^6 CFU.

4.4.4 Genomic DNA extraction for transposon sequencing

Mice were euthanized and wounds excised at the indicated time points. Wounds were subsequently homogenized in 1 mL of PBS. To reduce biological variance, 500 µL from two wound homogenates containing the same transposon mutants were pooled together into 4 mL of BHI to a final volume of 5 mL for 3 hours. The remaining wound homogenates were subjected to CFU enumeration on BHI agar (Acumedia, USA) supplemented with Rifampicin or BHI agar without any antibiotics to check for the presence of contamination. Homogenates containing contaminants were excluded from subsequent library preparation. *In vitro* comparator pools were made by incubating 10 µL of normalized transposon overnight cultures into 4 mL of

BHI broth for 3 hours as well. To recover as much transposon mutants as we can, we adopted an enrichment step of transposon pools for all mice samples by incubating the mixture at 37 °C for 3 hours and DNA were extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, Germany). A total of 3 biological replicates of DNA samples were made from 6 wounds per transposon pool.

4.4.5 Transposon library construction and sequencing

DNA extracted was used for DNA library construction using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, USA) according to the manufacturer's instructions. DNA was subjected to acoustic shearing to obtain fragment sizes of approximately 300 bp using g-Tube (Covaris, USA). We adopted and modified the TraDIS protocol published by Barquist *et al* (Barquist et al., 2016) by using the proposed splinkerette design for adapter ligation and subsequent enrichment of transposon pools using an amplicon based sequencing approach. TraDIS adapters were used for adapter ligation to obtain sequences with splinkerettes to allow for better specificity during the PCR enrichment step (**Appendix Table 4.2**) . PCR amplification was done using normalized DNA samples with 21 cycles of amplification using primers targeting the 15 bp at end of the transposon sequence and the P7 splinkette sequence using Q5® polymerase (New England Biolabs, USA). PCR cycle numbers were kept as low as possible to prevent PCR bias by subjecting constructs to linear amplification. Following PCR amplification, constructs were used as input for final library construction using the Nextera® XT DNA kit (Illumina, USA) as per manufacturer's instructions. Constructs were normalized and sequenced as 150 bp single read using the MiSeqV3.

4.4.6 Analysis of transposon sequencing results

Reads obtained from sequencing were checked using FastQC (version 0.11.5) and adapter trimmed using bbduk from the BBMap tools (version 34.49) (Bushnell,

2016). Trimmed reads containing the 15 bp transposon sequence at the 5' region were obtained using a customized python script and subsequently trimmed to obtain sequences for mapping. Reads were mapped onto the *E. faecalis* OG1RF (NCBI accession: GCF_000172575.2) reference genome using BWA (version 0.7.15-r1140) (Li & Durbin, 2009). Reads mapping to predicted open reading frames of each genome were quantified using HTSeq (Anders, Pyl, & Huber, 2015) and differential gene expression analysis was performed under the R environment (version 3.4.4) using Bioconductor package, edgeR (Robinson, McCarthy, & Smyth, 2010). Reads were normalized based on sequencing depth, scaled for the respective library sizes using trimmed mean of M-values (TMM), with common and tagwise dispersions being estimated for downstream analysis. Genes were considered differentially expressed under false discovery rate (FDR) <0.05 under correction by the Benjamin-Hochberg procedure. Gene annotation was performed using the database from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Shortlisted listed genes were viewed using the IGV genome browser (version 2.3.94) (Thorvaldsdóttir, Robinson, & Mesirov, 2013) to check if aligned reads had position-independent decrease of reads between the input reference pool and reads obtained from infection experiments. Transposon hits were then individually streaked out from the Mariner Transposon library and subjected to competitive infection experiment as described under **Mouse wound excisional model** in **Chapter 2** for validation.

4.5 Authors contribution

I designed the study of this project, performed, and analyzed the *in vivo* experiments for identifying *E. faecalis* fitness factors in the *in vivo* wound model, assisted by Eliza Soh Ye Chen. Tze Horng Liew constructed the $\Delta mprF1mprF2$ deletion mutant. Muhammad Hafiz Bin Ismail and I optimized the transposon

sequencing library construction and bioinformatics pipeline, assisted by Swaine Lin Chen, Fenlon Shannon Nicole, and Varnica Khetrapal.

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Chapter 5: Polymicrobial *Enterococcus faecalis* and *S. aureus* wound infections

5.1 Introduction

Polymicrobial infections are prevalent in a majority of wound infections where the average number of bacterial species cultured is ~4.7 to 4.9 per diabetic wound (Bowler, Duerden, & Armstrong, 2001; Bowler & Davies, 1999; Sapico et al., 1984). Enterococci can constitute a significant proportion of the wound microbial population, as revealed through metagenomic sequencing (Azevedo, Almeida, Melo, & Azevedo, 2014; Dworniczek et al., 2012; Galvan, Mateyca, & Ielpi, 2016; Giacometti et al., 2000; Holá, Ruzicka, & Horka, 2010; Macleod & Stickler, 2007; Wolcott et al., 2016). *E. faecalis* are often isolated from wounds, including diabetic ulcers, burns and surgical wounds, together with other common wound pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Corynebacterium spp*, and *Enterobacteriaceae spp* (Bowler et al., 2001; Citron et al., 2007; Giacometti et al., 2000). In addition to polymicrobial wound infections, *E. faecalis* mixed species interactions in urinary tract infections have also been reported and studied. Uropathogens frequently co-isolated with *E. faecalis* include *Proteus mirabilis* (co-isolated in almost 40% of *E. faecalis* biofilms) (Macleod & Stickler, 2007), *Escherichia coli*, and *Klebsiella pneumonia* (co-isolated on catheters) (Galvan et al., 2016), as well as *Pseudomonas aeruginosa* and *Candida albicans* (Holá et al., 2010).

Interspecies relationships can be antagonistic, where one species inhibits the growth of co-inhabiting organisms; mutualistic, where all species benefit in the host environment; or parasitic, where one species benefits at the expense of the other co-infecting organism. It is widely accepted that these polymicrobial interactions are context-specific and dependent on the host microenvironment. Given the frequency with which these organisms are co-isolated with enterococci, understanding interspecies interactions could prove pivotal in combating complex infections.

E. faecalis can modulate biofilm formation by other organisms in an antagonistic or mutualistic manner. For example, *E. faecalis* bacterocin secretion suppresses the growth of *Clostridium perfringens* and other non-bacterocin tolerant *E. faecalis* strains (Gilmore et al., 2015; Han et al., 2014). *E. faecalis* antagonism can be extended to inter-kingdom inhibition of biofilms as well, in which the secretion of enterocin EntV inhibits *C. albicans* hyphal morphogenesis and biofilm formation (Cruz et al., 2013; Graham, Cruz, Garsin, & Lorenz, 2017). Conversely, *E. faecalis* can exist with co-infecting organisms. In one study, *E. faecalis* was shown to suppress the inflammatory response to allow dissemination of non-pathogenic *E. coli* during catheter associated urinary tract infections (Tien et al., 2017). During wound infections and in the context of iron limitation, *E. faecalis* can induce *E. coli* siderophore production which in turn augments *E. coli* titers in wounds (Hughes & Winter, 2016; Keogh et al., 2016). Additionally, *E. faecalis* can produce AI-2 that chemoattracts *E. coli* to promote mixed species biofilm formation (Laganenka & Sourjik, 2017). *E. faecalis* can also induce increased production of Psl and Pel by *P. aeruginosa*, which promoted *P. aeruginosa* biofilm formation *in vitro* (Lee, Lee, Kim, & Yoon, 2017; Mottola et al., 2016).

