Mechanism of immune modulation in macrophages by *Enterococcus faecalis*

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**MECHANISM OF IMMUNE MODULATION IN
MACROPHAGES BY *ENTEROCOCCUS FAECALIS***

BRENDA TIEN YIN QI

Interdisciplinary Graduate School

HealthTech NTU (NTU institute for Health Technologies)

Nanyang Technological University

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MACROPHAGES BY *ENTEROCOCCUS FAECALIS***

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

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

2020

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.

9 Mar 2020

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BRENDA TIEN YIN QI

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.

9 Mar 2020

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KIMBERLY KLINE

Authorship Attribution Statement

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

Parts of results from Chapter 2 and 3 is published as Tien B. Y. Q., Goh H. M. S., Chong K. K. L., Bhaduri-Tagore S., Holec S., Dress R., Ginhoux F., Ingersoll M. A., Williams R. B. H., & Kline K. A. (2017). *Enterococcus faecalis* Promotes Innate Immune Suppression and Polymicrobial Catheter-Associated Urinary Tract Infection. *Infection and immunity*, 85(12), e00378-00317. doi:10.1128/IAI.00378-17

The contributions of the co-authors are as follows:

- Prof Kline provided the initial project direction and edited the manuscript drafts.
- I wrote the drafts together with Dr Goh and Dr Tagore. The manuscript was revised by Dr Chong and Dr. Goh.
- I co-designed the study with Prof Kline and Dr Tagore and optimised, performed and analysed all the *in vitro* laboratory work at Singapore Centre for Environmental Life Sciences Engineering. The multiplex cytokines and chemokines assay was performed by me with the help of Dr Chong.
- Dr Goh performed and analysed all the *in vivo* mice experiments.
- Dr Holec assisted in the collection of the RNA from *in vivo* mice experiments.
- Dr Williams assisted in the analysis and interpretation of the RNAseq data.
- Dr Chong assisted in analysis of the multiplex cytokines and chemokines assays.
- Dr Ginhoux, Dr Ingersoll and Dr Dress assisted in editing of the manuscript drafts.

9 Mar 2020

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BRENDA TIEN YIN QI

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Mechanism of immune modulation in macrophages by

Enterococcus faecalis

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Summary

Enterococcus faecalis is a member of the human gastrointestinal microbiota and is a gram-positive, opportunistic pathogen associated with hospital acquired wound, bloodstream, and urinary tract infections. *E. faecalis* can suppress or evade immune-mediated clearance by macrophages which promotes persistent infection. However, the mechanism by which *E. faecalis* modulates host immune function is not well understood. The overarching aim of this thesis is to fully characterize the interaction between *E. faecalis* and macrophages. I hypothesised that *E. faecalis* can modulate macrophage activation during infection thus affecting the host innate immune response *in vivo*. I established an *in vitro* model to study the immune-modulatory interaction of *E. faecalis* and macrophages. I observed that *E. faecalis* actively prevents NF- κ B signalling in mouse RAW-Blue macrophages in the presence of Toll-like receptor agonists and during polymicrobial infection with *Escherichia coli* in a dose-dependent manner, resulting in global downregulation of cytokines and chemokines during enterococcal infection. To elucidate the bacterial factor(s) that are involved in this macrophage suppression, various *E. faecalis* virulence mutants were tested. I showed that lactic acid, secreted by *E. faecalis*, is essential for macrophage suppression. Moreover, I showed

that lactate dehydrogenase-mediated immune suppression promotes *E. coli* survival during polymicrobial wound infection. Finally, to identify the pathways which *E. faecalis* modulates to suppress macrophage activation, I performed western blot analysis and RNA analysis. I observed that *E. faecalis* subverts macrophage activation by suppressing I κ B α and ERK level, preventing p65 phosphorylation and potentially activating Hif-1 α . In addition, *E. faecalis* infection at high doses also prevents polarisation of macrophages towards a M1 and M2 phenotype. Taken together, these results suggest that *E. faecalis* requires a high bacterial density to subvert the innate immune response, that *E. faecalis*-derived lactic acid is involved in this subversion, resulting in altered pathogenic outcomes. Understanding the interaction between *E. faecalis* and the host immune response may be helpful in developing alternative approaches or improved treatment strategies to prevent recalcitrant *E. faecalis* infection.

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Abbreviations

ACE	Adhesion to collagen of <i>E. faecalis</i>
AD	Atopic dermatitis
AMP	Adenosine monophosphate
AMPs	Antimicrobial peptides
AP-1	Activating protein-1
APCs	Antigen-presenting cells
Arg1	Arginase 1
AS	Aggregation substance
BHI	Brain heart infusion
CARD	Caspase activation and recruitment domain
CAUTI	Catheter-associated urinary tract infection
CBP	p300/co-activating proteins
c-di-AMP	Cyclic dimeric adenosine monophosphate
CWSS	Cell wall sorting signal
Cyl	Cytolysin
DAMPs	Damage-associated molecular patterns
DhaK	Dihydroxyacetone kinase
DHAP	Dihydroxyacetone phosphate
EII	Sugar-specific enzymes II
Ebp	Endocarditis and biofilm-associated pili
EC2S	Elongin BC-cullin 2-suppressor of cytokine signalling-box

ERKs	Extracellular signal regulated kinases
Ers	Enterococcal regulator of survival
ESAT-6	Early secreted antigenic target protein 6
ESP	Enterococcal surface protein
ExoY	Exoenzyme Y
Fyb	Fyn-binding protein
GAS	Group A <i>Streptococcus</i>
GelE	Gelatinase
GI	Gastrointestinal
GldA1	NAD ⁺ -dependent glycerol dehydrogenase
GlpO	Glycerol-3 oxidase
GM-CSF	Granulocyte macrophage colony-stimulating factor
HAT	Histones acetyltransferase
HDAC	Histones deacetylase
Hip1	Hydrolase important for pathogenesis 1
hpi	Hours post infection
HPr	Histidine-containing phosphocarrier protein
IFN	Interferon regulatory transcription factor
IFN γ	Interferon gamma
IFNs	Type I interferons
IKK	I κ B Kinase
IL	Interleukin
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element

JNKs	Jun amino-terminal kinases
LB	Luria bertani
LDH	Lactate dehydrogenase
LF	Lethal factor
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharides
LRRs	Leucine-rich repeats
LTA	Lipoteichoic acids
MAP	Mitogen-activated protein
MAPKs	Mitogen-activated protein kinase
MDA5	Melanoma differentiation associated factor 5
MEKK1	MAPK/ERK kinase kinase 1
MKK	MAP kinase kinase
MprF	Multiple peptide resistance factor protein
NACHT	NAIP, CIITA, HET-E and TP1 proteins
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding oligomerization domain
NF- κ B	Nuclear factor kappa-light chain enhancer of activated B cells (NF- κ B)
NIK	NF- κ B inducing kinase
NleC	Non-locus of enterocyte effacement encoded effector C
NLRs	Nucleotide oligomerization domain-like receptors
OD	Optical density
OspF	Outer shigella protein F

PAMPs	Pathogen-associated molecular patterns
pApA	Phosphoadenylyl adenosine
PDK1	3-phosphoinositide-dependent protein kinase 1
PDLIM2	Postsynaptic density 65-discs large-zonula occludens 1- abnormal cell lineage 11-islet 1- mechanosensory abnormal 3 domain-containing protein 2
PEP	Phosphoenolpyruvate
PG	Phosphatidylglycerol
PI3K	Phosphatidylinositol 3-kinase
PIP ₃	Phosphatidylinositol (3,4,5)-triphosphate
PML	Promyelocytic leukaemia
PPRs	Pathogen recognition receptors
PSM α 1- α 3	Phenol-soluble modulins α 1- α 3
PTS	Phosphoenolpyruvate phosphotransferase system
RANK	Receptor activator of NF- κ B
RIG-I	Retinoic acid-inducible gene-I
RIPK2	Receptor-interacting serine/threonine-preotein kinase 2
RLRs	RIG-1-like receptors
SAPKs	Stress-activated protein kinases
SepA	Staphylococcus efflux pump A
SIC	Streptococcal inhibitor of complement
SLO	Streptolysin O
SOCS1	Suppressor of cytokine signalling 1
SOCS3	Suppressor of cytokine signalling 3

SpeB	Streptococcal pyrogenic exotoxin B
SPI-2	<i>Salmonella</i> pathogenicity island 2
SprE	Serine protease
SSLs	Staphylococcal superantigen-like proteins
STING	Stimulator of interferon genes
T3SS	Type III secretion system
TAD	Transactivation domain
TAK1	Transforming growth factor beta-associated kinase 1
TCA	Tricarboxylic acid
Tcps	TIR-domain containing proteins
TcpF	TIR-domain containing proteins in <i>E. faecalis</i>
TIR	Toll/IL1-receptor
TirS	Staphylococcal TIR-domain protein
TLRs	Toll-like receptors
TNFR	Tumour necrosis factor receptor
TNF α	Tumour necrosis factor alpha
UTI	Urinary tract infection
VEGF	Vascular endothelial growth factor
VRE	Vancomycin-resistant Enterococcus
VopS	Vibrio outer protein S

Chapter 1: Introduction

Background and Significance

1.1 Research Motivation

Enterococci are common, commensal gram-positive bacteria that are ubiquitous in the gut of mammals and insects (Van Tyne & Gilmore, 2014). Since the 1980s, *Enterococcus faecalis* and *Enterococcus faecium* have emerged as opportunistic pathogens and are an etiological agent for bacteraemia, endocarditis, urinary tract infection (UTI), wound infections and gastrointestinal (GI) tract-associated infections (Arias & Murray, 2012; Fisher & Phillips, 2009; Flores-Mireles, Pinkner, Caparon, & Hultgren, 2014; Gilmore, Lebreton, & van Schaik, 2013; Lebreton et al., 2017; Morrison, Woodford, & Cookson, 1997; Murray, 1990). Alarming, Enterococcal strains that are intrinsically resistance to antibiotics such as β -lactams have acquired resistance to last resort antibiotics like cephalosporin, daptomycin and vancomycin (Arias & Murray, 2012; Miller, Munita, & Arias, 2014; Munoz-Price, Lolans, & Quinn, 2005; Paulsen et al., 2003). Notably, *E. faecalis* can survive for a prolonged period of time in mouse and human macrophages and hence may use macrophages as a mode of dissemination beyond the site of infection (Baldassarri et al., 2001; Gentry-Weeks et al., 1999; Sabatino et al., 2015; Zou & Shankar, 2016). In addition, during urinary tract infection (UTI), where the urinary catheter elicits a strong inflammatory response on its own, addition of *E. faecalis* results in fewer activated macrophages than non-activated macrophages and a robust colonization of the bladder, indicating that *E. faecalis* may be able to modulate macrophage activation (Guiton et al., 2013). Taken together, these

data suggest that *E. faecalis* may be capable of evading or modulating the inflammatory response (Baldassarri et al., 2005; Sabatino et al., 2015; Zou & Shankar, 2015, 2016).

Despite many studies that investigate the *E. faecalis* factors that contribute to infection, fundamental aspects of its interactions with host cells during enterococcal infections have not been well characterized. Bacterial infections are traditionally recognised by pathogen recognition receptors (PPRs) such as Toll-like receptors. Once pathogen-associated molecular patterns (PAMPs) are bound to TLRs, one of the downstream transcriptional pathways that is activated is the nuclear factor kappa-light chain enhancer of activated B cells (NF- κ B) signalling pathway, which is one of the prototypical pro-inflammatory signalling pathways (Lawrence, 2009; Moynagh, 2005a). Since *E. faecalis* can modulate immune activation during catheter-associated urinary tract infection (CAUTI) as well as to survive for a prolonged period in macrophages, we hypothesized that this occurs because *E. faecalis* is able to modulate the NF- κ B activation in macrophages during infection.

The overarching goal of this thesis is to fully characterize the interaction between *E. faecalis* and macrophages. The scope of work includes identifying potential bacterial factors involved in the survival of *E. faecalis* in macrophages as well as the mechanism through which this immunomodulation is achieved. Finally, an understanding of the *E. faecalis* immunomodulatory mechanism in macrophages as well as its effects on host response in the context of infection may potentially help in the development of new, alternative, immunomodulatory treatments to prevent recalcitrant enterococcal infections in the long term.

1.2 *Enterococcus faecalis* infections

1.2.1 *E. faecalis* as an opportunistic pathogen

E. faecalis is a non-spore forming, gram-positive, facultative anaerobe that commonly resides within the gastrointestinal tract of mammals (Fisher & Phillips, 2009). Together with *E. faecium*, they make up about less than 1% of the adult intestinal microbiota (Noble, 1978; Sghir et al., 2000; Tendolkar, Baghdayan, & Shankar, 2003). *E. faecalis* is classified as Group II and III within the five classification groups of enterococci based on its ability to hydrolyse arginine as well its ability to form acid in mannitol broth (Gilmore et al., 2002). Enterococci have a genomes size ranging from 2.7Mb to 3.6Mb with a low GC content of DNA ranging from 37 to 45% (Gaechter, Wunderlin, Schmidheini, & Solioz, 2012; Palmer et al., 2012; Qin et al., 2012; van Schaik & Willems, 2010). In addition to its residence in the mammalian GI tract, *E. faecalis* is also commonly found in ecological habitats such as water and dairy products. *E. faecalis* is able to colonize a variety of niches due to its ability to survive in harsh environments such as an extensive range of temperatures (optimum at 35°C), pHs (4.5-10, with optimum growth at 7.5), and in high salinity (Fisher & Phillips, 2009; Gentry-Weeks et al., 1999; Johnson, 1994; Shepard & Gilmore, 2002). *E. faecalis* is also intrinsically resistant to various classes of antibiotics including beta-lactams and aminoglycosides, and their malleable genome allows them to easily acquire resistance to additional antibiotics (Chow, 2000).