Organisms in polymicrobial communities can specially organize and/or segregate to occupy a niche, a concept sometimes referred to as “biogeography” (Stacy et al., 2016). In an *in vitro* model of mixed species biofilms, *E. faecalis* spatially segregate for *P. aeruginosa* where *E. faecalis* are found at the base of the biofilm and *P. aeruginosa* at the top (Lee, Lee, Kim, & Yoon, 2017). However, in polymicrobial wound infections, *P. aeruginosa* infects deeper in wounds than *E. faecalis* (Dalton et al., 2011; Stacy et al., 2016). Interestingly, no species capable of enhancing *E. faecalis* biofilm formation has been identified thus far. Taken together,

E. faecalis can inhibit or enhance biofilm formation of other co-inhabiting organisms, where their spatial organisation can be influenced by the microenvironment.

The most famous interspecies interaction with *E. faecalis* pertains to the transfer of vancomycin resistance to *S. aureus*. Vancomycin-resistant *S. aureus* (VRSA) arose from the acquisition of *E. faecalis* *vanA*, encoding vancomycin resistance, which is believed to have happened within wound biofilms (Chang et al., 2003; Weigel et al., 2007). Two mechanisms have been proposed to mediate vancomycin resistance in *S. aureus*. Firstly, *S. aureus* can maintain the *E. faecalis*-derived plasmid which encodes *vanA* within the transposon element. Secondly, in some *S. aureus* strains, the *E. faecalis*-derived plasmid can act as a suicide gene vector for transposition of the transposon element into another *S. aureus* plasmid or chromosome *via* recombination. (Courvalin, 2006; Périchon & Courvalin, 2009). In biofilms, it is speculated that *S. aureus* displays increased retention of the enterococcal plasmid (preventing its degradation or dispersion), or that the close proximity of these organisms in biofilms facilitate foreign plasmid acquisition by *S. aureus* (Niederhäusern et al., 2011; Périchon & Courvalin, 2009), thus promoting emergence of VRSA. Besides antibiotic resistance gene transfer, nothing is known about other interactions between *E. faecalis* and *S. aureus* in wounds.

Therefore, in this chapter, we characterized the interactions between *E. faecalis* and *S. aureus* using *in vitro* biofilm models and an *in vivo* model of infection. We found that *E. faecalis* biofilm growth and wound infection titers are augmented in the presence of *S. aureus*. Transcriptomic data showed that, in co-culture, *S. aureus* L-arginine anabolic genes are induced, which may provide free arginine as an energy source for *E. faecalis*. A transposon library screen identified *S. aureus* mutants in a predicted membrane transporter that no longer augment mixed species biofilm growth. MALDI analysis of *S. aureus* supernatants revealed a decrease in arginine in

the predicted transporter mutant, suggesting that arginine may be the substrate for this system. Therefore, we hypothesize that *E. faecalis* utilizes arginine secreted by *S. aureus* to promote its growth during biofilm formation. Taken together, our data suggests that *S. aureus* is able to potentiate *E. faecalis* growth by providing it with different metabolic sources during biofilm formation and is postulated to synergistically maintain its growth *in vivo*.

5.2 Results

5.2.1 *E. faecalis* is augmented in polymicrobial biofilms with *S. aureus*

To model the interactions between *E. faecalis* and *S. aureus*, we used a previously characterized mixed species macrocolony assay (Keogh et al., 2016). Because *E. faecalis* are often co-isolated with *S. aureus* from wounds, we hypothesized that they can survive together and may synergistically influence one another. We co-cultured *E. faecalis* with *S. aureus* at a 1:1 ratio, and observed an “egg-yolk” phenotype in which *E. faecalis* appears to surround the *S. aureus* in macrocolonies (**Fig 5.1**). Furthermore, we observed the same “egg-yolk” phenotype at an input ratio of 19:1 (**Fig 5.1**) and in both TSB and BHI media (**Appendix Fig 5.1**). To test if the spatial restriction of *S. aureus* by *E. faecalis* (the “egg-yolk” phenotype) affects the growth of either species, we enumerated CFU from macrocolonies. We observed that both *S. aureus* and *E. faecalis* CFU were decreased 10-fold in mixed species macrocolonies compared to either single species macrocolony at 1 dpi (**Fig 5.2**). Interestingly, the initial drop in *E. faecalis* CFU was not sustained and instead we observed a 10-fold increase in *E. faecalis* titers, while *S. aureus* CFU were still lower in the mixed macrocolonies at 5 dpi (**Fig 5.2B**). To test if CFU differences between the two different time points was similar in other biofilm models, we grew single and mixed species biofilms of *E. faecalis* and *S. aureus* using a 96-well plate microtiter plate assay. We observed an increase in biofilm biomass, as measured by

crystal violet absorbance, of more than 50% in the mixed species biofilm compared to the single species at 5 dpi (**Fig 5.3**). Together, these results suggest that *S. aureus* is able to augment *E. faecalis* growth in biofilms.

E. faecalis

S. aureus

1_{SA}:1_{EF}

E. faecalis

S. aureus

19_{SA}:1_{EF}

Figure 5.1. *E. faecalis* and *S. aureus* co-culture results in an “egg-yolk” macrocolony phenotype regardless of inoculation ratios. Macrocolonies of *E. faecalis*, *S. aureus*, mixed 1_{EF}:1_{SA} ratio, or mixed 19_{SA}:1_{EF}, were grown on TSB agar for 5 days. Images shown are representative of three independent experiments.

Figure 5.2. *E. faecalis* CFU are augmented in the presence of *S. aureus* during co-culture in macrocolonies. Macrocolonies of *E. faecalis*, *S. aureus*, and mixed 1:1 ratio, were grown on TSB and enumerated at (A) 1 dpi, and (B) 5 dpi. N=3, n=3. Bar graphs depict the mean and standard deviation. Statistical analysis was performed using Mann-Whitney test comparing corresponding CFU of mixed species to single species. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

Figure 5.3. Biofilm biomass was augmented in mixed species co-culture of *E. faecalis* and *S. aureus*. *E. faecalis*, *S. aureus*, and mixed 1:1 ratio, were grown in TSB media and biofilm biomass were determined after 5 days. N=2-4, n=4-6. Bar graphs were plotted showing the mean and error bars represent standard deviation. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

To further characterize the spatial restriction of *S. aureus* by *E. faecalis*, we used a cross-streak assay which has been previously described to characterize *S. aureus* mixed species interactions (Hammer et al., 2014). We cross-streaked *E. faecalis* and *S. aureus*, streaking *E. faecalis* across *S. aureus* and vice versa. We observed that the orange pigmented *S. aureus* was spatially confined in areas where *E. faecalis* streaks occur (Fig 5.4A). Interestingly, we did not observe any difference in spatial localization of *S. aureus* in areas where there is *E. faecalis* carryover from the first streak (Fig 5.4B), suggesting that *E. faecalis* density may be important for restriction of *S. aureus* spatial localization.

Figure 5.4. *S. aureus* is inhibited locally at the point of *E. faecalis* contact. Cross-streaking of *E. faecalis* (blue arrow) and *S. aureus* (orange arrow) on TSB agar. Solid arrowhead denotes the first streak, thin arrowheads denotes the second streak. Arrow direction represents the streak direction. Images shown are representative of three independent experiments.

5.2.2 Polymicrobial *S. aureus* and *E. faecalis* infection results in increased titers of *E. faecalis* and an increased pro-inflammatory response

Because we observed that *S. aureus* was able to augment *E. faecalis* growth in biofilms, we hypothesized that this mixed species interaction may contribute to synergistic virulence during infection. To investigate if *S. aureus* is able to enhance *E. faecalis* growth during *in vivo* wound co-infection, we inoculated mouse wounds with equal amounts of *S. aureus* and *E. faecalis* using a 10^6 inoculum. At 8 hpi, we observed 10-fold fewer *S. aureus* in the mixed species infection compared to *S. aureus* single species infection (**Fig 5.5A**). However, consistent with our *in vitro* findings, *E. faecalis* CFU were >10-fold greater in polymicrobial wound infections at 24 hpi compared to *E. faecalis* single species infection (**Fig 5.5B**). We postulated that higher numbers of *E. faecalis* during polymicrobial infections at 24 hours could either be due to a lack of *E. faecalis* clearance between 8 and 24 hpi, or *S. aureus* augments CFU at 24 hpi. To test the hypothesis that an impaired host response during polymicrobial infection leads to higher *E. faecalis* CFU at 24 hpi, we performed cytokine, growth factor, and chemokine analyses on supernatants of polymicrobial infected wound homogenates. Contrary to our hypothesis, we observed that infection by both organisms resulted in higher levels of most cytokines compared to the single species controls, demonstrating that co-infection with *S. aureus* and *E. faecalis* results in a greater inflammatory response (**Fig 5.6**). Overall, our data suggests that *E. faecalis* grows better during co-infection with *S. aureus* regardless of the strong pro-inflammatory response.

Figure 5.5. *S. aureus* augments *E. faecalis* titers in wounds. Wounds were inoculated with either *E. faecalis*, *S. aureus*, or a mixed 1_{EF}:1_{SA} ratio and harvested at **(A)** 8 hours, and **(B)** 24 hours post-inoculation and the colony forming units (CFU) enumerated on selective agar. Each dot represents one mouse, and the solid horizontal lines indicate the median, N=1-2, n=5. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = $p < 0.05$.

Figure 5.6. Co-infection of *E. faecalis* and *S. aureus* in wounds results in a modest host pro-inflammatory response. Mice were wounded and infected with either *E. faecalis*, *S. aureus*, or mixed 1_{EF}:1_{SA} ratio. After 24 hours, wounds were processed into single cell suspensions and subjected to cytokine analysis. Cytokine concentrations are shown in pg/mL on the radial axis. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons where * = $p < 0.05$ represents comparison between mixed species to *E. faecalis* single profiles; and where # = $p < 0.05$ represents comparison between *S. aureus* single to *E. faecalis* single profiles.

5.2.3 *S. aureus*-derived arginine may promote *E. faecalis* growth during mixed species growth

To interrogate the mechanism underlying *E. faecalis* growth augmentation during co-culture with *S. aureus*, we performed RNA sequencing on 5 day old *E. faecalis* and *S. aureus* single and mixed species macrocolonies. We hypothesized that *E. faecalis* may take advantage of *S. aureus* secreted or released factors that could serve as a nutrient source or otherwise promote its growth. Transcriptomic data revealed the top gene categories upregulated in *S. aureus* mixed macrocolonies

include ribosomes, two-component systems, purine metabolism, pyrimidine metabolism, biotin metabolism, and arginine biosynthesis (**Fig 5.7, Appendix Fig 5.2, Appendix Table 5.1**). Comparatively in *E. faecalis*, we observed pathways related to oxidative phosphorylation, ribosome synthesis, and amino acid biosynthesis induced in the presence of *S. aureus* (**Appendix Table 5.2**). These data suggest that although we observe a ~2-fold decrease in *S. aureus* CFU in 5 day mixed species macrocolonies (**Fig 5.2**), the high level of metabolic gene transcription suggests that they are actively turning over. However, these data were not highly informative as to the *S. aureus* factor responsible for *E. faecalis* growth augmentation.

Figure 5.7. *E. faecalis* and *S. aureus* metabolic and ribosomal genes are upregulated in mixed macrocolonies. Transcriptomic comparison of mixed 1_{EF}:1_{SA} macrocolonies to (A) *E. faecalis*, or (B) *S. aureus* monospecies macrocolonies, grown to 5 days. Data is plotted as log₂ fold change (log₂FC). Genes and functional categories were considered differentially regulated when FDR < 0.05. The black line indicates the median value for each functional category; each circle represents each individual gene differentially regulated in the corresponding functional group, with different colors representing different gene categories. N=3, n=3.

To test if *E. faecalis* is augmented by *S. aureus* metabolite production, I collaborated with another postdoctoral fellow, Dr. Ch'ng Jun Hong, who used a transposon screening approach to elucidate *E. faecalis* and *S. aureus* interactions. We used a *S. aureus* USA300 transposon library of 1920 mutants to screen for *S. aureus* mutants that were unable to augment *E. faecalis* biofilm growth using the 96 well microtiter plate assay (Bose, Fey, & Bayles, 2013; Fey et al., 2013). Shortlisted *S. aureus* mutants that were unable to augment *E. faecalis* growth compared to the wild type control were subjected to secondary screening by normalizing and adding equal CFU of *E. faecalis* and *S. aureus* prior to biofilm quantitation, with additional growth kinetics assays to rule out false positives in the screening assays. Screening of 1920 mutants yielded one *S. aureus* mutant which has an insertion in a hypothetical glycolipid permease (NE462) that gave a 33% reduction in crystal violet readings (**Fig 5.8A**). Since we hypothesized that the *S. aureus* factor that promoted *E. faecalis* biofilm growth would be a secreted factor, we tested spent supernatants from the permease mutant for its ability to augment *E. faecalis* biofilm and we observed that indeed the permease supernatants yielded less biofilm biomass than *S. aureus* wild type spent media (**Fig 5.8B**). Together these data show that *S. aureus* is able to augment mixed species biofilm formation with *E. faecalis*, and suggests that *S. aureus* secreted products are responsible for the augmentation. To find differences in composition between wild type *S. aureus* and permease mutants secreted products, we subjected supernatants from both strains to mass spectrometry analysis. We observed that arginine levels in wild type *S. aureus* supernatants were ~2-4 times higher than supernatants from the permease mutant (**Table 5.1**). This finding corroborated our mixed species macrocolony transcriptomic data where we observed increased transcription of arginine anabolic genes *argB*, *argC*, *argJ*, *argG*, and *argH* in *S. aureus* (**Table 5.2, Appendix Fig 5.2, Appendix Table 5.1**) (Nuxoll et al., 2012).

However, we observed a decrease in the same permease gene expression in the transcriptomic data. Nonetheless, our data are consistent with a model in which, in a mixed-species biofilm, *S. aureus* increases synthesis of arginine which we postulate *E. faecalis* can make use of as an energy source to promote biofilm growth.

Figure 5.8. *S. aureus* permease (NE462) mutant is attenuated in mixed species biofilm formation and *E. faecalis* biofilm augmentation. *E. faecalis* were grown in (A) mixed 1:1 ratio with either wild type *S. aureus* or *S. aureus* permease mutants (*S. aureus* permease::tn) in TSB media, or in (B) supernatants derived from either wild type *S. aureus* or *S. aureus* permease mutants (*S. aureus* NE462::tn) cultured after 1 day post inoculation in TSB media. Biofilm biomass was assessed after 5 days post inoculation. N=2-3, n=4-6. Data was normalized (A) relative to *E. faecalis* single species biofilm biomass, or (B) relative *E. faecalis* biofilm biomass grown in wild type *S. aureus* supernatants. Bar graphs were plotted showing the mean and error bars represent standard deviation. Statistical analysis was performed using Student's unpaired t-test. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Data generated by Dr Ch'ng Jun Hong.

L-Arginine quantification	Concentration (mM)	Concentration (µg/mL)	Fold change relative to wild type
<i>S. aureus</i> wild type	1.79	311	1
<i>S. aureus</i> NE462::tn mutant	0.42	72	0.23

Table 5.1. Arginine levels are higher in supernatants of wild type *S. aureus* compared to supernatants from the *S. aureus* permease (NE462). Wild type *S. aureus* or the *S. aureus* permease mutants (*S. aureus* NE462::tn) were grown overnight in TSB media for 24 hours. Supernatants were then subjected to mass spectrometry identification and analysis. Concentrations are listed in mM and µg/mL, with L-arginine values normalized to *S. aureus* wild type levels. N=3. Data generated by Dr Ch'ng Jun Hong

Locus tag	Log ₂ FC	Gene	Annotation
SAUSA300_0184	0.989	argB	Acetylglutamate kinase
SAUSA300_0186	0.812	argC	N-acetyl-gamma-glutamyl-phosphate reductase
SAUSA300_0864	2.303	argG	Argininosuccinate synthase
SAUSA300_0863	2.5084	argH	Argininosuccinate lyase
SAUSA300_0185	0.990	argJ	Arginine biosynthesis bifunctional protein ArgJ
SAUSA300_0917	0.990	NE462	Permease mutant from transposon screen

Table 5.2. Upregulation of arginine biosynthesis genes in mixed macrocolonies compared to *S. aureus* single species. Table showing arginine biosynthesis genes in *S. aureus* from transcriptomic analysis comparing mixed macrocolonies with *S. aureus* monospecies macrocolonies. Differentially regulated genes are expressed as log₂ fold change (Log₂FC) after filtering with FDR < 0.05. KEGG locus tags and annotations are shown to the respective genes.