Enterococci have emerged as opportunistic pathogens in the nosocomial environment due to their ability to cause infections in susceptible individuals especially in immunocompromised and cancer patients, with mortality rates exceeding 50% (Arias & Murray, 2012; Schmidt-Hieber et al., 2007). *E. faecalis* is one of the major pathogens

in hospital-acquired infection, accounting for 80% to 90% of all enterococcal clinical isolates, followed by *E. faecium* (Gordon et al., 1992; Grayson et al., 1991; Lewis & Zervos, 1990; Patterson et al., 1995; Ruoff et al., 1990). *E. faecalis* can cause infections such as bacteraemia, endocarditis, surgical wound infection, UTI, hepatobiliary sepsis, and neonatal sepsis (Fisher & Phillips, 2009; Gentry-Weeks et al., 1999; Hidron et al., 2008). Various *E. faecalis* isolates that are resistant to last resort antibiotics such as daptomycin and vancomycin have also been isolated since mid-1980s, which increases the mortality rate up to 75% (Arias & Murray, 2012; Arias et al., 2011; Bearman & Wenzel, 2005; Munoz-Price et al., 2005). Taken together, these characteristics render the treatment of *E. faecalis* infection complicated, necessitating new treatment strategies or control mechanisms for these common, serious, and often antibiotic resistant infections.

1.2.1.1 Enterococcal wound and urinary tract infections

Enterococcal infections are often polymicrobial in nature with two or more species present at the infection site, especially in CAUTI and wound infections. Polymicrobial infection are usually associated with increased severity of infection due to synergistic interactions between species of microbes, making it harder to treat (Hughes & Winter, 2016; Keogh et al., 2016; Lavigne et al., 2008). *E. faecalis* is one of the main bacteria that are commonly isolated from wound infections together with *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Citron et al., 2007; Dowd et al., 2008; Giacometti et al., 2000; Gjodsbol et al., 2006). From 2006 to 2007, enterococci were the second most commonly isolated bacteria from surgical wound infections in the US (Hidron et al., 2008; Sievert et al., 2013). Furthermore, following surgery, vancomycin resistant *E. faecalis* has also been co-isolated with *E.*

coli and *P. aeruginosa* from femoral wounds (Dombrádi et al., 2009). In chronic venous ulcers, *E. faecalis* (found in 71.7% of the ulcers) is also commonly co-isolated with *S. aureus*, *Proteus spp*, *P. aeruginosa*, and other anaerobes (Gjodsbol et al., 2006).

In addition to wound infection, another most common enterococcal infections occurs in the urinary tract where upper urinary tract infections may lead to bacteraemia, especially in older men (Agudelo & Huycke, 2014). UTI represent 35% to 40% of all nosocomial infections with *E. coli* being the most commonly isolated bacteria and *E. faecalis* associated with 7.2% of hospital associated UTI (Foxman, 2002; Haley et al., 1985; Klevens et al., 2007; Weiner et al., 2016). Approximately 15 to 25% of hospitalized patients received a urinary catheter during their stay with increased duration of catheterization associated with increased rate of UTI (Al-Hazmi, 2015; Letica-Kriegel et al., 2019; Weber et al., 2011; Weinstein et al., 1999). Catheterization of the urinary tract increases the chances of bacteriuria by 3 to 10% while risk of bacteraemia is increased by 3 to 36 times (Hooton et al., 2010; Nicolle, 2009; Nicolle, 2014). In fact, 21% of health care acquired bloodstream infection were from urinary source in Quebec hospitals (Fortin et al., 2012). *E. faecalis* (7.2%) is the fifth most commonly isolated bacteria from catheter-associated urinary tract infection (CAUTI), out of which 8% are resistance to vancomycin treatment in 2014 (Sievert et al., 2013; Weiner et al., 2016) in the US. Given the high prevalence of enterococci infections and their propensity to be a part of polymicrobial infections which can exacerbate infection outcomes, more studies of *E. faecalis* pathogenesis in this context need to be conducted.

1.2.2 Metabolism of *E. faecalis*

1.2.2.1 Sugar metabolism

The ability of *E. faecalis* to thrive in diverse environments can be partly attributed to its ability to metabolise at least 13 different sugar sources for carbohydrate fermentation. *E. faecalis* are homofermentative with L-lactic acid being the primary end-product of carbohydrate metabolism – the Embden-Meyerhof Parnas (glycolysis), Entner-Doudoroff, and pentose phosphate (phosphogluconate) pathways (Ramsey, Hartke, & Huycke, 2014; Sokatch & Gunsalus, 1957). Hexose sugars are converted by aldolases to form glyceraldehyde-3-phosphate in both glycolysis and the Entner-Doudoroff pathway, generating ATP via substrate phosphorylation. In addition, hexoses, pentoses, and other sugar acids can be fermented for energy via the pentose phosphate pathway which generates nicotinamide adenine dinucleotide phosphate (NADPH) as well as channelling pentoses into nucleotide biosynthesis (Goddard & Sokatch, 1964).

In comparison to the pentose phosphate pathway and Entner-Doudoroff pathway, the phosphoenolpyruvate phosphotransferase system (PTS) is better characterized. PTS sense sugar in the environment and utilise phosphorylation for carbohydrate transportation (Postma, Lengeler, & Jacobson, 1993). Substrates for PTS are derived from most sugar sources that are metabolised by *E. faecalis*. The first step in PTS involves the autophosphorylation of enzyme I (E1) by phosphoenolpyruvate (PEP) which subsequently phosphorylates His-12 in histidine-containing phosphocarrier protein (HPr) (**Figure 1.1**). Histidyl-phosphorylated HPr then passes the phosphoryl group to sugar-specific enzymes II (EIIs). Lastly, EII enzymes phosphorylate sugar during the uptake process. Mannitol-, maltose-, and gluconate-specific enzymes II are the only EIIs that have been characterized for enterococci (Brockmeier et al., 2009;

Fischer, von Strandmann, & Hengstenberg, 1991; Le Breton et al., 2005). EIIs is made up of three to four subunits (A, B and C or A, B, C and D), where EIIC and EIID are hydrophobic integral membrane subunits (Deutscher, Bauer, & Sauerwald, 1993). Apart from their function in sugar metabolism to provide energy, PTS also help to regulate glycerol metabolism, virulence, and may provide resistance to environmental stress factors (Deutscher et al., 1993; Paulsen et al., 2003; Peng et al., 2017). Knockout of *ptsI* and *pts2*, which impairs only the function of EIICDs that helps in carbohydrate translocation and allowing EIIB domain to phosphorylate the sugar, results in increased susceptibility to hydrogen peroxide and acid as well as decreased survivability in macrophages and *Caenorhabditis elegans* (Peng et al., 2017). Furthermore, deletion of the maltose-specific permease of the phosphoenolpyruvate (PEP), MalT, reduces *E. faecalis* ability to use maltose and maltotriose as a source of energy for growth and reduces its ability to colonize and survive in kidneys and liver of intraperitoneally infected mice (Sauvageot et al., 2017). Recently, a study showed that knockout of PTS system involved in gluconate uptake leads to failure to metabolise gluconate, decreased intracellular survival within macrophages, as well as decreased pro-inflammatory cytokine secretion by colon tissue using a colitis mice model (Fan et al., 2019). Finally, the ability to ferment mannitol sets *E. faecalis* apart from *E. faecium*. EIIM^{II} and mannitol-1-phosphate dehydrogenase are part of the mannitol phosphotransferase system which catalyses the uptake and phosphorylation of mannitol in *E. faecalis* (Fischer et al., 1991).

Figure 1.1 Phosphoenolpyruvate phosphotransferase pathway of *E. faecalis*.
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In addition to utilising carbohydrate for sugar metabolism, glycerol can also be used as a carbon/energy source for lipid and lipoteichoic acid synthesis in enterococci (Hancock, Murray, & Sillanpaa, 2014). In *E. faecalis*, glycerol catabolism can occur under aerobic and anaerobic conditions via two different pathways (Bizzini, Zhao, Auffray, & Hartke, 2009; Gunsalus & Sherman, 1943). *E. faecalis* can phosphorylate glycerol to glycerol-3-P by glycerol kinase (GlpK/ EF1929) with the use of ATP before oxidation to dihydroxyacetone phosphate (DHAP) by glycerol-3 oxidase (GlpO/EF1928). Alternatively, oxidation of glycerol to dihydroxyacetone by NAD⁺-dependent glycerol dehydrogenase (GldA1/EF1358) can occur. Dihydroxyacetone is subsequently phosphorylated by dihydroxyacetone kinase (DhaK/EF1360) to DHAP which enters the glycolysis pathway (Ramsey et al., 2014). In anaerobic conditions, *E. faecalis* reoxidation of NADH is dependent on fumarate which functions as an electron acceptor. *E. faecalis* can utilise either the GlpK or DhaK pathway during aerobic conditions depending on the modulation of gene expression rather than differing gene

content between different strains (Bizzini et al., 2010). For example, JH2-2 utilises both GlpK and DhaK pathway simultaneously while TX0104 and MMH594 utilise preferentially the DhaK and GlpK pathways for glycerol metabolism (Bizzini et al., 2010). However, in anaerobic growth, glycerol catabolism solely depends on DhaK pathway (Bizzini et al., 2010).

E. faecalis can also ferment citrate as the sole source of carbon and energy by cleaving citrate into acetyl-coA and oxaloacetate by citrate lyase before decarboxylation into pyruvate (Ramsey et al., 2014). Considering that *E. faecalis* commonly resides within gastrointestinal tracts of mammals, in addition to their ability to metabolize non-absorbed sugar in the gastrointestinal tracts, *E. faecalis* can also use mucins that are produced by epithelial cells as a carbon source (Ramsey et al., 2014). Therefore, the ability to catabolise different sugar for energy allows *E. faecalis* to thrive in different environments.

1.2.2.2 Homolactic fermentation and heterofermentative pathway

E. faecalis can metabolize pyruvate, a methyl- α -keto acid product, as a source of energy for growth in the absence of oxygen allowing it to grow in anaerobic conditions. Pyruvate formation occurs in at least four of the metabolic pathways including carbohydrate fermentation, citrate fermentation, malate metabolism and glycerol metabolism for *E. faecalis* (Deibel & Niven, 1964). The last step of glycolysis involves pyruvate kinase catalysing the dephosphorylation of phosphoenolpyruvate, produced by sugar fermentation, to pyruvate leading to the generation of ATP (**Figure 1.2**). In addition, malic enzyme can also break down malate to release energy, resulting in the formation of pyruvate.

Dehydrogenation of pyruvate by *E. faecalis* can follow several pathways that lead to the formation of at least five different end-products, depending on the growth condition: acetate, acetoin, ethanol, formate and lactate (**Figure 1.2**) (Snoep, Joost, Neijssel, & de Mattos, 1991). Interestingly, enzymes necessary for tricarboxylic acid (TCA) cycle, such as aconitase, fumarase and isocitrate dehydrogenase, are not evident in *E. faecalis* and other enterococci genome databases suggesting a lack of TCA cycle in this genus. *E. faecalis* is considered strict fermenters due to the lack of TCA cycle and this may contribute to their ability to survive in both aerobic and anaerobic environment (Pessione, 2012). Instead, pyruvate dissimilation occurs through one of the three reactions leading to the production of acetyl-coA (**Figure 1.2**). Subsequently, ATP is generated following a two-step reduction of acetyl-coA. Alternatively, acetyl-coA can directly enter fatty acid biosynthesis. The pyruvate dehydrogenase complex (*pdh*) is one way in which oxidative phosphorylation of pyruvate can occur. Under anaerobic conditions, *E. faecalis* converts pyruvate and CoA to formate and acetyl-coA by pyruvate formate-lyase (*pflB*). One additional molecule of ATP can be generated through the reaction of acetylphosphate and CoA formation from acetyl-CoA by phosphotransacetylase (*eutD*) and acetate kinase (*ackA*) (Ramsey et al., 2014; Wolfe, 2005, 2010).

Figure 1.2 Pyruvate metabolic pathway in *E. faecalis*.
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Under nutrient-limiting conditions, formate, lactate and ethanol are produced during the fermentation of glucose or mannitol by *E. faecalis* (Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2001; Snoep et al., 1991). A gene similar to multifunctional alcohol dehydrogenase (*adhE*) that is found in *Escherichia coli* and *Lactobacillus lactis* might be involved in the process of ethanol formation in *E. faecalis* by catalysing the conversion of acetyl-CoA to ethanol with the generation of NAD⁺ (Arnau et al., 1998). Acetoin is another end product of pyruvate decarboxylation from a two-step process starting with α-acetolactate synthase (*alsS*) followed by α-acetolactate decarboxylase (*alsD*) (Mehmeti et al., 2011). Acetoin is a neutral compound

and hence, allows *E. faecalis* growth without considerable acidification of media (Dolin & Gunsalus, 1951; Sarantinopoulos et al., 2001).