5.3 Discussion and conclusion

E. faecalis and *S. aureus* are common pathogens frequently co-isolated during polymicrobial wound infections (Bowler et al., 2001; Bowler & Davies, 1999; Giacometti et al., 2000). Both *E. faecalis* and *S. aureus* in wounds can impede the wound healing process. Furthermore, *E. faecalis* has the potential to transfer glycopeptide resistance genes to *S. aureus* in wounds, which could further increase *S. aureus* persistence and limit treatment options in wounds. In this chapter, we aimed to characterize the interspecies interactions between *E. faecalis* and *S. aureus* and determine the mechanisms underlying those interactions in wounds.

We show that *S. aureus* is able to augment *E. faecalis* growth during *in vitro* biofilm assays and *in vivo* in a mouse model of wound infection. Importantly, we show that mixed species infection *in vivo* resulted in elevated pro-inflammatory cytokine, chemokine, and growth factor expression compared to single species infection controls.

We next sought to determine the mechanism underlying *E. faecalis* growth augmentation mediated by *S. aureus*. We showed that L-arginine may be the *S. aureus* extracellular product utilized used by *E. faecalis*. Furthermore, a *S. aureus* permease mutant attenuated for promoting *E. faecalis* biofilm augmentation showed lower levels of L-arginine in its supernatants. We therefore hypothesize that this putative permease is important for the export of L-arginine. RNA sequencing data showing upregulation of arginine anabolism genes in mixed species macrocolonies supports the observation of higher arginine levels in wild type supernatants compared to the permease mutant. Because, glutamate are precursors for arginine biosynthesis, we hypothesize that glutamate flux in *S. aureus* is being shuttled towards arginine production in the presence of *E. faecalis*. While we observe lower arginine levels in supernatants from the putative permease mutant, we also observe lower transcription

of the same gene in mixed species macrocolonies. The bioavailability of L-arginine in wounds can influence the wound healing process. In one study, plasma L-arginine levels were lower in infected wounds compared to non-infected wounds (Debats et al., 2006). Though there is no study directly comparing the metabolites between diabetic and non-diabetic wounds, supplementation of external L-glutamate and L-arginine in diabetic wounds can promote wound healing through increasing proline synthesis needed for collagen production and deposition (Piatti et al., 2001; Thangavel et al., 2017; Jankovic et al., 2016; Jones et al., 2014). Moreover, addition of L-arginine can normalize nitric oxide in diabetic wounds to non-diabetic levels, consequently reducing the amount of reactive oxidative species and increasing insulin sensitivity (Piatti et al., 2001; Jankovic et al., 2016). We do not yet understand the significance of this, but speculate that this is specific to the macrocolony assay where we performed RNA sequencing and may not be observed in the biofilm assay, which will be tested by qPCR. Alternatively, its expression may be growth phase dependent.

Altogether, in this chapter we characterize *E. faecalis* and *S. aureus* interactions during biofilm formation and *in vivo* wound infection, and speculate that *S. aureus*-derived L-arginine augments *E. faecalis* growth and/or biofilm formation in these environments. Given the prevalence of polymicrobial infections containing *E. faecalis* and *S. aureus*, coupled with clinical data that VRSA emergence is a risk during wound infections, further studies on the mechanisms underlying these polymicrobial infections based on this work is needed.

5.4 Materials and Methods

5.4.1 Bacterial growth and strains

Enterococcus faecalis, OG1RF (ATCC 47077) was grown at 37 °C statically for 15-18 hours in Brain Heart Infusion media (BHI; Neogen, USA). *Staphylococcus*

aureus, USA300 LAC strain was grown at 37 °C statically for 15-18 hours in Tryptic Soy Broth (TSB; Oxoid, USA).

5.4.2 Macrocolony assay for mixed species biofilm growth

Colonies of *E. faecalis* and *S. aureus* were inoculated into BHI and TSB respectively and grown statically overnight at 37 °C. Overnight bacterial suspensions were washed twice in phosphate buffered saline (PBS), centrifuged at 5,000 x g for 4 mins. Optical density of 600 nm (O.D_{600nm}) of *E. faecalis* and *S. aureus* suspensions were adjusted to 0.7 and 0.8 respectively, which corresponds to 2 x 10⁸ CFU/mL for each organism. Macrocolonies were made by spotting 5 µL of normalized cultures onto 100 mm dishes containing TSB or BHI agar with *E. faecalis* single, *S. aureus* single or mixed ratios of both species and incubated at 37 °C. Separate macrocolonies were grown at 3.5 cm apart. To perform colony forming units (CFU) enumeration, macrocolonies were excised using a sterile blade and dislodging in PBS by vortexing. Bacterial suspensions were then serially diluted and enumerated onto selective agar; BHI containing 25 µg/mL of Rifampicin (Sigma Aldrich, USA) for *E. faecalis* and MRSASelect™ II Agar (Biorad, USA) for *S. aureus*.

5.4.3 RNA sequencing of *in vitro* macrocolonies

Macrocolonies were grown as described and dislodged in RNAprotect™ bacteria reagent (Qiagen, USA) containing PBS for 15 minutes at the respective time points. Fixed bacteria were pelleted at 5,000 x g for 10 mins and supernatant discarded with pellets stored at -80 °C for subsequent RNA extraction. RNA was extracted using the TRIzol™-chloroform method (Ambion®, USA) according to manufacturer's instructions. Briefly, bacterial pellets were resuspended in TRIzol™, subjected to mechanical shearing using Lysing Matrix D beads (MP Biomedicals, USA) and RNA extracted using chloroform and ethanol precipitation. Extracted RNA

was subjected to TURBO DNA-free™ kit (Ambion®, USA) for removal of DNA to <10%. DNase-treated RNA was subjected to rRNA removal using Ribo-Zero™ rRNA removal kit (Illumina, USA) and subjected to cDNA synthesis using NEBNext® RNA First Strand and NEBNext® Ultra Directional RNA Second Strand synthesis modules (New England Biolabs, USA). Library preparation was done by the sequencing facility of the Singapore Centre for Environmental Life Sciences Engineering (SCELS) and sequenced as 250 bp paired read using HiSeq 2000.

5.4.4 Transcriptomic analysis

Reads obtained from sequencing were checked using FastQC (version 0.11.5) and adapter trimmed using bbdut from the BBMap tools (version 34.49) (Bushnell, 2016). Trimmed reads were mapped onto the *E. faecalis* OG1RF (NCBI accession: GCF_000172575.2) or *S. aureus* USA300_FPR3757 (NCBI accession: GCF_000013465.1) reference genomes using BWA (version 0.7.15-r1140) (Li & Durbin, 2009). Reads mapping to predicted open reading frames of each genome were quantified using HTSeq (Anders, Pyl, & Huber, 2015) and differential gene expression analysis was performed under the R environment (version 3.4.4) using Bioconductor package, *edgeR* (Robinson, McCarthy, & Smyth, 2010). Genes were considered differentially expressed under false discovery rate (FDR) < 0.05. Gene annotation was performed using the database from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Pathway analysis was done using the R packages clusterProfiler (version 3.4.4) (Yu, Wang, Han, & He, 2012) and visualized using pathview (version 1.16.7).

5.4.5 Cross-streak assay

Cultures of *E. faecalis* and *S. aureus* were normalized to an O.D_{600nm} of 0.7 and 0.8 as described. Sterile 10 mL inoculating loops were dipped into the normalized

culture and used to streak across TSB agar, with 2 streaks perpendicular to each other. Plates were then incubated at 37 °C and photographed at 24 hours post streaking.

5.4.6 Microtiter 96-well plate biofilm assay

E. faecalis and *S. aureus* were assessed for their ability to form *in vitro* biofilms in co-culture using a crystal violet assay. Bacterial suspensions were normalized as described, and diluted 1:25 into 96-well polystyrene microtiter plates (Thermo Fisher Scientific, USA) containing 200 µL of final volume of TSB. Total bacterial CFU were kept constant during mixed species inoculation with 4 µL of each organism inoculated into a well. Plates were incubated statically at 37 °C until the desired time point for crystal violet staining. After incubation, wells were decanted to get rid of supernatants and wells were washed twice with PBS. Microtiter plates were blotted dry on absorbent hand towels to remove excess liquid. Biofilms were stained with 0.1% crystal violet (Sigma Aldrich, USA) for 30 mins at 4 °C, excess crystal violet was removed by washing twice with PBS. Solubilisation of crystal violet was done using ethanol-acetone (4:1) for 45 mins at room temperature and absorbance at O.D_{595nm} was measured with the Infinite® 200 PRO (Tecan Trading AG, Switzerland).

5.4.7 Polymicrobial infections of *E. faecalis* and *S. aureus* in a mouse wound excisional model

Mouse wound infection procedures were performed as stated in **Chapter 2** with the following modifications. A 6-mm biopsy punch (Integra Miltex, New York) was used to create a full-thickness wound and 10 µL of the respective bacteria or mixed species inoculum was applied. The wound site was then sealed with a transparent dressing (Tegaderm, 3M, St Paul Minnesota). At indicated time points, mice were euthanized and a 1 cm by 1 cm square piece of skin surrounding the wound

site was excised into sterile phosphate buffered saline (PBS). Excised wounds were homogenized and viable bacteria enumerated by plating onto selective agar; BHI containing 25 µg/mL of Rifampicin (Sigma Aldrich, USA) for *E. faecalis* and MRSASelect™ II Agar (Biorad, USA) for *S. aureus*. TSB agar without antibiotics was used to check for contamination. Mice with contaminants were excluded from datasets and subsequent analysis.

5.4.8 Cytokine Luminex® xMAP® analysis

Luminex MAP analysis was performed using the Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, California, USA) as per manufacturer's specifications.

5.4.9 Nebraska *S. aureus* transposon mutant library screen

The *S. aureus* USA300 LAC mariner transposon library containing 1920 mutants was obtained and glycerol stocked in 96-well microtiter plates (BEI Resources, USA) (Bose et al., 2013). The transposon mutants were cultured with a VIAFLO 96 (Integra Biosciences, Switzerland) into 200 mL TSB media for static overnight culture at 37 °C. *E. faecalis* was normalized to an O.D_{600nm} of 0.7 in PBS. A primary screen of the *S. aureus* transposon mutant library was performed by inoculating 8 µL of the normalized *E. faecalis* culture with 5 µL of the overnight *S. aureus* transposon mutants in TSB media to make up a final volume of 200 µL in 96-well titer plates. The microtiter plates were incubated for 5 days at 37 °C statically inside a moistened chamber to prevent loss of volume through evaporation. Biofilm was quantified using crystal violet staining as described above. Mutants with reduced signal compared to 3 standard deviations from the wild type were validated in the same assay with two independent biological replicates for each mutant. Slow overnight growers of *S. aureus* transposon mutants in TSB media were excluded from

the analysis. Secondary validations of the transposon mutants were performed to quantify biofilm growth using normalized 1_{SA}:1_{EF} ratios of *S. aureus* mutants.

5.4.10 Mass spectrometry profiling of L-arginine

S. aureus was cultured for 24 hours overnight statically at 37 °C and supernatants were filtered using a 0.2 µm filter. Supernatants were lyophilized using a FreeZone freeze dryer (Labconco, USA) and stored at -80 °C. Samples were then sent to the Singapore Phenome Centre (LKCmed, Singapore) for liquid chromatography and mass spectrometry profiling.

5.4.11 Statistical analysis

Statistics indicated in the figure legends were performed using GraphPad Prism software (Version 6.0f for Mac OS X, California).

5.4.12 Ethics statement

All procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee in Nanyang Technological University (ARF SBS/NIEA0198Z).

5.5 Authors contribution

I designed the study of this project, performed, and analyzed the experiments for characterization of *E. faecalis* and *S. aureus* interactions in this dissertation, assisted by students Nicole Yap Shao Jun and Yong Luo Yan. I analyzed the RNA-seq data from this study. Dr. Ch'ng Jun Hong initiated and performed the *S. aureus* transposon screening and supernatant assays for this part of the dissertation.

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Chapter 6: Conclusion and future perspectives

6.1 What do we currently know about *Enterococcus faecalis* wound infections?

The overarching goal of this thesis was to bridge the knowledge gap in understanding *E. faecalis* wound infections. Epidemiological studies show that *E. faecalis* are enriched within diabetic polymicrobial wounds. We hypothesized that *E. faecalis* will increase its pathogenic potential during wound infection in diabetics, where host immunity is impaired. Moreover, synergism with co-infecting organisms within wounds might increase the overall microbial burden and/or virulence in wounds. To investigate *E. faecalis* pathogenic mechanisms in wounds, we i) established a mouse wound infection model to characterize *E. faecalis* virulence; ii) performed comparative genomics to gain insight into relationships between gene content with phenotypic virulence traits; iii) determined *E. faecalis* virulence factors involved during wound infection; and iv) characterized the mechanisms underlying polymicrobial wound infections with *E. faecalis* and *S. aureus*. In this final chapter, I will review the findings and discuss how this thesis sets the foundation for future studies of *E. faecalis* wound infections.

We first sought to characterize and model *E. faecalis* wound infections to better understand their bacterial-host interactions. There is no study on *E. faecalis* wound pathogenic mechanisms and we aimed to fill this knowledge gap. We initiated this study by developing a mouse infection model of excisional wounds. First, we reported the minimum colonization and infection doses in both healthy and diabetic mice, and characterized the infection dynamics at both doses. *E. faecalis* infection in wounds resulted in early replication with overt inflammation that resolved to a persistence phase with limited inflammation. We observed *E. faecalis* microcolonies on the wound surfaces, similar to the morphology of bacterial biofilms reported in clinical wounds. Moreover, *E. faecalis* persistence is localized at the wound edge and

wound bed. *E. faecalis* wound infection consequently resulted in a poorer wound healing compared to uninfected wounds. We profiled the immune response to acute *E. faecalis* infection and found that an increased infiltration of neutrophils and macrophages to the site of infection. This suggests that the host immune system is able to respond to high titer *E. faecalis* in wounds and mounts an early pro-inflammatory response. Interestingly, *E. faecalis* titers were still relatively high despite the lack of inflammation after the initial immune-mediated clearance. We speculate that *E. faecalis* might actively modulate the immune response which could contribute to its persistence in wounds. Taken together, these observations set the groundwork for characterization of *E. faecalis* pathogenic mechanisms in wounds [Chapter 2].