Under anaerobic conditions in the presence of excess glucose, homolactic fermentation is the predominant pathway involved in sugar metabolism. Pyruvate is reduced to lactate by lactate dehydrogenase (*ldh*) to generate NAD⁺ for ongoing glycolysis. There are two *ldh* genes found in *E. faecalis*, *ldh1* and *ldh2*, with the former being more biochemically active and playing the major role in lactate production during growth in hexose sugars (Jönsson, Saleihan, Nes, & Holo, 2009; Mehmeti et al., 2011). These enzymes biochemical activity are not only regulated by intracellular pH and phosphate concentration, but can also be activated by the glycolytic intermediate effects of fructose-1,6-bisphosphate (Feldman-Salit et al., 2013). Since lactate is an anionic molecule at physiological pH (pKa=3.8), therefore lactate efflux occurs in symport with protons by a carrier-mediated process that translocates the lactic acid (Harold & Levin, 1974). The production of lactate results in the acidification of the environment. Considering that LDH2 alone is not able to metabolise large amounts of pyruvate form during glycolysis, the deletion of *ldh1* leads to upregulation of the mixed acid fermentation pathway resulting in the production of more acetoin, ethanol and formate, to maintain intracellular pH through the proton motive force. (Jönsson et al., 2009; Mehmeti et al., 2011). Despite the lack of *ldh1*, 25% of normal lactate levels is still produced as compared to wildtype suggesting that *ldh2* is not a redundant gene. In addition, this also suggests that *ldh1* and *ldh2* plays a different function with the latter playing a less essential role in lactate production. Considering that *ldh1* plays a more important role in lactate production, it is not surprising that *ldh2* knockout mutant does not show a significant decrease in lactate production (Jönsson et al., 2009). Interestingly,

besides being involved in detoxifying pyruvate reduction to generate lactate, LDH may contribute to bacterial virulence since a *ldhI* knockout mutant is more susceptible to environmental stresses, such as hydrogen peroxide treatment, is attenuated for colonization host organs in murine model of systemic infection (Clarke & Knowles, 1980; London, 1968; Pritchard & Wimpenny, 1978). Therefore, given that LDH is not just an enzyme that is important for the metabolic process but also potentially plays a role in virulence, more investigation is needed to study the interaction of this mutant with the host.

1.2.3 Enterococcal virulence factors involved in infection

Despite having a relatively small genome size of 2.7 million base pairs, as compared to *E. faecalis* V583 of 3.2 million base pairs, *E. faecalis* OG1RF possess a surprising array of virulence factors that allows it to colonize and cause infections (Bourgogne et al., 2008). These virulence factors are either surface-associated or secreted (Arias & Murray, 2012). Gelatinase (GelE), a secreted bacterial factor, is a matrix metalloprotease with hydrolytic capabilities and which degrades host extracellular matrix such as fibrinogen and collagen (Thurlow et al., 2010). It facilitates innate immune evasion by cleaving anaphylatoxin C3, C3a and C5a of the complement system to reduce opsonization and to decrease neutrophil recruitment (Arias & Murray, 2012; Park et al., 2007; Park et al., 2008; Thurlow et al., 2010). Other secreted virulence factors include cytolysin and serine protease (SprE). *sprE* is downstream of and co-transcribed with *gelE* (Qin, Singh, Weinstock, & Murray, 2000, 2001). Together, GelE and SprE regulate biofilm formation and thus, may play a role in pathogenesis (Garsin, Frank, & Silanpää, 2014; Qin et al., 2000). Cytolysin (Cyl) is a type-A lantibiotic and has a lytic effect on immune cells such as macrophage and neutrophils and contributes

to virulence in infection models (Coburn & Gilmore, 2003; Day, Cove, & Phillips-Jones, 2003; Garsin et al., 2014).

Aggregation substance (AS), autolysin, and endocarditis and biofilm-associated pili (Ebp), adhesin to collagen of *E. faecalis* (Ace), and enterococcal surface protein (Esp) are additional well-studied surface-associated virulence factors. AS, Ebp and Ace are cell wall sorting signals (CWSS)-containing proteins and are anchored to the cell wall by sortases (Spirig, Weiner, & Clubb, 2011). The CWSS consist of a positively charged tail, a transmembrane domain and a C-terminal LPXTG motif. CWSS-motif containing proteins is cleaved between threonine and glycine bond by sortases before transferring the protein to cell wall precursor lipid II during transpeptidation (Spirig et al., 2011). Since sortases are involved in anchoring surface proteins to the cell wall, sortase A, a housekeeping sortases found in all Gram-positive bacteria, is essential for adherence and the formation of robust biofilm *in vitro* as well as virulence in a murine CAUTI model (Guiton et al., 2010; Guiton et al., 2009).

AS is a LPXTG-containing protein that aids binding to host protein such as fibronectin and type I collagen, as well as colonization during endocarditis leading to increased mortality (Chow et al., 1993; Hirt, Schlievert, & Dunny, 2002; Rozdzinski et al., 2001; Schlievert et al., 2010). AS also promotes phagocytosis and internalization into macrophages via interaction with complement receptor type 3. After internalization AS can resist superoxide killing leading to increased survival in macrophages (Sussmuth et al., 2000). Ace, another LPXTG-containing protein, aids in binding to host proteins such a collagen, laminin and fibrinogen (Nallapareddy, Singh, & Murray, 2008; Rich et al., 1999; Xiao, Hook, Weinstock, & Murray, 1998). Furthermore, Ace is essential for successful colonization within the host at sites including heart valves and the UTI (Hubble et al., 2003; Singh, Nallapareddy, Sillanpää, & Murray, 2010). *E. faecalis* pili

facilitate binding to host collagen and fibrinogen, primarily via the pilus tip adhesin protein EbpA (Flores-Mireles et al., 2014; Nallapareddy et al., 2011), and biofilm formation (Nallapareddy et al., 2006; Nielsen et al., 2012). Lastly, Esp is also involved in biofilm formation and an Esp deficient mutant is attenuated in *in vivo* UTI models (Arias & Murray, 2012; Shankar et al., 2001; Tendolkar, Baghdayan, Gilmore, & Shankar, 2004; Toledo-Arana et al., 2001). Taken together, all these factors help *E. faecalis* colonization in the host and promote infection.

1.3 Innate Immunity

For *E. faecalis* to establish an infection, it must first overcome the host response to colonize, persist and cause infection. The innate immune system is the first line of defence against invading pathogen and depends on cytokines, chemokines and phagocytic cells that recognise conserved proteins on pathogens. Upon pathogen recognition, the innate immune system triggers an inflammatory response which helps to recruit cells that aid in pathogen clearance (Albert, Johnson, & Lewis, 2002). In addition, the complement system of the innate immune system is also activated during infection. The complement system consists of inactive proteins that can be triggered during infection to enhance the ability of antibodies and immune cells to phagocytose pathogens through opsonization, to promote inflammation, and to increase the susceptibility of invading pathogens to immune cells clearance (Janeway, Travers, & Walport, 2001). Cells that comprise the innate immune system include mast cells, macrophages, neutrophils, dendritic cells, basophils, eosinophils, natural killer cells and $\gamma\delta$ T cells (Murphy & Weaver, 2017).

Upon entry into the host, the recognition of pathogens by immune cells and subsequent inflammatory response is triggered by the binding of pathogen-associated

molecular patterns (PAMPs) from microorganisms to the pathogen-recognition receptors (PRRs) on host cells. PRRs consist of NOD-like receptors (NLRs), Toll-like receptors (TLRs), and retinoic acid-inducible gene-I-like receptors (RLRs) (Janeway & Medzhitov, 2002). The binding of PRRs with PAMPs triggers phagocytosis of pathogens as well as production and secretion of cytokines or chemokines. Pro-inflammatory cytokines are usually secreted, and they affect the behaviour of nearby cells bearing the appropriate receptors. Secreted chemokines attract cells bearing chemokine receptors such as neutrophils and monocytes from the bloodstream to the local site of the infection. This leads to inflammation at the infection site including swelling, heat, and pain due to vasodilation, increased vascular permeability. Innate immune activation also plays a role in inducing an adaptive immune response. For example, macrophages and dendritic cells are also antigen-presenting cells (APCs), which can travel to nearby lymph node to present the pathogen antigen to T lymphocytes to activate pathogen-specific adaptive immunity response. Unlike the innate immune response that targets different pathogen based on recognition of PAMPs, the adaptive response is highly specific to the pathogen that induced them and can provide long-lasting protection against the specific pathogen due to the formation of memory B and T cells. After exposure to a new pathogen, adaptive immune responses take a longer time to develop, typically a week before the response is effective, due to the time taken for specific B and T cells to become activated and expand. Therefore, during the initial stages of infection, the innate immune systems play a more critical role in protecting the host from the invading pathogens despite the adaptive immunity high specificity and ability to destroy pathogens and any toxic molecules produced by the pathogen.

1.3.1 Macrophages

The macrophage is a specialised cell of the immune system, and is the mature form of monocytes. Macrophages are involved in the detection, phagocytosis, and killing of invading pathogens (Elhelu, 1983). Together, macrophages and monocytes make up one of the three types of phagocytes in the immune system. Macrophages are present in the tissue and beneath the mucosal surfaces (Davies, Jenkins, Allen, & Taylor, 2013; Smith et al., 2011). They act as sentinels against infection due to their longer lifespan and ability to produce inflammatory cytokines that play an important role by bridging the innate immune response to adaptive immune response. Upon engulfing invading pathogens, the phagosomes fuse with lysosomes containing hydrolases to form phagolysosomes for pathogen degradation (Huynh & Grinstein, 2007). Since macrophages can also function as antigen-presenting cells (APCs), they can also migrate to nearby draining lymph nodes to present antigens to T cells to initiate specific T cells response of the adaptive immune system (Weiss & Schaible, 2015). Excluding their role in innate immunity, macrophages are also involved in other homeostatic functions such as tissue regeneration, iron recycling, erythropoiesis, surfactant homeostasis, thermogenesis, and insulin sensitivity (Gordon & Martinez-Pomares, 2017).

Macrophages can acquire different functional phenotypes, broadly classified as classically activated (M1) or alternatively activated (M2), through polarisation in response to pathological and physiological cues (Ginhoux et al., 2015; Martinez & Gordon, 2014; Murray et al., 2014). Importantly, polarization is not static. Macrophages are adequately plastic and flexible such that the phenotype of polarized macrophages can be reversed *in vitro* and *in vivo* in response to environmental changes (Mosser & Edwards, 2008; Sica & Mantovani, 2012).

Polarisation to M1 macrophages generally arises during inflammatory settings and are typically associated with pathogen invasion leading to high production of pro-inflammatory cytokines, reactive nitrogen intermediates, and strong microbicidal and tumoricidal activity (Martinez & Gordon, 2014; Murray et al., 2014). Cytokines and chemokines such as interferon gamma (IFN γ), tumour necrosis factor alpha (TNF α), and granulocyte macrophage colony-stimulating factor (GM-CSF) can also induce M1 activation (Mills et al., 2000; Shapouri-Moghaddam et al., 2018).

On the other hand, M2 macrophage polarization typically occurs during T_H2 immune responses triggered by immunity to helminths, or during tissue remodelling and immunoregulatory functions. M2 macrophages are considered the benign opposite of M1 macrophages and are characterized by the upregulation of galactose and mannose receptors, arginase pathway, expression of scavenging molecules, and increased phagocytic activities (Gordon & Martinez, 2010; Mantovani et al., 2002). However, M2 macrophages can also result in allergic inflammation, aid tumour growth, and can act as a cellular reservoir to several pathogens (Sica & Mantovani, 2012). Based on the stimuli and the resultant transcriptional activity, M2 macrophages can be further classified into different subtypes: M2a, M2b, M2c and in some case M2d (Ferrante et al., 2013; Martinez & Gordon, 2014; Murray et al., 2014; Porcheray et al., 2005). The M2a subtype is defined as alternatively activated macrophages or wound-healing macrophages and is triggered in response to IL4 and IL13 stimulation and are involved in mounting T_H2 response (Mosser & Edwards, 2008). Type 2 macrophages, M2b, are stimulated by immune complexes and bacterial lipopolysaccharides (LPS) which leads to upregulation of CD86 and MHCII as well as increased production of both pro- and anti-inflammatory cytokines IL-1, IL-6, IL-10, IL-12 and TNF α (Mantovani et al., 2004; Mosser & Edwards, 2008; Yue et al., 2017). M2c subtype macrophages, also known as

deactivated macrophages, are induced by IL-10, glucocorticoids and TGF β , resulting in upregulation of CD163 and CD206 and increased production of IL-10, TGF β , MERTK, and ECM which helps in tissue repair and remodelling (Lurier et al., 2017). Lastly, M2d macrophages are activated in response to IL-6 and adenosine and are characterized by upregulation of VEGF-A, IL-10, IL-12, TNF α and TGF β (Ferrante et al., 2013; Porcheray et al., 2005). Given the plasticity of macrophages, the polarisation state of macrophages during *E. faecalis* infection is an open question.

1.3.2 Recognition of foreign particle by macrophage

1.3.2.1 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are transmembrane proteins, which is one type of pattern-recognition receptor (PPR) present on macrophages for the recognition of invading pathogens (Janeway, 1989). There are a total of ten TLRs in humans and thirteen in mice. TLR ligands are typically lipids, nucleic acids, or proteins from microbes (Kaisho & Akira, 2006). Upon activation of TLRs, a T_H1 response is triggered via the activation of APCs resulting in the production of inflammatory cytokines or type I interferons (IFNs). The leucine-rich extracellular domain of the TLRs is involved in ligand binding while the intracellular domain, also known as Toll/IL-1-receptor (TIR) domain, is essential for signal transduction upon activation.