At the outset of this study, there were few sequenced *E. faecalis* wound isolates available, which motivated us to collect and sequence human wound isolates in an attempt to identify common genetic traits that may contribute to wound virulence in these isolates. We collaborated with Tan Tock Seng Hospital and obtained 49 *E. faecalis* wound isolates for our analyses. The majority of *E. faecalis* wound isolates clustered into two major groups, ST16/179 and CC2/6. We observed *E. faecalis* wound isolates from CC2/6 were able to form biofilms better than the other isolates, and were enriched in a putative collagen-binding gene *ecbA*. Interestingly, we observed a majority of the isolates from ST16/179 groups could form thicker biofilms when we supplemented additional glucose into the media, but this was not observed for the CC2/6 group. Overall, our data suggests that the presence of *ecbA* can be predictive of biofilm formation under low glucose conditions, and that higher glucose levels may promote biofilm formation in wounds. These findings suggest that the majority of *E. faecalis* wound isolates may share genomic relatedness and can be grouped by their capacity to form biofilms. Since it

has been speculated that wounds serve as a nidus for bacteremia, it will be interesting to see if these same *E. faecalis* clonal types are also enriched in disseminated infections. [Chapter 3]

Next, we sought to identify *E. faecalis* virulence factors that are important for wound infection and persistence. We tested known mutants of *E. faecalis* based on their roles in surface attachment, immune defense, and biofilm formation in other niches. We also performed an unbiased transposon sequencing screen for *in vivo* *E. faecalis* fitness factors in the wound model established in **Chapter 2** (Van Opijnen & Camilli, 2013). We observed that *E. faecalis* defense against cationic antimicrobial peptides is important, but sortases are dispensable for *E. faecalis* persistence in wounds. We reported from our initial transposon sequencing that disruption of a PTS transporter, predicted to transport mannose/fructose/sucrose, impaired *E. faecalis* fitness in wound persistence. Taken together, our results suggest that the ability to resist immune defenses, and to use carbohydrates as an energy source, are important for *E. faecalis* wound persistence. We are the first to report *E. faecalis* fitness factors that can contribute to its infection and persistence in wounds. [Chapter 4]

Finally, we characterized the interactions between *E. faecalis* and *S. aureus*, which are frequently co-isolated from human wounds. We reported that *E. faecalis* and *S. aureus* can spatially distribute themselves in an “egg-yolk” phenotype during macrocolony formation. In addition, we observed that *E. faecalis* titers were higher during co-culture with *S. aureus* during *in vitro* biofilm growth and *in vivo* wound infections. Through a transposon screening approach and mass spectrometry identification, we showed *S. aureus* derived arginine is able to augment *E. faecalis* growth during *in vitro* co-culture. Moreover, we observed an increased expression of arginine producing genes in *S. aureus* during co-culture with *E. faecalis*. Taken together, our findings suggest that *E. faecalis* may benefit from *S. aureus* co-culture

by importing L-arginine as an energy source. It is of future interest to study the role of L-arginine *in vivo* and to identify the *E. faecalis* transporter for arginine import. Understanding these polymicrobial wound interactions can provide us insights into treating these infections. [Chapter 5]

Overall, we have achieved the overarching goal of this thesis. These findings set the foundation for studying *E. faecalis* monomicrobial and polymicrobial wound infections. We have developed a robust *in vivo* wound model to characterize and understand host-pathogen interactions. Furthermore, we have developed genetic tools enabling us to identify *E. faecalis* virulence factors *in vivo*. Our findings highlight that *E. faecalis* can infect and persist in wounds by resisting the immune system and importing carbohydrates during monomicrobial wound infection. In addition, our comparative genomic analyses identified a majority of *E. faecalis* isolates from human wounds are biofilm-positive and are genomically related. Finally, we showed that *S. aureus* can promote the growth of *E. faecalis* during polymicrobial wound infection. These findings will serve as a basis for future studies discussed in the next section.

6.2 Future perspectives

Mechanisms of *E. faecalis* wound pathogenesis still remain largely unexplored, especially in the context of biofilm formation and progression, as well as the host-pathogen mechanisms involved during chronic wound infections. We showed that *E. faecalis* microcolonies are present on the wound bed but have yet to identify factors that contribute to their formation and maturation. In general, human wound biofilms can form thick structures consistent with biofilm maturation, and are considered a hallmark of chronic infection (Hurlow, Blanz, & Gaddy, 2016; Hurlow et al., 2015; Metcalf & Bowler, 2015). We have not yet elucidated if *E. faecalis* is able to form mature complex structures in wounds or whether *E. faecalis* wound

biofilms are limited to microcolony formation, similar to what has been reported in *E. faecalis* gut biofilms (Barnes et al., 2017; Hirt et al., 2018). The progression of *E. faecalis* biofilms can be monitored by performing more extensive histological examination over a time course through a combination of histological sectioning, fluorescence *in situ* microscopy (FISH), and scanning electron microscopy on *E. faecalis* clinical wound samples. In addition, host factors may contribute to *E. faecalis* wound biofilm formation. In our studies, we observed “thread-like” structures around *E. faecalis* wound microcolonies. We hypothesized that these “thread-like” structures are indicative of either host fibrin bundles or neutrophil extracellular traps (NETS), both of which are involved in defenses against invading pathogens. Immunofluorescence analysis can be used to identify target of interest, or specifically look for factors involved in wound clotting or markers of NETS. Taken together, understanding *E. faecalis* wound biofilm formation can help us in diagnosis and treatment of these infections in wounds.

One of the outstanding questions from this work is how the host responds to *E. faecalis* wound infection in diabetics. We can apply the same flow cytometry methods used in this dissertation to earlier time points in normal mice, to investigate host responses during *E. faecalis* chronic wound infections in normal and in diabetic mice. Furthermore, we can use our transposon sequencing method (**Chapter 4**) to identify *E. faecalis* fitness factors in chronic wounds in normal and diabetic backgrounds. One of the limitations to transposon sequencing is that the contribution of secretory products may be masked due to sharing of public goods. To compensate for this limitation, we can complement transposon sequencing with transcriptomic analysis to gain additional information about the *in vivo* situation. “Dual-seq” approaches can also be used to determine gene expression profiles from both host and pathogen at the same time within the same infection (Westermann & Vogel, 2018). Therefore, we can

utilize both genetic approaches to identify pathways involved during *E. faecalis* wound infection.

Comparative genomics analyses of clinical *E. faecalis* wound isolates suggest that *E. faecalis* in wounds are genetically related. However, a drawback of our study is that we do not have any source information of the wound environment from which these strains were isolated. Future studies should examine associations between different types of chronic wounds (e.g. pressure, diabetic, or venous ulcers), and the genomic content of *E. faecalis* each wound type. More importantly, we have not determined if *E. faecalis* wound infection can lead to disseminated bacteremia in patients. Hence, we propose to collect paired strains isolated from patients with both *E. faecalis* wound and bloodstream infections. In addition, with the increasingly available genomic sequencing capacity, we can begin to draw correlations between virulence profiles and look for genomic patterns of dissemination during *E. faecalis* wound infection on a global scale.

This dissertation has just begun to scratch the surface of understanding *E. faecalis* polymicrobial wound infections. With a developed model, we can now begin to study the underlying mechanisms involved in polymicrobial wound infections. Recent metagenomic approaches are trying to correlate skin microbiota changes to wound chronicity (Byrd, Belkaid, & Segre, 2018; Grice & Segre, 2011; Pereira, Moura, Carvalho, & Empadinhas, 2017). Since vancomycin resistant Enterococci has been isolated on the skin of intensive care unit patients, this raise the possibility that skin colonization can serve as a reservoir for *E. faecalis* wound infection particularly in vulnerable populations. We and others have shown that *E. faecalis* can be mutualistic with other co-infecting organisms during wound infections. However, we do not yet understand how *E. faecalis* contributes to polymicrobial infections where more species are present. Hence there is a challenge in overcoming the difficulty in

understanding these polymicrobial infections, where the complexity in studying these systems is proportionate to the number of species at the site of infection. However, I am confident that eventually as we understand the contribution of different microbial communities to development of chronic wounds, we will be able to build a framework to understand, diagnose, and propose treatment to these polymicrobial wound infections.

Our *in vivo* model can also serve as a platform to test antimicrobial compounds against *E. faecalis* monomicrobial and polymicrobial wound infections. We have begun using this model to test antimicrobial compounds against other microbes, and can be extended to looking at polymicrobial wound infections (not covered in this dissertation) (Lim et al., 2018). Our model gives us the flexibility to test different compounds (e.g. hydrogels, prophylaxis, and different wound dressings), and the ability to assess the bacterial burden and look at changes in wound healing markers post-treatment. Therefore, our model provides us the ability to validate different antimicrobials for clinical translational studies, while studying the dynamics and response of these bacterial wound infections.