TLRs are located at different domains of cells depending on the ligands that they recognised. Lipid and protein recognising TLRs are localized on the plasma membrane while TLRs that binds nucleic acids are typically found on the endosome membrane (Kawasaki & Kawai, 2014). TLR2 and TLR4 recognise bacteria cell wall components such as lipoteichoic acids (LTA) found in gram-positive bacteria and lipopolysaccharides (LPS) from gram-negative bacteria, respectively (Beutler, 2000).

Notably, purified *E. faecalis* LTA is recognised by TLR2 leading to activation of downstream signalling cascade such as mitogen-activated protein (MAP) kinase signalling pathway for immune activation against infection (Park et al., 2013; Zou & Shankar, 2015). In addition, an assortment of proteins can also be recognised by TLRs. For example, TLR5 and TLR11 recognise flagellin, a subunit of bacterial flagella, or protozoan-derived profilin-like protein respectively. Finally, bacterial and viral nucleic acids containing a high level of unmethylated CpG motifs can be recognised by TLR9. Single-stranded virus RNA is recognised by TLR7 and TLR8, while double-stranded RNA is recognised by TLR3. The expression of TLR3,7,8 and 9 inside the endosome prevents self-DNA from binding and therefore, prevents autoimmunity (Barton, Kagan, & Medzhitov, 2006). Interestingly, TLR9 can be expressed at the cell surface of human peripheral blood mononuclear cells and in freshly isolated human and mouse primary polymorphonuclear leukocytes suggesting the CpG motifs containing nucleic acids do not need to be accumulated in subcellular compartments in order for TLR9 binding and signalling to occur (Eaton-Bassiri et al., 2004; Lindau et al., 2013).

1.3.2.2 Nod-like receptors (NLRs)

In addition to TLRs, other PRRs have been identified including the nucleotide oligomerization domain (Nod)-like receptors (NLRs). NLRs were first identified in plants to play a role in disease resistance against invading pathogens and are highly conserved with homologs present in vertebrates and some invertebrates such as the sea urchin (Hibino et al., 2006; Jones & Dangl, 2006; Motta, Soares, Sun, & Philpott, 2015). Unlike the TLRs, NLRs are expressed only intracellularly and can respond to an extensive variety of intracellular PAMPs and damage-associated molecular patterns (DAMPs) from pathogen or host resulting in induction of innate immune response

(Jeong & Lee, 2011). There are 23 known NLR family members in humans, many of which are associated with diseases, and at least 34 in mice (Franchi, Warner, Viani, & Nuñez, 2009; Motta et al., 2015; Ting et al., 2008; Zhong, Kinio, & Saleh, 2013). NLR proteins have a common domain organization with a central nucleotide-binding NOD domain, also known as NAIP, CIITA, HET-E and TP1 proteins (NACHT) domain, NH₂-terminal effector domain for binding downstream signalling molecules and C-terminal leucine-rich repeats (LRRs) (Ting et al., 2008). Upon activation, NLRs activate signalling pathways involved in promoting inflammation, inflammasome assembly, activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, autophagy as well as pathways involved in developmental processes (Kufer & Sansonetti, 2011; Motta et al., 2015). The NLRs that are commonly associated with bacterial recognition are NOD1 and NOD2. NOD1 is involved in the recognition of meso-diaminopimelic acid-containing PGN fragments of mainly Gram-negative bacteria while NOD2 recognises muramyl dipeptide which are found in PGN of nearly all Gram-positive and Gram-negative bacteria (Chamaillard et al., 2003; Girardin, Boneca, Carneiro, et al., 2003; Girardin, Boneca, Viala, et al., 2003; Inohara et al., 2003). Interestingly, although NLRs are predominantly thought to be expressed only in the cytosol, a recent study revealed that a fraction of NOD1 and NOD2 can be found on the plasma membrane during bacterial infection (Kufer et al., 2008). NOD1 activation promotes neutrophil chemotaxis *in vivo* and both NOD1 and NOD2 signalling activates antimicrobial peptide production (Boughan et al., 2006; Masumoto et al., 2006; Uehara, Fujimoto, Fukase, & Takada, 2007; Voss et al., 2006; Zilbauer et al., 2007).

1.3.2.3 RIG-like receptors (RLRs)

Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are another family of innate immune receptors. RLRs are cytoplasmic helicases which play a critical role in recognition of viral or self RNA and initiating antiviral response by producing type I and type III interferon (IFN) (Kawai & Akira, 2006; Moore & Ting, 2008; Onoguchi, Yoneyama, & Fujita, 2011). There are currently 3 RLR members that have been identified: laboratory of genetics and physiology 2 (LGP2), melanoma differentiation associated factor 5 (MDA5) and RIG-I. RLRs are broadly expressed in most tissues in a variety of cell types other than immune cells at low levels in resting cells but is upregulated upon IFN exposure or after viral infection (Imaizumi et al., 2005; Kang et al., 2004; Yoneyama et al., 2005; Yoneyama et al., 2004). Upon RLR activation, the downstream signalling cascade is similar to TLR signalling pathway leading to activation of transcription factors such as interferon regulatory transcription factor 3 (IRF3), IRF7, and NF- κ B resulting in production of IFN and upregulation of interferon-stimulated genes (ISGs) (Kell & Gale, 2015). Aberrant RLR signalling is now associated with autoimmune disease development in addition to affecting susceptibility to virus infection (Hall & Rosen, 2010).

1.3.2.2 NF- κ B signalling pathway

TLR activation results in activation of various pathways leading to upregulation of transcription factors such as NF- κ B and IRFs (Kawai & Akira, 2007; Moynagh, 2005b) inducing inflammation. NF- κ B is a family of inducible transcription factors that function as key regulators of the immune and inflammatory response by binding to the κ B enhancer to drive the production of inflammatory cytokines and chemokines like tumour necrosis factor alpha (TNF α) and IL-6 (Lawrence, 2009). NF- κ B family is

composed of five different subunits including NF- κ B1 (p50), NF- κ B2 (p52), Rel A (p65), RelB, and c-Rel. Importantly, during resting state, NF- κ B dimers consisting of p65 and p50 are usually sequestered in the cytoplasm by inhibitory proteins such as I κ B family proteins. Since there are five different subunits of the NF- κ B family, there are also five inhibitory protein – I κ B α , I κ B β , I κ B ϵ , p105, and p100. NF- κ B subunits are able to dimerize with each other in various combinations to form homo- or heterodimers that are essential for DNA binding (Hoffmann, Natoli, & Ghosh, 2006).

TLRs activate NF- κ B via myeloid differentiation-primary response protein 88 (MyD88) dependent pathway, which is essential for M1 macrophage polarisation and pro-inflammatory cytokines production (Yu et al., 2014). Briefly, upon PAMP recognition, the toll/interleukin-1 receptor (TIR) domain of activated TLRs binds to MyD88, and this dimer subsequently interacts with and phosphorylates IL1-receptor-associated kinase 4 (IRAK4) and IRAK1. Next, IRAK1 binds to TNF receptor-associated factor 6 (TRAF6), which has a really interesting new gene (RING) protein domain that confers E3 activity, and subsequently activates transforming growth factor beta-activated kinase 1 (TAK1). Upon TAK1 activation by ubiquitination, both mitogen-activated protein kinases (MAPKs) and I κ B kinase (IKK) complex consisting of IKK α , IKK β and IKK γ are phosphorylated and activated. Lastly, activated IKK complex phosphorylates I κ B α which leads to its polyubiquitination and proteasomal degradation, and subsequently allows free NF- κ B dimers to translocate to the nucleus to bind to the κ B enhancers for expression of target genes (**Figure 1.3**) (Lawrence, 2009; Liu, Zhang, Joo, & Sun, 2017). In addition, aside from MyD88 dependent pathway, TLRs can also activate NF- κ B and IRFs via a MyD88 independent pathway since MyD88-deficient mice are still able to respond to LPS (Kawai et al., 1999). MyD88-independent pathways involve signalling through toll/IL-1R domain-containing

adaptor-inducing IFN- β (TRIF) resulting in interferon- β (IFN- β) production (Oshiumi et al., 2003; Yamamoto et al., 2002). Upon binding of PAMPs to TLRs, TRIF is recruited and activated via phosphorylation. Interferon regulatory factor 3 (IRF3) is subsequently recruited and phosphorylated before translocating to the nucleus to bind to the interferon-stimulated response element (ISRE) together with p300/co-activating proteins (CBP) coactivator to activate IFN-regulated genes (Hiscott et al., 1999; Lin, Heylbroeck, Pitha, & Hiscott, 1998; Weaver, Kumar, & Reich, 1998; Yoneyama, Suhara, & Fujita, 2002). In addition, TRIF is also able to activate NF- κ B via direct binding to TRAF6 causing its autoubiquitination and phosphorylation resulting in activation of the downstream signalling pathway similar to that observed in MyD88 dependent pathway leading to production of pro-inflammatory cytokines (Jiang, Mak, Sen, & Li, 2004; Sato et al., 2003).

The canonical NF- κ B pathway responds to PAMPs via their interactions with PRRs, which eventually activates the p50-p65 NF- κ B dimer. On the contrary, the non-canonical NF- κ B pathway involves the recognition of a specific subset of ligands, such as CD40 and receptor activator of NF- κ B (RANK), by the tumour necrosis factor receptor (TNFR) superfamily and selectively activates NF- κ B2 p52 and RelB (**Figure 1.3**)(Sun, 2017). Activation of the non-canonical NF- κ B pathway depends on the tightly regulated inducible phosphorylation of p100, which functions as an I κ B-like molecule, that regulates RelB activation (Xiao, Harhaj, & Sun, 2001). Upon activation of TNFR, TNFR bind and activate NF- κ B inducing kinase (NIK) (Senftleben et al., 2001). Activated NIK, together with IKK α , mediates the phosphorylation of p100 leading to p100 ubiquitination and proteasomal degradation of the C-terminal I κ B-like structure (Liang, Zhang, & Sun, 2006; Xiao et al., 2001). The degradation of the C-terminus of

p100 results in production of mature NF- κ B2 and the NF- κ B2-RelB complex is now able to translocate to the nucleus for the activation of its target genes (Sun, 2017).

Figure 1.3 Canonical and non-canonical NF- κ B signalling pathway.
P indicates phosphorylation while degradation is accomplished by proteasomes.

Considering that NF- κ B plays an essential role in multiple facets of the immune response as well as the pathogenesis of inflammatory diseases and cancer, NF- κ B cannot be constitutively activated and must be regulated to prevent tissue damage, increased

risk of cancer, and autoimmune disease all which may arise due to uncontrolled inflammation (Karin & Greten, 2005). One mechanism to terminate NF- κ B activity occurs when newly synthesized I κ B α , which is also a NF- κ B target gene, enters the nucleus and removes NF- κ B from DNA before translocating back into the cytoplasm (Hayden & Ghosh, 2004; Pahl, 1999). Post-translational modification of activated NF- κ B can also lead to the termination of NF- κ B signalling by regulating the displacement and degradation of NF- κ B dimer as well as altering cofactor binding. Histones acetyltransferase (HAT) and histone deacetylase (HDAC) regulate acetylation of p65 and hence regulate the termination of NF- κ B response (Ghosh & Hayden, 2008; Kiernan et al., 2003). Acetylation of p65 by HAT negatively impact its DNA-binding affinity while deacetylation of p65 by HDAC promotes binding of p65 with I κ B α resulting in nuclear export and hence terminates NF- κ B response (Calao et al., 2008; Kiernan et al., 2003). Alternatively, ubiquitin ligases such as suppressor of cytokine signalling 1 (SOCS1) and postsynaptic density 65-discs large-zonula occludens 1-abnormal cell lineage 11-islet 1- mechanosensory abnormal 3 domain-containing protein 2 (PDLIM2) can lead to ubiquitination-dependent proteasomal degradation of DNA-bound p65 (Ryo et al., 2003; Saccani, Marazzi, Beg, & Natoli, 2004). In addition to triggering proteasomal degradation of NF- κ B, PDLIM2 can also transport p65 to promyelocytic leukaemia (PML) nuclear bodies leading to intranuclear sequestration and transcriptional silencing of p65 (Tanaka, Grusby, & Kaisho, 2007). Interestingly, besides regulating the activation of NF- κ B, IKK α is also important for promoting turnover of p65 and c-Rel leading to their dissociation from NF- κ B gene promoter (Lawrence et al., 2005). As the mechanisms of NF- κ B termination are not fully elucidated and therefore, further investigation in this area is needed to have a better understanding of function of NF- κ B.

1.3.2.3 MAPK signalling pathways

TLRs can also activate the MAPK pathway via TAK1. In mammals, the MAPKs can be classified into extracellular signal regulated kinases (ERKs), Jun amino-terminal kinases (JNKs), and stress-activated protein kinases (SAPKs), also known as p38. MAPKs are activated by a variety of factors such as environmental stresses, inflammatory cytokine, and growth factors involved in cell differentiation, cell proliferation and cell death in eukaryotes (Keshet & Seger, 2010; Morrison, 2012; Qi & Elion, 2005; Raman, Chen, & Cobb, 2007). In addition, JNK family proteins play a role in cytokine production, inflammation and metabolism while p38 regulates inflammation and cell cycle (Cuadrado & Nebreda, 2010; Cuenda & Rousseau, 2007; Huang, Shi, & Chi, 2009; Rincon & Davis, 2009). Activation of MAPK pathway induced by TLR activation is typically via TRAF6 or TAK1. Activation of TRAF6 leads to phosphorylation of MAPK/ERK kinase kinase 1 (MEKK1), followed by MAP kinase kinase 3 or 6 (MKK3/6) phosphorylation and subsequent p38 activation to promote pro-inflammatory cytokine production (Yoshida et al., 2008). In addition, activated TAK1 can also activate MAP kinase kinase 4 or 7 (MKK4/7) to activate JNK (Lim & Staudt, 2013; Morrison, 2012). Finally, ERK can be activated by TLRs which leads to downstream p105 phosphorylation and p105 subsequently undergoes partial degradation to form p50. MEK kinase TPL2, under unstimulated conditions, is bound to p105 and is therefore, inactivated. Upon partial degradation of p105, TPL2 is activated which in turn activates ERK via MAPK/ERK kinase 1 or 2 (MEK1/2) (Beinke, Robinson, Hugunin, & Ley, 2004; Loniewski, Patial, & Parameswaran, 2007). ERK and JNK are able to drive the transcription of *cFos* and *cJun*, respectively, leading to increases production of cFos and cJun, which are part of the Jun family of proteins (cJun,

JunB and JunD) and Fos family of proteins (cFos, FosB, Fra1 and Fra2) that make up the activating protein-1 (AP-1) transcription family factor (Herdegen & Leah, 1998; Karin, 1995; Morgan & Curran, 1991). Therefore, the relative amount of specific Jun/Fos family factors present in a given cell at any time can determine the functional activity of AP-1. In addition, JNK can also directly phosphorylate cJun at its stimulatory N-terminal sites (Deng & Karin, 1994; Gupta, Campbell, Derijard, & Davis, 1995; Hibi et al., 1993). Subsequent binding of AP-1 to DNA drives expression of genes important in inflammation, cell proliferation, cell differentiation and apoptosis.

1.4 Pathogen – host interaction

The innate immune system that exists in most eukaryotic organisms is highly complex and tightly regulated to protect the host from invading pathogens. The ability of a pathogen to establish infection is directly dependent on its ability to evade or subvert immune surveillance and clearance. Therefore, pathogens and immune system are constantly locked in evolutionary arms race to outcompete each other. Despite a rapid host signalling cascade to activate immune genes upon pathogen recognition, bacteria have evolved different counter-strategies such as cytotoxicity toward host cells, subversion of killing by antimicrobial peptides, expression of bacterial cell surface factors to mask PAMPs from PRRs and targeting the intracellular immune signalling pathways and hence affects the downstream cytokines and chemokines secretion to thwart host defence which will be discussed more in details below (Finlay & McFadden, 2006; Reddick & Alto, 2014) (**Figure 1.4**).

Figure 1.4 Bacterial factors from Gram-positive bacteria involved in immune system subversion. Upon PAMPs binding to TLRs, stimulated TLR binds to MyD88 and activates the downstream NF-κB signalling pathways. Bacteria such as *Staphylococcus aureus* (Blue), *Staphylococcus pneumoniae* (Red) and *Enterococcus faecalis* (green) can produce capsules to shield the PAMPs from recognition by TLRs. SpeA and V8 protease from *S. aureus*, M1 protein from Group A *Streptococcus* (GAS) (purple), GelE and SprE from *E. faecalis* can cleave AMPs LL-37. Furthermore, staphylokinase from *S. aureus* can inhibit α-defensins to protect *S. aureus* from antimicrobial killing. MprF found in various bacteria can also help bacteria to repel cationic AMPs. In addition to block AMPs, bacteria can also target the intracellular signalling pathway to subvert immune response. SSL3 from *S. aureus* binds and inhibit TLR2 signalling while PSMα1-α3 inhibits TLR4. In addition, Hip1 from *Mycoplasmata tuberculosis* (brown) dampens TLR2 signalling. TirS from *S. aureus* interfere with TLR2 signalling leading to reduced host inflammatory response. In addition, TcfF from *E. faecalis* directly interact with MyD88 to block downstream signalling pathway. InlA from *M. tuberculosis* binds IKKα to block downstream kinases activation. SLO from GAS can inhibit phosphorylation of IκBα, p65 and cFos. PPE18 from *M. tuberculosis* can indirectly inhibit IκBα activation by activating SOCS3. Apart from activating the NF-κB pathway, TLR activation can also activates MAPK pathway through the activation of MAPKKK. LF from *Bacillus anthracis* (pink) can cleaves the amino terminus of MEK1/2 and p38 to downregulate MAPK pathway.

1.4.1 Resistance to antimicrobial peptides

One of the most ancient and conserved forms of protection against pathogen infection is through antimicrobial peptides (AMPs) expressed by host cells such as epithelial cells of the skin and lung (Bahar & Ren, 2013). Some of the AMPs secreted include defensins, gramicidins, and cathelicidins which protect the host from pathogen invasion by forming pores in the bacterial membrane which leads to death of the

pathogen (Bahar & Ren, 2013; Raj & Dentino, 2002). Bacteria have evolved different mechanisms to abrogate and avoid lytic activity induced by AMPs. One of the most common methods employed by bacteria is to modify their cell surface by incorporating positively charged residues to repel AMPs since most AMPs are cationic in nature. For example, multiple peptide resistance factor protein (MprF), which was first identified in *S. aureus*, found in both Gram-positive and Gram-negative bacteria including *E. faecalis*, *Mycoplasma tuberculosis*, and *P. aeruginosa* provides resistance to defensin-like cationic peptides by modifying the phosphatidylglycerol (PG) head group in the bacterial membrane with the addition of a positively charged amino acid residues to increase the negative charge of the membrane and subsequently help to repel cationic defensin molecules (Ernst & Peschel, 2011; Ernst et al., 2009). Similarly, MprF1 and MprF2 in *E. faecalis* can also reduce negative net charge of the membrane protein through aminoacylation of PG and therefore lead to repulsion of cationic AMPs (Bao et al., 2012; Kandaswamy et al., 2013; Rashid et al., 2017). Furthermore, D-alanylation of teichoic acid by D-alanine-D-alanyl carrier protein ligase, encoded by the *dltA* operon, in Gram-positive bacteria including *S. aureus*, Group A *Streptococcus* (GAS), *E. faecalis* and *L. plantarum* helps in repulsion of cationic AMPs, promoting resistance to neutrophil killing and increasing adherence to eukaryotic cells (Fabretti et al., 2006) (Attieh et al., 2019; Kristian et al., 2005; Peschel et al., 1999).

Besides modifying bacterial surface charge to subvert AMPs killing, bacteria can also cleave AMPs to avoid immune clearance. For example, *S. aureus*-derived proteinases such as aureolysin, staphylococcus efflux pump A (SepA), and V8 protease; *Bacillus anthracis*-derived metalloprotease; and GAS-derived secreted cysteine protease streptococcal pyrogenic exotoxin B (SpeB) and streptococcal inhibitor of complement (SIC) protein can cleave cathelicidin LL-37 to inactivate it to protect

bacteria against LL-37 antimicrobial action (Fernie-King, Seilly, & Lachmann, 2004; Nyberg, Rasmussen, & Björck, 2004; Sieprawska-Lupa et al., 2004; Thwaite, Hibbs, Titball, & Atkins, 2006). In addition, staphylokinase from *S. aureus* can neutralise α -defensins to protect *S. aureus* (Jin et al., 2004). M1 protein from GAS can bind and sequester LL-37 and is therefore associated in promoting resistance of GAS to antimicrobial killing (Lauth et al., 2009). Finally, in *E. faecalis*, proteases such as GeLE and serine protease (SprE) can also degrade LL-37 to subvert antimicrobial action (Park et al., 2007; Schmidtchen et al., 2002). Therefore, inhibition of AMPs is a major mechanism that is employed by bacteria to subvert the innate immune system.

1.4.2 Bacteria subverting immune recognition by PPRs

The innate immune response is triggered upon recognition of PAMPs by PRRs presented on host cells. It is unsurprising that bacteria have evolved to mask the recognition of PAMPs by PRRs using various strategies. Expression of surface polysaccharide capsule is one strategy utilised by bacteria such as *E. faecalis*, *S. aureus*, *S. pneumoniae*, and GAS to avoid detection by PRRs and subsequent clearance by host immune system (Cress et al., 2014; Huebner et al., 1999; Hufnagel et al., 2004; O'Riordan & Lee, 2004). *E. faecalis* can produce one of two capsular serotypes (C or D) grouped based on serospecificity (Hufnagel et al., 2004). Capsular polysaccharide (*cps*), which is encoded by a nine gene operon (*cpsC-cpsK*) is currently the only locus that is associated with capsule production (Hancock & Gilmore, 2002; Xu, Murray, & Weinstock, 1998). Capsule production in *E. faecalis* helps to mask LTA from detection by agglutinating antibodies as well as to mask complement molecules such as C3 deposited on the bacterial surface to prevent opsonic killing (Thurlow, Thomas, Fleming, & Hancock, 2009). Encapsulated serotypes C and D are more resistant to neutrophilic

killing as compared to unencapsulated serotypes A and B, with most pathogenic strains of *E. faecalis* belonging to serotype C (Hufnagel et al., 2004; McBride et al., 2007) .

Another strategy to subvert PRR recognition is to modify the structure or composition of PAMPs in a way that interferes with PRR binding. Hydrolase important for pathogenesis 1 (Hip1), a cell envelope-associated serine hydrolase, from *M. tuberculosis* dampens TLR2 signalling, probably due to masking cell surface interaction between TLR2 and PAMPs on *M. tuberculosis*, leading to a decrease in pro-inflammatory cytokine production (Madan-Lala, Peixoto, Re, & Rengarajan, 2011). Finally, other approaches include directly inhibiting TLR signalling via secreted bacterial factors or to activate an alternative pathway to counteract the TLR signalling pathway. For example, staphylococcal superantigen-like proteins (SSLs) secreted by *S. aureus* can modulate the immune response. SSL3 binds to TLR2 and inhibits the activation of TLR signalling pathway resulting in decreased secretion of pro-inflammatory cytokine IL-8 (Bardoel et al., 2012; Yokoyama et al., 2012). Lastly, phenol-soluble modulins $\alpha 1$ - $\alpha 3$ (PSM $\alpha 1$ - $\alpha 3$) of *S. aureus* act as antagonists of TLR4 by directly ligating with TLR4 leading to inhibition of NF- κ B activation (Chu et al., 2018).

1.4.3 Bacterial targeting of NF- κ B signalling pathways

Bacteria have evolved to target the NF- κ B signalling pathway at multiple levels to subvert the immune response during infection. Given that most of the bacterial factors described to date that target the NF- κ B signalling cascade are in Gram-negative bacteria, more investigation to elucidate how Gram-positive bacteria target this immune signalling pathway is necessary. Regardless, here we will summarise how Gram-positive bacteria can manipulate the NF- κ B signalling pathways. Some examples of how

virulence factors from Gram-negative bacteria can manipulate the signalling pathway will be provided instead if there are currently no known Gram-positive bacteria factors involved.

1.4.3.1 Preventing recruitment or activation of adaptor proteins

Upon activation of TLRs, adaptor protein such as MyD88 and TRIF are recruited and activated via phosphorylation which leads to activation of downstream signalling molecules (Takeda & Akira, 2004). Importantly, the TIR domain, an intracellular conserved region found in all TLRs and adaptors, is crucial for mediating recruitment and activation of adaptor proteins (Watters, Kenny, & O'Neill, 2007). Therefore, bacteria can subvert the immune response by targeting these adaptor proteins either via degradation or interfering with TIR-TIR interactions. For example, TIR-domain containing proteins (Tcps) have been identified from many bacteria such as *E. faecalis*, *E. coli* and *S. aureus*. In *E. faecalis*, the TIR-domain containing protein in *E. faecalis* (TcpF) can directly interact with MyD88 and therefore block downstream activation of the NF- κ B pathway which contributes to survival of *E. faecalis* in macrophages (Zou, Baghdayan, Payne, & Shankar, 2014). In addition, staphylococcal TIR-domain protein (TirS), whose TIR domain is homologous to the human TIR-domain, is secreted by *S. aureus* and attenuates TLR2 signalling and hence attenuates NF- κ B and JNK activation (Askarian et al., 2014). TirS-expressing *S. aureus* is more virulent leading to increased *S. aureus* load in multiple organs such as kidney, spleen, heart, brain and blood during murine intravenous infection (Askarian et al., 2014).

1.4.3.2 Interfering with kinases involved in NF- κ B signalling pathway

Virulence factors from Gram-positive bacteria can interfere with kinases, such as TAK1, IKK and I κ B α , involved in NF- κ B signalling pathway, although the exact pathway in which most of the factors act is uncharacterized. Internalin proteins (InlA) from *Listeria monocytogenes* can directly bind to IKK α resulting in impairment of phosphorylation of I κ B α and delay of I κ B α degradation, therefore limiting TNF α production by impairing NF- κ B (Gouin et al., 2010). Furthermore, the PPE18 protein of *M. tuberculosis* is able to indirectly inhibit I κ B α by first upregulating the expression and phosphorylation of suppressor of cytokine signalling 3 (SOCS3) (Nair, Pandey, & Mukhopadhyay, 2011). Phosphorylated SOCS3 subsequently binds I κ B α and inhibits the phosphorylation of I κ B α at serine 32/36 residues by IKK, resulting in inactivation of NF- κ B and preventing pro-inflammatory cytokine production (Nair et al., 2011).