Overall, we have characterized and determined mechanisms underlying *E. faecalis* monomicrobial and polymicrobial wound infections. Moreover, my dissertation provides insight into the pathogenic potential of *E. faecalis* in wounds and how *E. faecalis* is able to persist in this niche. These findings will serve as the foundation for future studies in understanding mechanisms of *E. faecalis* infections and provide insights into designing diagnostics and treatments against these *E. faecalis* chronic wound infections.

6.3 References

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Appendix figures and tables

This section contains the appendix figures for this dissertation. Figures shown in the appendix are directly referenced in **Chapters 2-5**. The appendix is broken up into the four respective chapters with their respective labels for each chapter.

Chapter 2 appendix

Appendix Figure 2.1 *E. faecalis* infection downregulates neutrophil cytokines and chemokines, IL-2, IL-5, IL-10, IL-12p70, CCL11, IFN- γ and CSF2. Male C57BL/6 mice were wounded and infected with 10² CFU or 10⁶ CFU *E. faecalis*, or

mock-infected with PBS. Wounds were harvested at **(A)** 8 hours or **(B)** 3 dpi, and homogenized, clarified, and supernatants were analyzed by Luminex MAP analysis to determine absolute cytokines levels in pg/mL. Each dot represents one mouse and the solid horizontal lines indicate the median. n=5. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$. **(C)** Principal component analysis was done for all analytes measured at all time points. Principal component1 (PCA1) and principal component2 (PCA2) represent 41.5% and 32.3% of the variations of all analytes respectively. Each dot represents one mouse and are clustered plotted according to their positions in PCA1 and PCA2. Clustering was performed to group analyte profiles of the same timepoints together and represented as follows: 10^6 CFU 3 dpi; red circle, 10^2 CFU 3 dpi; yellow triangle, PBS 3 dpi; green square, 10^6 CFU 8 hpi; cross, 10^2 CFU 8 hpi; box with cross, PBS 8 hpi; star. Ellipses represent the standard error of variation by each group.

Appendix Figure 2.2. *E. faecalis* modulates the soluble and cellular host response at the wound site. Mice were wounded and infected with 10^6 CFU of *E. faecalis* or mock-infected with PBS. Wounds were harvested at (A) 1 dpi and (B) 3 dpi, and processed into single cell suspensions. Total number of monocytes Ly-6C^{low} (CD45⁺ MHCII⁻ CD11b⁺ Ly6C^{low} CD64⁻), monocytes Ly-6C^{high} (CD45⁺ MHCII⁻ CD11b⁺ Ly6C^{high}), monocyte-derived-cells (CD45⁺ MHCII⁺ CD11b⁺ CD64⁺ Ly6C^{low-int}), conventional dendritic cells 1 (CD45⁺ MHCII⁺ CD24⁺ CD11b⁻), conventional dendritic cells 2 (CD45⁺ MHCII⁺ CD11b⁺ Ly6C⁻ CD64⁻) and Langerhan cells (CD45⁺ MHCII⁺ EpCAM⁺ CD11b^{int}) infiltrating into and accumulating in the skin were analyzed by flow cytometry. N=2, n=5. Error bars represent standard error of mean. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-hoc test comparing infected against mock-infected wounds. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

<i>E. faecalis</i> strain (resistance, if known) ^a	Antibiotics concentration used for selection	Source
OG1RF (Rif, Fus)	Rifampicin (25 µg/ml)	
V583 (Van)		
OG1X (Str)	Streptomycin (500 µg/ml)	
EF_001		Clinical isolate from wound
EF_002		Clinical isolate from wound
EF_003		Clinical isolate from wound
EF_004		Clinical isolate from wound

^aRif, Rifampicin; Fus, Fusidic acid; Van, Vancomycin; Str, Streptomycin.

Appendix Table 2.1. Strains used in Chapter 2.

Molecule	Clone	Vendor
MHC II (IA/IE)	M5/114.13.2	BD Biosciences
Ly6G	1A8	Biolegend
CD64	X54-5/7.1	Biolegend
F4/80	BM8	Biolegend
EpCAM	G8.8	Biolegend
CD45	30F11	eBioscience
Ly6C	HK1.4	eBioscience
CD24	M1/69	eBioscience
CD11b	M1/70	eBioscience

Appendix Table 2.2. Flow cytometry antibodies used in Chapter 2.

Appendix Figure 2.3. *E. faecalis* infection in diabetic wounds results in a sustained pro-inflammatory response. Diabetic *db/db* mice were wounded and infected with 10² CFU or 10⁶ CFU *E. faecalis*, or mock-infected with PBS. Wounds were harvested at (A) 8 hours or (B) 3 dpi, and homogenized, clarified, and supernatants were analyzed by Luminex MAP analysis to determine absolute cytokines levels in pg/mL. Each dot represents one mouse and the solid horizontal lines indicate the median. n=5. Statistical analysis was performed using Kruskal-Wallis test with Dunn's post-test to correct for multiple comparisons. * = p < 0.05, ** = p < 0.01.using Kruskal-wallis test with Dunn's post-hoc test comparing infected against mock-infected wounds.

Chapter 3 appendix

Isolate name	<i>E. faecalis</i> (Pure or Mixed growth)	Collection site
1-EF	Mixed	L Foot
2-EF	Mixed	Perianal Abscess
3-EF	Mixed	Ear cyst wound Swab
4-EF	Mixed	Scrotal Abscess Swab
5-EF	Mixed	Right foot wound Swab
6-EF	Mixed	Peripancreatic fluid
7-EF	Mixed	Wound swab
8-EF	Mixed	L Foot tissue
9-EF	Mixed	Suprapubic wound
10-EF	Mixed	Nasopharyngeal wound
11-EF	Mixed	Right lateral foot
12-EF	Mixed	Supraorbital wound
13-EF	Mixed	Buttock wound
14-EF	Mixed	F forefoot tissue
15-EF	Pure	L periauricular abscess
16-EF	Mixed	Wound swab
17-EF	Mixed	Sacral sore
18-EF	Mixed	Sacral swab
19-EF	Mixed	Index finger
20-EF	Mixed	Toe
21-EF	Mixed	Bile wound swab
22-EF	Mixed	Wound swab
23-EF	Mixed	Wound swab
24-EF	Mixed	Perianal Abscess
25-EF	Mixed	Wound swab
26-EF	Mixed	Toe wound
27-EF	Mixed	Bile
28-EF	Mixed	Leg wound
29-EF	Pure	Pancreatic abscess
30-EF	Mixed	Sacral swab
31-EF	Mixed	Foot swab
32-EF	Mixed	Stump
33-EF	Mixed	Wound swab
34-EF	Mixed	Heel w.s
35-EF	Mixed	Leg wound
36-EF	Mixed	Gluteal pus
37-EF	Mixed	Peripancreatic fluid
38-EF	Mixed	Inguinal abscess
39-EF	Mixed	Tissue R forefoot
40-EF	Mixed	Wound swab
41-EF	Mixed	Tissue left lateral leg
42-EF	Mixed	WS bedsore
Table continued on next page		

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EF_001	Mixed	Wound swab
EF_002	Mixed	Wound swab
EF_003	Mixed	Wound swab
EF_004	Mixed	Wound swab
EF_005	Mixed	Wound swab
EF_1008	Mixed	Wound swab
EF_1010	Mixed	Wound swab

Appendix Table 3.1. Clinical isolates used for this study.

Species	Primer name	Sequence (5' to 3')	Product size (bp)
<i>E. faecalis</i>	FL1	ACTTATGTGACTAACTTAACC	30
	FL2	TAATGGTGAATCTTGGTTTGG	

Appendix Figure 3.1. PCR-based identified *E. faecalis* species via amplification of *sodA* gene. Genomic DNA from isolates were extracted and subjected to PCR amplification with primers targeting the superoxide dismutase (*sodA*) gene in *E. faecalis*. Presence of a 360 base pair band is indicative of the *E. faecalis* strain. *E. coli* and *S. aureus* were included as negative controls. A subset of all 49 clinical *E. faecalis* wound isolates is shown here. Primer sequences used for species-specific targeting of *sodA*.