Unlike in Gram-positive bacteria, there are many more Gram-negative bacterial factors that have been identified to directly interact with kinases involved in NF- κ B signalling pathway. For example, YopJ, an effector protein injected into the host cell cytoplasm during *Yersinia pestis* infection, mediates serine/threonine acetylation of TAK1 leading to subsequent blocking of downstream signalling (Paquette et al., 2012). In addition, *E. coli* uses the type III secretion system (T3SS) to secrete NleE and NleB, which enhances the effect of NleE, to prevent IKK β phosphorylation, possibly by inactivation of upstream kinases such as TAK1 although the exact mechanism is currently unknown (Nadler et al., 2010).

1.4.3.2 Interfering with NF- κ B function

Upon release from the inhibitory I κ B α complex, NF- κ B translocates to nucleus to bind appropriate κ B sites and initiate transcription of target genes (Newton & Dixit). NF- κ B family comprises five different subunits such as NF- κ B1 (p50), NF- κ B2 (p52), Rel A (p65), RelB, and c-Rel where the members form different combination of hetero- and homo-dimers, with p65-p50 heterodimer being the most abundant (Hayden, West, & Ghosh, 2006; Moynagh, 2005a). Importantly, p50 and p52 lack a transactivation domain (TAD) that is required for transactivation of genes, while p65, RelB, and c-Rel possess TAD in C-terminal which is required for transcription of target gene (Li & Verma, 2002). Therefore, bacteria can interfere with subunit activation to regulate transcription of NF- κ B target genes. GAS not only induces more activation of non-classical p65-p52 heterodimer instead of the classical p65-p50 subunit but also reduces p65 nuclear translocation, as compared to *S. aureus* and *E. coli*, leading to lower production of proinflammatory cytokines although, again, the exact mechanism is unknown (Wu et al., 2016). p100, the precursor of p52, is characterised as a fourth inhibitory protein of p65 which plays a role in inhibiting the classical p65 subunit to lower pro-inflammatory cytokines production (Basak et al., 2007).

Another strategy employed by bacteria to modulate NF- κ B activation is to target NF- κ B phosphorylation and subsequent translocation into the nucleus. For example, GAS secretes streptolysin O (SLO), a cholesterol-dependent cytolysin, which can decrease phosphorylation of p65, I κ B α and cFos to suppress osteoclast activation leading to bone destruction upon GAS infection (Yi et al., 2019). In addition, bacterial species used in probiotics, such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, mediate anti-inflammatory effects by suppressing NF- κ B activation (Virginia

Rodriguez & Griet, 2016). Secreted metabolites found in conditioned media of *L. acidophilus* or *Bifidobacterium infantis* can prevent IL-1 β -induced I κ B α degradation and NF- κ B translocation resulting in inhibition of NF- κ B signalling pathways and decreased inflammation (Guo et al., 2015). In addition, an uncharacterised secreted factor from *Bifidobacterium animalis subsp. lactis* can inhibit NF- κ B activation to inhibit TNF α -induced IL8 secretion in colonic epithelial cells (Heuvelin et al., 2009). Therefore, while we are beginning to understand how Gram-positive bacteria suppress NF- κ B signalling, more investigation is required to elucidate the bacterial factors involved in suppression as well as the molecular details in how NF- κ B is being suppressed.

1.4.4 Manipulating MAPK signalling pathway

In addition to modulating the NF- κ B pathway, bacteria are also able to manipulate the MAPK pathway in order to thwart immune responses and establish infection in host. Lethal factor (LF), a metalloprotease which is one component of the anthrax toxin secreted by *B. anthracis*, can prevent p38 MAPK activation by cleaving between the N-terminal and the catalytic domain of p38 resulting in macrophage apoptosis (Ali et al., 2011; Park, Greten, Li, & Karin, 2002). In addition, LF can also cause proteolysis of MEK1 and MEK2 N-termini which reduces the affinity of MEK proteins with their respective substrates leading to downregulation of MAPK pathway (Chopra, Boone, Liang, & Duesbery, 2003; Vitale et al., 1998).

Taken together, given that understanding microbial pathogenesis provides insight into the Achilles heel of the host immune response, more studies are necessary to provide better understanding and to inform new therapeutics to prevent infectious disease.

1.4.5 Dissemination and intracellular survival capability of *E. faecalis*

Despite being a commensal bacterium in a healthy host, *E. faecalis* can disseminate into the bloodstream, liver, and spleen, from the intestinal lumen and can translocate across monolayers of polarized human epithelial cells *in vitro* as well as in an antibiotic-treated murine model of superinfection (Wells, Erlandsen, Dunn, & Simmons, 1991; Wells, Jechorek, & Erlandsen, 1990; Wells, Jechorek, Maddaus, & Simmons, 1988; Zeng, Teng, Weinstock, & Murray, 2004). Enterococci that enter the sterile abdominal cavity first encounter cells in the innate immune system, such as macrophages, which function as the first line of defence during an invasion by external pathogens. The balance between the ability of macrophage to clear the pathogens and the pathogen's ability to evade or modulate macrophage signalling expression of immunomodulatory factors can determine the outcome of an infection.

E. faecalis needs to be able to escape clearance by macrophages to enter the bloodstream or draining lymph node to cause systemic infection. Despite traditionally being considered as an extracellular bacterium, *E. faecalis* can survive and persist within eukaryotic cells such as intestinal epithelial cells, cervical epithelial cells, endothelial cells, osteoblasts and even in macrophages and monocytes (Baldassarri et al., 2005; Baldassarri et al., 2001; Bertuccini, Ammendolia, Superti, & Baldassarri, 2002; Campoccia et al., 2016; Gentry-Weeks et al., 1999; Millan, Chiriboga, Patarroyo, & Fontanilla, 2013; Olmsted, Dunny, Erlandsen, & Wells, 1994; Wells et al., 1991; Wells et al., 1990). Interestingly, *E. faecalis* is capable of survival within macrophages for up to 72 hours post infection (hpi) (Baldassarri et al., 2005; Gentry-Weeks et al., 1999). Unlike *E. faecalis*, the survival rate of *E. coli* in the study dropped quickly within 6 to 24 hours post infection (Gentry-Weeks et al., 1999). *E. faecalis* may also utilize

macrophage translocation as a platform to spread to distant sites within the host (Gentry-Weeks et al., 1999). Furthermore, *E. faecalis* can survive intracellularly for an extended period up to 24 hours in human monocytes and 48 hours in rat peritoneal macrophages (Baldassarri et al., 2005; Sabatino et al., 2015). In addition, biofilms appear to be implicated in aiding the intracellular survival as compared to planktonic enterococci by stimulating lower levels of pro-inflammatory cytokines production (Daw, Baghdayan, Awasthi, & Shankar, 2012; Mathew, Yaw-Chyn, & Kishen, 2010). Moreover, some studies have examined the interaction of enterococci with macrophages and reported that bacterial factors such as transcriptional regulators including enterococcal regulator of survival (Ers), a PrfA-like regulator of *E. faecalis*, may contribute towards prolonged survival within macrophages (Gentry-Weeks et al., 1999; Hartke et al., 2006). Finally, intracellular survival of *E. faecalis* is achieved by *E. faecalis* containing vacuoles in macrophages resisting acidification and the inhibition of LC3-II production to inhibit autophagy following phagocytosis (Zou & Shankar, 2016). Although many studies have shown that *E. faecalis* is able to survive in host cells, the exact mechanism of how it does so remain to be characterized.

1.4.6 Immunomodulatory functions of *E. faecalis* during *in vivo* infection

Using a CAUTI model, *E. faecalis*-infected mice have more basophils and eosinophils as compared to catheterized but uninfected mice (Guiton et al., 2013). By contrast, the number of dendritic cells, mast cells and lymphocytes isolated were the same for both infected and uninfected controls. Interestingly, *E. faecalis*-infected mice have significantly more non-activated macrophages to activate macrophages as compared to catheterized but un-infected mice suggesting that *E. faecalis* might modulate macrophage activation (Guiton et al., 2013). Furthermore, heat-killed *E.*

faecalis has been shown to be an alternate treatment option for atopic dermatitis (AD), which can be caused by immune dysregulation, due to its immunomodulatory effects (Choi, Iwasa, Han, Kim, Tang, Hwang, et al., 2016). Taken together, these findings suggest that *E. faecalis* may be able to modulate the immune system via an unknown mechanism.

1.5 Thesis outlines and aims

Although *E. faecalis* can exist as a commensal bacterium, it can become an opportunistic pathogen in immunocompromised or otherwise susceptible individuals leading to infections. Our group and others have shown that *E. faecalis* is able to persist in wounds and in macrophages, suggesting that they may be able to modulate the host immune system to avoid clearance during infection. Therefore, we hypothesized that *E. faecalis* can modulate the NF- κ B pathway, thus affecting the host immune response. The aim of this thesis is to 1) characterize the immune-modulatory interaction of *E. faecalis* and macrophage further in relation to previous findings, 2) determine the *E. faecalis* factors involved in macrophage immunomodulation, and 3) determine the macrophage pathways which *E. faecalis* modulates to suppress NF- κ B. This thesis is comprised of three different chapters to address each specific aim:

Aim 1: Characterise the immune-modulatory interaction of *E. faecalis* and macrophages. Our group has previously shown that *E. faecalis* is able to suppress NF- κ B activation at high doses *in vitro*. Building on this finding, here we used this *in vitro* model to further study the immune-modulatory interaction of *E. faecalis* and macrophages. In addition, since *E. faecalis* is commonly isolated with *E. coli* in CAUTI and wound infection, we investigated mixed infection of macrophages with *E. faecalis* with *E. coli* to determine whether *E. faecalis* can suppress *E. coli*-induced NF- κ B

activation in this physiologically relevant setting. To characterize the immune response of macrophages to *E. faecalis* infection, we used multiplex ELISAs. This work is described in **Chapter 2**.

Aim 2: Determine the *E. faecalis* factors involved in macrophage immunomodulation. Based on the results in **Chapter 2** where we identified that extracellular bacteria play an essential role in immune suppression, we hypothesized that bacterial secreted factor(s) play a role in immune suppression. We used bacterial supernatants to investigate whether the supernatant (containing the secreted factor(s)) can recapitulate the suppression of LPS-induced NF- κ B activation in macrophages using live bacteria. Since immune suppression is dose-dependent and secreted virulence factor(s) may play a role in this immunomodulation, we postulated that deletion mutants involved in quorum sensing and host immunity resistance, described in other *E. faecalis* infection models, may be important for macrophage suppression phenotype at high MOI. Furthermore, *E. faecalis* is a lactic acid producing bacteria and lactic acid has been implicated in immune suppression in tumour environments, so we hypothesized that lactate dehydrogenase (LDH), the gene responsible for lactic acid production, may be important for *E. faecalis* suppression of NF- κ B signalling. In addition to the identification of *ldhI*, which validates our hypothesis-driven findings, additional mutants, identified from an unbiased transposon library screen, that were both attenuated in lactic acid production and macrophage suppression were also examined. We further characterized mutants that failed to suppress NF- κ B activation in macrophages for their ability to survive under oxidative stress conditions, in macrophages *in vitro*, as well as their ability to cause infection *in vivo*. Our lab has previously shown that co-infection of *E. faecalis* with *E. coli* promotes the colonization

of *E. coli* as compared to mono-species wound infection. Using this polymicrobial wound model, we assessed the ability of an *E. faecalis* *ldh1/2* mutant to promote the colonization of *E. coli*, as compared to wildtype *E. faecalis*, during mice polymicrobial wound model. This work is described in **Chapter 3**.

Aim 3: Determine the macrophage pathways which *E. faecalis* modulates to suppress NF- κ B. Using the *in vitro* macrophage infection model established in **Chapter 2**, we used western blot analysis to determine which point in the NF- κ B pathway is affected by *E. faecalis* infection. In addition, we also used western blot analysis to determine other immune signalling pathways that are affected by *E. faecalis* infection apart from NF- κ B pathway. To gain further insights into the molecular mechanism of *E. faecalis* infection, transcriptomic analyses were performed to identify the polarisation of macrophages during infection at high doses. These findings will be described in **Chapter 4**.

Chapter 5 of this thesis summarizes and synthesizes all of the findings reported here, and explores the future directions that can be performed. Altogether, this work provides a mechanistic understanding of *E. faecalis*'s ability to modulate immune activation of macrophages which has not been well characterized before. We show that *E. faecalis* can prevent NF- κ B activation at high doses even in the presence of TLR agonist such as LPS. We identified bacterial factors that are involved in macrophage NF- κ B suppression and at which stage these bacterial factors act. In conclusion, this work expands our knowledge of *E. faecalis* pathogenic mechanisms and may potentially help in the development of new alternative treatments to prevent recurrent enterococcal infection.

Chapter 2: *Enterococcus faecalis* promotes innate immune suppression in a dose-dependent manner

2.1 Introduction

E. faecalis is an early colonizer in infants and a ubiquitous member of the human gut microbiome (Gilmore, Clewell, Ike, & Editors., 2014). *E. faecalis* has recently emerged as opportunistic pathogen in nosocomial environments and is also associated with up to 70% of wound infections, nearly 10% of bloodstream infections, and up to 30% of catheter-associated urinary tract infections (CAUTI) (Gjodsbol et al., 2006; Hidron et al., 2008; Maki & Tambyah, 2001; Wisplinghoff et al., 2004). Many of these infections are polymicrobial in nature, either in origin or in manifestation (Bakaletz, 2004; Brogden, Guthmiller, & Taylor, 2005; Nelson et al., 2012). During CAUTIs, *E. faecalis* is frequently isolated as part of the polymicrobial community which typically includes *E. coli* (Flores-Mireles, Walker, Caparon, & Hultgren, 2015). Similarly, in surgical site and wound infections, *E. faecalis* are often part of the polymicrobial community that is present (Dowd et al., 2008; Giacometti et al., 2000). *E. faecalis* can promote polymicrobial infection by increasing the resistance of co-infecting organisms, such as *P. aeruginosa* and *P. mirabilis*, to clearance by antibiotics and thus promoting their survival during UTIs (Tsuchimori et al., 1994; Yamasaki, Arakawa, & Kamidono, 1991). Furthermore, upon exposure to peptidoglycan of Gram-positive bacteria, *P. aeruginosa* increase production of the antimicrobial phenazine (Korgaonkar & Whiteley, 2011). In addition, mixed species biofilm consisting of *P. aeruginosa* and *E. faecalis* results in enhanced biofilm thickness due to upregulation of genes involved in exopolysaccharide production of *P. aeruginosa* (Lee, Lee, Kim, & Yoon, 2017). As a result, polymicrobial infection by *E. faecalis* and *P. aeruginosa* can more often lead to

aggravated pyelonephritis, compared to monomicrobial infection (Tsuchimori, Hayashi, Shino, Yamazaki, & Okonogi, 1994).

To successfully colonize and persist in the host, *E. faecalis* must withstand, modulate or evade immune-mediated clearance mechanisms. *E. faecalis* invokes multiple strategies to persist within the host, including the formation of biofilms that prevent phagocytosis by immune cells, and the ability to survive within macrophages and neutrophils for extended periods of time (Baldassarri et al., 2005; Gentry-Weeks et al., 1999; Rakita et al., 1999; Roilides, Simitsopoulou, Katragkou, & Walsh, 2015; Zou et al., 2014; Zou & Shankar, 2016). While *E. faecalis* infection at low doses results in activation of MAPKs and NF- κ B, leading to the production of pro-inflammatory cytokines, other studies have shown that several *E. faecalis* strains isolated from the GI tract of healthy human infants can suppress MAPK and NF- κ B signalling, and IL-8 expression in intestinal epithelial cells *in vitro* (Wang, Hibberd, Pettersson, & Lee, 2014; Wang, Ng, Chow, & Lee, 2008; Zou & Shankar, 2015).

The immunomodulation capabilities of *E. faecalis* was first hinted in CAUTI models as well as *in vitro* experiments. During UTI, the cellular immune response to *E. faecalis* infection is primarily monocytic and independent of TLR2 (Kau et al., 2005). Furthermore, in a CAUTI model, the presence of a urinary catheter alone was sufficient to elicit a strong pro-inflammatory response and histological abnormalities in the bladder composed of neutrophils and monocyte-derived cells (Delnay et al., 1999; Guiton et al., 2013; Guiton et al., 2010; Peychl & Zalud, 2008; Rousseau et al., 2016). Yet despite the presence of the strong inflammatory response induced by catheterization, the number of both non-activated and activated bladder-associated macrophages was lower in catheterized bladders infected with *E. faecalis* as compared to catheterized but uninfected bladder resulting in the development of high titer catheter-associated

biofilms and bladder infection (Guiton et al., 2013). Together, these observations suggest that *E. faecalis* can subvert immune-mediated killing to persist within the infected bladder.

Despite various studies supporting that *E. faecalis* can modulate macrophage immune response which aids in its prolonged survival within host cells, the exact mechanism on how *E. faecalis* is able to modulate the NF- κ B response has not been well characterized (Baldassarri et al., 2005; Sabatino et al., 2015; Zou & Shankar, 2015, 2016). Therefore, we hypothesized that *E. faecalis* can modulate the NF- κ B activation in macrophages during infection via specific *E. faecalis* interactions with the specific elements of the NF- κ B pathway, thus affecting the host immune response.

In this Chapter, I have fully optimized an *in vitro* model of *E. faecalis* infection model using RAW-Blue macrophages and have successfully used it to characterize the interactions between macrophages and *E. faecalis*. I have shown that *E. faecalis* is able to actively suppress TLR agonist-mediated NF- κ B activation in a dose-dependent manner using an *in vitro* model. In addition, *E. faecalis* was also able to prevent *E. coli* mediated NF- κ B activation during polymicrobial infection. Furthermore, the modulation of immune activation by *E. faecalis* was dependent on TLRs. Using cytokine luminex® xMAP® analysis, I have shown that *E. faecalis* modulated NF- κ B dependent cytokine and chemokines production in macrophages in a dose-dependent manner. Finally, this *in vitro* model would serve as a foundation for the investigation of macrophage pathways which *E. faecalis* modulates in **Chapter 3** as well as the bacterial virulence factor(s) involved in macrophage modulation in **Chapter 4**.

2.2 Results

2.2.1 *E. faecalis* prevents LPS- or LTA-mediated NF- κ B activation in macrophages in a dose-dependent manner

Various strains of *E. faecalis* can persist intracellularly in mouse and human macrophages for prolonged periods of time (Baldassarri et al., 2001; Gentry-Weeks et al., 1999; Sabatino et al., 2015). Here, we hypothesized that *E. faecalis* OG1RF has immunomodulatory capabilities in order to promote their survival during infection. To characterize whether *E. faecalis* can modulate murine macrophages response during infection, we infected RAW-Blue cells using different multiplicities of infection (MOIs) of *E. faecalis* for 6 hours. *E. faecalis* was first cultured overnight in brain heart infusion (BHI) media. Subsequently, *E. faecalis* is cultured in cell culture media during infection to keep the growth conditions as close to physiological condition as possible, while not impacting the growth of macrophages and *E. faecalis*. RAW-Blue cells are RAW 264.7 cells that encode a secreted embryonic alkaline phosphatase (SEAP) reporter that secretes SEAP upon NF- κ B/AP-1 activation. The SEAP levels quantified by QUANTI-BLUE™ correlated to the level of NF- κ B transcription induction.

Both *E. faecalis* strain OG1RF (**Figure 2.1A**) and the multidrug-resistant strain V583 (**Figure 2.2A**) activated NF- κ B at low multiplicities of infection (MOI) as previously reported (Zou & Shankar, 2015). In contrast, neither *E. faecalis* OG1RF (**Figure 2.1A**) nor V583 (**Figure 2.2B**) activated NF- κ B signalling at high MOIs. Therefore, this suggests that *E. faecalis* is able to prevent NF- κ B activation at higher MOI and this modulation of NF- κ B is dependent on the dosage of *E. faecalis*. We also carried out LDH assays to test the viability of RAW-Blue cells after infection with *E. faecalis*. Neither the LPS used nor *E. faecalis* at different MOIs used affected the RAW-Blue cell viability (**Figure 2.1B**).

E. faecalis can attenuate pro-inflammatory cytokine secretion in intestinal epithelial cells (Wang et al., 2008). To determine whether *E. faecalis* actively prevented NF- κ B-mediated transcription, or simply failed to induce NF- κ B-mediated transcription at high MOIs in macrophages, we tested whether *E. faecalis* could prevent NF- κ B-driven activation in the presence of TLR agonists that initiate NF- κ B signalling. We exposed macrophages to lipopolysaccharide (LPS) or lipoteichoic acid (LTA) simultaneously with *E. faecalis* for 6 hours, quantified both NF- κ B-mediated transcription and LDH release, and observed a dose-dependent inhibition of LPS- and LTA-induced NF- κ B activation by *E. faecalis* (**Figure 2.1C, 2.1E and Figure 2.2C**) in the absence of cytotoxicity (**Figure 2.1D, 2.1F and Figure 2.2D**). Taken together, our results show that *E. faecalis* can suppress or prevent macrophage LPS- and LPS-mediated NF- κ B response in a dose dependent manner using an *in vitro* infection model.

Figure 2.1 *E. faecalis* prevents NF-κB-driven macrophage activation.

Mouse RAW-Blue macrophages were infected with live *E. faecalis* OG1RF alone, or treated concurrently with either LPS (100 ng/mL) or LTA (100 ng/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and cytotoxicity (LDH activity). (A) NF-κB-driven SEAP reporter activity and (B) LDH activity of RAW-Blue macrophages infected by *E. faecalis* alone. (C) NF-κB-driven SEAP reporter activity and (D) LDH activity in the presence of *E. faecalis* and LPS. (E) NF-κB-driven SEAP reporter activity and (F) LDH activity in the presence of *E. faecalis* and LTA. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS or LTA represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ as compared to media alone (-) controls; and where # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ among all of the MOIs as compared to MOI 100.

Figure 2.2 *E. faecalis* vancomycin-resistant strain V583 prevents NF-κB activation in mouse macrophages.

Mouse RAW-Blue macrophages were infected with either live *E. faecalis* alone, or treated concurrently with LPS (100 ng/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and percentage cytotoxicity (LDH activity). (A) NF-κB-driven SEAP reporter activity and (B) LDH activity of RAW-Blue macrophages infected by *E. faecalis* alone. (C) NF-κB-driven SEAP reporter activity and (D) LDH activity in the presence of *E. faecalis* and LPS. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed with post-hoc Tukey's multiple comparison test between all conditions where ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as compared to media alone (-) controls; and where ## $P < 0.01$ and ### $P < 0.001$ among all of the MOIs as compared to MOI 100.

2.2.2 *E. faecalis* actively modulates NF- κ B activation

To determine whether the *E. faecalis* immunomodulatory capability is an active process, we examined the macrophage response to heat-killed or UV-killed *E. faecalis*. Heat-killed bacteria are lysed completely with cell content released, whereas UV-killed bacteria are dead bacteria with intact cell membrane and cell wall. We observed that both heat-killed and UV-killed *E. faecalis* activated NF- κ B at all MOIs, in an inverse manner to that of live intact cells (**Figure 2.3A**) in the absence of cytotoxicity (**Figure 2.3B**). Together, these data suggest that *E. faecalis* actively prevented NF- κ B activation and that the bacteria need to be alive in order to modulate the immune activation. Furthermore, factor(s) that were present on the bacterial cell wall were not required for modulation of NF- κ B activity since UV-killed bacteria stimulates NF- κ B at high MOI instead of suppressing NF- κ B activation.

Figure 2.3 Immunomodulation activity of *E. faecalis* is an active process.

Mouse RAW-Blue macrophages were infected with either live, heat-killed or UV-killed *E. faecalis* alone at specific MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and percentage cytotoxicity (LDH activity). (A) NF-κB-driven SEAP reporter activity and (B) LDH activity upon stimulation with live, heat-killed or UV-killed *E. faecalis* at the indicated MOI. Culture supernatants post infection period were collected for SEAP reporter assays and LDH assays. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA test with Tukey's multiple comparison test where * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ as compared to media alone (-) controls.

2.2.3 *E. faecalis* prevents *E. coli*-mediated immune activation of macrophages in a polymicrobial infection

To investigate whether *E. faecalis*-mediated immune suppression contributed to polymicrobial UTI, we first tested its ability to suppress NF- κ B activity in the presence of *E. coli* in vitro. We determined that RAW-Blue macrophages infected with *E. coli* K12 strain MG1655 at a MOI of 1 or *E. coli* UTI89 at an MOI of 0.125 induced NF- κ B activation (**Figure 2.4 and 2.5C**) in the absence of cytotoxicity (**Figure 2.4B and 2.5D**), whereas higher MOIs were toxic to the mammalian cells (**Figure 2.4B and 2.5D**). In addition, we observed minimal NF- κ B activity for *E. coli* UTI89 at high MOI, consistent with previous reports that the same strain was able to suppress the cytokine response in bladder epithelial cells and suggesting a general immunomodulatory capacity of this strain as well (**Figure 2.4C and 2.5D**) (Hunstad et al., 2005). We then simultaneously infected macrophages with *E. faecalis* and *E. coli* at these pre-determined MOIs and observed that, while both *E. coli* strains MG1655 and UTI89 mono-infection induced NF- κ B reporter activity equal to LPS alone, *E. faecalis* prevented *E. coli*-induced NF- κ B activity in a dose-dependent manner (**Figure 2.5**).

Figure 2.4 *E. coli* activates NF-κB at low MOI.

Mouse RAW-Blue macrophages were stimulated with live *E. coli* K12 strain MG1655 or UTI89 for 6 hours. (A) NF-κB-driven SEAP reporter activity and (B) LDH activity of RAW-Blue macrophages infected by MG1655 alone. (C) NF-κB-driven SEAP reporter activity and (D) LDH activity of RAW-Blue macrophages infected by UTI89 alone. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA test with Tukey's multiple comparison test where * $P < 0.05$, **** $P < 0.0001$, as compared to media (-) control

Figure 2.5 *E. faecalis* suppress *E. coli* induced immune activation *in vitro*.

Mouse RAW 267.4 macrophages were stimulated simultaneously with *E. faecalis* OG1RF and *E. coli* MG1655 (MOI 1) prior to measurement of (A) NF-κB-driven SEAP reporter activity and (B) LDH activity. Mouse RAW 267.4 macrophages were co-infected with *E. faecalis* OG1RF and *E. coli* UT189 (MOI 0.125) before measuring (C) NF-κB-driven SEAP reporter activity and (D) LDH activity. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments. Statistical analysis was performed using one-way ANOVA test with Tukey's multiple comparison test where ***P<0.001, ****P<0.0001 as compared to media alone (-) controls; and where #P<0.05, ###P<0.001, ####P<0.0001 among all of the MOIs as compared to MOI 100.