Appendix Figure 3.2. Growth kinetics of *E. faecalis* clinical wound isolates with and without glucose supplementation. *E. faecalis* clinical wound isolates were grown in (A) TSB media (TSB) or (B) TSB media supplemented with 0.25% glucose (TSB(G)). *E. faecalis* OG1RF is a biofilm positive laboratory strain. *E. faecalis* $\Delta srtA\Delta ebpABC$ is a biofilm negative strain. Baseline readings are indicated by BLANK, containing only the indicated media.

Strain	Gentamicin (mg/mL)	Amoxicillin (mg/mL)	Daptomycin (mg/mL)	Vancomycin (mg/mL)
1-EF	48	1.0	0.50	1.0
2-EF	384	1.0	0.38	1.0
3-EF	16.0	0.75	0.75	1.5
4-EF	12.0	0.50	0.50	1.5
5-EF	48.0	1.0	0.50	1.5
6-EF	12.0	0.75	0.50	1.5
7-EF	> 1024	0.75	0.75	0.8
8-EF	> 1024	0.50	0.38	1.0
9-EF	> 1024	1.0	0.50	1.5
10-EF	> 1024	0.75	0.38	1.0
11-EF	> 1024	1.00	0.75	3.0
12-EF	16.0	0.75	1.00	2.0
13-EF	> 1024	0.75	1.00	2.0
14-EF	> 1024	0.75	1.00	0.8
15-EF	> 1024	0.75	1.00	0.5
16-EF	> 1024	0.50	0.38	1.0
17-EF	24.0	0.75	0.50	1.0
18-EF	> 1024	0.75	0.38	1.0
19-EF	> 1024	0.75	0.75	1.5
20-EF	16.0	0.75	1.00	1.0
21-EF	> 1024	0.75	0.75	1.0
22-EF	> 1024	0.50	0.75	1.0
23-EF	> 1024	0.75	0.50	1.0
24-EF	> 1024	0.38	0.50	1.0
25-EF	> 1024	0.50	0.19	1.0
26-EF	> 1024	0.38	0.50	2.0
27-EF	> 1024	0.75	0.38	0.75
28-EF	> 1024	0.75	0.50	0.5
29-EF	> 1024	1.0	0.19	1.0
30-EF	> 1024	0.064	0.38	1.0
31-EF	> 1024	0.75	0.38	1.0
32-EF	16.0	0.75	0.094	1.0
33-EF	> 1024	1.50	0.50	1.0
34-EF	> 1024	0.19	0.50	1.0
35-EF	16.0	0.75	0.50	3.0
36-EF	> 1024	0.75	0.50	1.0
37-EF	> 1024	1.0	0.38	1.0
38-EF	16.0	0.75	0.75	1.5
39-EF	> 256	0.75	0.38	1.0
40-EF	16.0	0.75	0.50	1.0
41-EF	> 256	0.75	0.38	1.0
42-EF	> 1024	0.75	0.50	1.0

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EF_001	16.0	1.0	0.75	1.0
EF_002	> 256	1.5	0.38	1.0
EF_003	> 1024	1.0	0.38	0.75
EF_004	> 256	1.0	0.25	2.0
EF_005	> 256	1.5	0.75	1.0
EF_1008	12.0	0.75	0.25	1.0
EF_1010	12.0	1.0	0.75	2.0

Appendix Table 3.2. Minimum inhibitory concentrations of *E. faecalis* wound isolates for gentamicin, amoxicillin, daptomycin, and vancomycin.

Appendix Figure 4.1. Host extracellular matrix binding proteins, Ace, Cna, and PavA are dispensable for *E. faecalis* wound infection. Wounds were infected with 1:1 ratio of *E. faecalis* strains OG1X:wild type OG1RF, or OG1X:OG1RF *ace::tn*, or OG1X:OG1RF *cna::tn*, or OG1X:OG1RF *pavA::tn*, at 10^6 CFU per inoculum. Wounds were harvested at 24 hpi and the recovered bacteria enumerated on selective media for each strain. Each dot represents one mouse and the solid horizontal lines indicate the median. $N \geq 1$, $n \geq 4$. Statistical analysis was performed using Mann-Whitney U test.

<i>E. faecalis</i> strain (resistance, if known) ^a	Antibiotics concentration used for selection
OG1RF (Rif, Fus)	Rifampicin (25 µg/ml)
OG1X (Str)	Streptomycin (500 µg/ml)
OG1RF $\Delta srtA\Delta srtC$ (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF $\Delta ebpABC$ (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF <i>ace::tn</i> (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF <i>cna::tn</i> (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF <i>pavA::tn</i> (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF_10020::tn (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF_10021::tn (Rif, Fus)	Rifampicin (25 µg/ml)
OG1RF $\Delta mprF1\Delta mprF2$ (Rif)	Rifampicin (25 µg/ml)

^aRif, Rifampicin; Fus, Fusidic acid; Van, Vancomycin; Str, Streptomycin.

Appendix Table 4.1. Strains used in Chapter 4.

Oligo Name	Sequence 5' to 3'
Spinkerette_top	G*AGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCC GATC*T
Spinkerette_botto m	[Phos]G*ATCGGAAGAGCGGTTCAGCAGGttttttttcaaaaaa *a
Transposon specific primer 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTATC AGCCAACCTGT
Transposon specific primer 2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATAG TCGGTTGGACA
3' Spinkerette primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAG ATCGGTCTCGGCATTCC

* denotes phosphorothioate linkages

[Phos] denotes phosphate group

Appendix Table 4.2. Primers and sequences used for enriching sequences containing transposon insertions.

Appendix Figure 5.1. *E. faecalis* and *S. aureus* co-culture forms a “egg-yolk” macrocolony phenotype independent of media tested. Macrocolonies of *E. faecalis*, *S. aureus*, and mixed 1_{SA}:1_{EF} ratio were grown on TSB or BHI agar for 5 days. Images shown are representative of three independent experiments.

Appendix Figure 5.2. Top categories differentially expressed in 1EF:1SA macrocolonies to *S. aureus* monospecies macrocolonies at 5 days post inoculation. Size of the circle represents the number of genes differentially expressed in the respective KEGG category. GeneRatio (x-axis) represents the ratio of the number of genes differentially expressed over the total number of unique genes in the KEGG category. Color of the circle represents the adjusted p-values. The corresponding table can be found as **Appendix Table 5.1**.

Pathway KEGG ID	Description	Set Size	NES	p.adjust
saa03010	Ribosome	57	2.531	0.031
saa02020	Two-component system	69	2.224	0.031
saa00230	Purine metabolism	60	1.934	0.031
saa00240	Pyrimidine metabolism	44	1.909	0.031
saa00780	Biotin metabolism	11	1.893	0.031
saa00220	Arginine biosynthesis	20	1.857	0.031
saa00010	Glycolysis / Gluconeogenesis	40	1.802	0.045
saa00910	Nitrogen metabolism	13	1.786	0.045
saa01120	Microbial metabolism in diverse environments	136	1.683	0.031

Set size: Number of genes in the pathway that are differentially regulated.

NES: Normalized enrichment score

p.adjust: Adjusted P values

Appendix Table 5.1. Gene set enrichment analysis comparing 1_{EF}:1_{SA} macrocolonies to *S. aureus* monospecies macrocolonies at 5 days post inoculation.

KEGG pathway ID	Description	p.adjust	Count
efi00190	Oxidative phosphorylation	0.029621354	19
efi03010	Ribosome	0.045433696	34
efi01230	Biosynthesis of amino acids	0.045955077	38

p.adjust: Adjusted p-values

Count: Number of genes in that pathway that are differentially regulated

Gene categories shown have p.adjusted values < 0.05.

Appendix Table 5.2. Enrichment analysis comparing 1_{EF}:1_{SA} macrocolonies to *E. faecalis* monospecies macrocolonies at 5 days post inoculation.