2.2.4 *E. faecalis* modulation of NF- κ B activation during infection is dependent on TLR4 but independent of TLR2 and TLR9

TLRs are a type of PRR present on the cell surface of primarily immune cells that is required for sensing pathogens during infection by detecting PAMPs (Creagh & O'Neill, 2006). We postulated that modulation of NF- κ B activity by *E. faecalis* is regulated by the TLR signalling pathway. To examine whether the immunomodulatory capability of *E. faecalis* is dependent on TLRs, we used HEK-Blue™ mTLR2 (for identifying the Gram-positive PAMP LTA), HEK-Blue™ mTLR4 (for identifying the Gram-negative PAMP LPS) as well as HEK-Blue™ mTLR9 (for identifying unmethylated cytosine-phosphate-guanine (CpG) motifs) that contain the NF- κ B controlled SEAP reporter. Unmethylated CpG motifs have stimulatory effects on mammalian immune cells and are commonly present in bacterial and viral DNA at a 20-fold higher frequency than mammalian DNA, which is mostly methylated and thus does not exert an immune-stimulatory effect (Hemmi et al., 2000; Krieg, 2007; Lipford, Heeg, & Wagner, 1998; Takeshita et al., 2001).

HEK cell lines are human embryonic kidney cells that do not express any TLRs except for the specific TLR encoded in the selection plasmid which were transfected into the cell. We infected the different HEK cell lines with *E. faecalis* for 6 hours at different MOIs before measuring the SEAP levels. We observed that recognition of *E. faecalis* required TLR2 and TLR9 but not TLR4 (**Figure 2.6A-C**). However, suppression of TLR agonist-induced NF- κ B activation could only be observed for HEK mTLR4 cells suggesting that only TLR4 may be involved in immunomodulatory capabilities of *E. faecalis* but not TLR2 and TLR9 (**Figure 2.6A-C**). We also carried out LDH assays to test the viability of HEK cells and *E. faecalis* infection did not significantly affect viability of cells (**Figure 2.6D-F**). Taken together, our results

suggest that modulation of NF- κ B activity by *E. faecalis* is dependent on TLR4 but independent of TLR2 and TLR9.

Figure 2.6 TLR2 is required for macrophage suppression by *E. faecalis*.

Mouse HEK-Blue™ mTLR2 cells were infected with either live *E. faecalis* alone, or treated concurrently with LTA (1 µg/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and percentage cytotoxicity (LDH activity). NF-κB-driven SEAP reporter activity for (A) HEK mTLR2 cells infected by *E. faecalis* with or without LTA, (B) HEK mTLR4 cells infected by *E. faecalis* with or without LPS (C) HEK mTLR9 cells infected by *E. faecalis* with or without CpG-oligodeoxynucleotides (ODN). LDH activity of (D) HEK mTLR2 cells, (E) HEK mTLR4 cells (F) HEK mTLR9 cells when infected by *E. faecalis* with the respective TLR agonist. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LTA/LPS/ODN represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA test followed with post-hoc Tukey's multiple comparison test between all conditions where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as compared to (+) control or (-) control for (B); and where ##### $P < 0.0001$ MOI100+ODN as compared to MOI 100.

2.2.5 Intracellular *E. faecalis* alone is unable to modulate NF- κ B activity

To determine whether internalization of *E. faecalis* is necessary for NF- κ B modulation, we first infected macrophages with *E. faecalis* at different MOIs for 3 hours. After 3 hours, we killed the extracellular bacteria using gentamicin and penicillin and further incubated the macrophages for 18 h with gentamicin and penicillin. Bacterial enumeration was also performed at 3 hpi and 24 hpi to assess the amount of bacteria internalised. We observed that NF- κ B is significantly activated for all MOIs in the presence of only intracellular *E. faecalis* without significant cell death (**Figure 2.7A and 2.7B**). In fact, *E. faecalis* infection leads to a higher NF- κ B activation as compared to LPS positive control (**Figure 2.7A**). Macrophage cell count were performed right before the infection assay to determine the number of *E. faecalis* to be used. We observed that at 3 hpi, the amount of intracellular *E. faecalis* is approximately 164 bacteria/macrophage for MOI 100 while at 24 hpi there are roughly 28.5 bacteria/macrophages (**Figure 2.7C and 2.7D**), assuming all macrophages were infected and that there is no significant macrophage replication at end of 24 hpi. Therefore, this preliminary result suggests that the intracellular *E. faecalis* does not play an important role in the suppression of NF- κ B activity.

Figure 2.7 Intracellular *E. faecalis* activates NF-κB in mouse macrophages.

Mouse RAW-Blue macrophages were infected with live *E. faecalis* alone at the specified MOI for 3 hours followed by gentamicin and penicillin treatment for 18 h prior to measurement of NF-κB-driven SEAP reporter activity and percentage cytotoxicity (LDH activity). (A) NF-κB-driven SEAP reporter activity and (B) LDH activity of RAW-Blue macrophages infected by *E. faecalis*. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Bacterial enumeration for internalised *E. faecalis* was performed at (C) 3 hours (D) 24 hours post-infection. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed with post-hoc Tukey's multiple comparison test between all conditions where ****P<0.0001 as compared to media alone (-) controls.

2.2.6 *E. faecalis* suppresses NF- κ B-dependent cytokines and chemokine production in RAW macrophages

E. faecalis suppresses cytokines such as IL-8, TNF α , and IL-1 β in intestinal epithelial cells (Wang, Hibberd, et al., 2014; Zou & Shankar, 2015). To investigate whether *E. faecalis* also suppresses cytokine production in infected macrophages, we measured release of a variety of cytokines and chemokines, whose expression is dependent on NF- κ B activation, in the absence of LPS. We observed an overall increase of both pro- and anti-inflammatory cytokines and chemokines at MOI 10 and 1, similar to that observed in LPS-treated cells (**Figure 2.8**). Strikingly, at MOI 100, we observed a global decrease in cytokine, chemokine, and growth factor expression as compared to MOI 10 or LPS exposure (**Figure 2.8**). Moreover, at MOI 100, we observed that most of the analytes (IFN- γ , CCL11, CSF2, IL-4, IL-17, IL-12p40, IL-12p70, IL-2, IL-1 β , CCL2, CXCL1, and IL-5) were present at levels similar to the media control (**Figure 2.8 and Figure 2.9A**). Principal component analysis (PCA) of analytes revealed that the profile of MOI 100 overlapped with the profile of uninfected macrophages, suggesting that analytes were not expressed despite greater numbers of *E. faecalis* (**Figure 2.9B**). Therefore, these data suggest that *E. faecalis* suppression of NF- κ B signalling at high MOI lead to an overall suppression of cytokines and chemokine expression.

Figure 2.8 *E. faecalis* suppresses NF- κ B-dependent cytokine and chemokine production in RAW macrophages.

Mouse RAW 267.4 macrophages were infected with live *E. faecalis* at the indicated MOI. (A) Spider plot showing the fold-change of cytokines, chemokines and growth factors detected in filtered supernatants collected 6 hpi at depicted conditions compared to media control. Data were normalized against the media control, represented in pink, to obtain fold-change. (B) Heat map depicting the log₂ transformed absolute values measured in pg/mL of the indicated cytokines, chemokines, and growth factors.

Figure 2.9 *E. faecalis* prevented NF- κ B driven cytokine and chemokine expression.

Mouse RAW 267.4 macrophages were stimulated with live *E. faecalis*, and (A) the absolute concentrations in pg/mL of cytokines from filtered supernatants collected 6 hpi at various conditions are shown. Horizontal bars represent the mean value for each condition. Statistical significance was determined using the Kruskal-Wallis test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as compared to media alone control. Open symbols colored red represents data points that are zero and plotted on the X-axis for representation. (B) Principal component analysis of cytokines and chemokines from filtered supernatants collected at 6 hpi from different conditions. Ellipses represent the distribution of each condition. The two dimensions measured explained a total of 84.3% of variability of all analytes measured.

2.3 Discussion

Bacterial immunomodulatory functions can alter infection sites leading to increased susceptibility to colonization and persistence (Tay, Chong, & Kline, 2016; Zou et al., 2014; Zou & Shankar, 2014). *E. faecalis* can augment the immune response in a variety of cell types, including intestinal epithelial and mouse macrophage cell lines (Wang, Hibberd, et al., 2014; Wang et al., 2008; Zou et al., 2014). Recently, it was shown that *E. faecalis* isolated from healthy newborns were able to suppress NF- κ B activation of intestinal epithelial cells at MOI 100 or 1000 (Wang, Hibberd, et al., 2014; Wang et al., 2008). By contrast, infection of RAW-Blue macrophages and bone marrow-derived macrophages with *E. faecalis* strain E99 at MOI 10 resulted in NF- κ B activation (Zou et al., 2014). These discrepant reports of NF- κ B activation and suppression by *E. faecalis* underscore the need for further investigation into *E. faecalis* immunomodulatory activities within macrophages. Therefore, in this chapter, we aimed to investigate the mechanism of immune modulation in the host innate immune response by *E. faecalis* during infection.

In this study, we resolved previous conflicting reports and showed that *E. faecalis* suppression of NF- κ B signalling in macrophages is dose-dependent where NF- κ B is suppressed at high MOI 100 but activated at low MOI. In addition, we showed that both *E. faecalis* strains V583 and OG1RF not only prevented NF- κ B activity in RAW-Blue macrophages in a dose-dependent manner but can also prevent TLR agonist-induced NF- κ B activation. The number of macrophages present in wound 1-day post-infection (dpi) is approximately 10^4 while the initial *E. faecalis* inoculum used was 10^6 with 10^7 CFU recovered from wound 1dpi (Chong et al., 2017). Immune modulation

was observed both in the wound infection (Chong et al., 2017) as well as CAUTI (Tien et al., 2017), therefore, it is possible for MOI 100 to occur during natural infection.

Interestingly, when only intracellular *E. faecalis* was present, there was significantly higher levels of NF- κ B activation as compared to LPS-mediated NF- κ B activation. This suggests that the intracellular bacteria are not essential for the modulation of immune response in macrophages. One limitation of this assay is that it does not directly elucidate whether the uptake of *E. faecalis* by macrophages is important for the suppression of NF- κ B activity by *E. faecalis*. A more standard assay to test whether bacteria uptake is important would be to block macrophage phagocytosis by using drugs such as cytochalasin D and Latrunculin A which are actin inhibitors. However, the drug treatment durations and concentrations, as suggested by literatures, used were not sufficient to inhibit *E. faecalis* uptake by macrophages at 6 hpi and hence we not able to conclude whether uptake of *E. faecalis* by macrophage is essential for NF- κ B suppression during *E. faecalis* infection (data not shown). One difference between our experimental setup as compared to that used by literatures would be that most literatures used a maximum of 2 hours incubation of macrophages with bacteria after treatment with phagocytosis inhibitor drugs (Baldassarri et al., 2005; Cortes-Perez et al., 2015; Zou & Shankar, 2014). However, we could not reduce the duration of infection assay to lesser than 6 hours incubation due to the limitation of the NF- κ B-driven SEAP reporter activity. An alternative way to overcome this problem would be to use a cocktail of phagocytosis inhibitor drugs such as cytochalasin D, Latrunculin A and Wortmannin instead of a single drug. Regardless, coupled with the observation that immunomodulation only happened during infection at high MOI using live bacteria, our results suggests that active extracellular *E. faecalis* may be essential for immune suppression of macrophage to occur.

Given that a large proportion of *E. faecalis* infections are polymicrobial and *E. faecalis* is commonly co-isolated with *E. coli* in UTI and wound infections, we also investigated the interactions of *E. faecalis* and *E. coli* *in vitro* (Cooper, 2013; Dowd et al., 2008; Ronald, 2003; Siegman-Igra, Kulka, Schwartz, & Konforti, 1994; Tay et al., 2016). We showed that *E. faecalis* can prevent *E. coli*-mediated NF- κ B activation in macrophages. Given the prevalence of *E. faecalis* in polymicrobial interactions in UTI, our lab also used *in vivo* experiments to determine the contribution of *E. faecalis* to co-infections. Indeed, *E. faecalis* was able to augment *E. coli* K12 strain MG1655 titres in the kidneys (Tien et al., 2017). The augmentation of *E. coli* titres may be due to the immunomodulatory capability of *E. faecalis* which may aid in promoting the survival of the less fit *E. coli* strain. Similarly, Gram-positive uropathogens *Staphylococcus saprophyticus* and Group B *Streptococcus* induce minimal pro-inflammatory responses in the urinary tract, while the latter limits uropathogenic *E. coli* (UPEC) pathogenesis in mice (Carlin, Lewis, Varki, & Nizet, 2007; Kline & Lewis, 2016; Kline et al., 2012). Here, our data suggests that the ability of Enterococci to modulate the immune response may aid in promoting the survival of other co-infecting pathogens, leading to increased susceptibility and poorer prognosis of the host.

TLRs found on macrophages are involved in the recognition of pathogens during infection by detecting PAMPs (Creagh & O'Neill, 2006). To investigate which TLRs are involved in the modulation of NF- κ B activity by *E. faecalis*, we tested the hypothesis that it may interfere with either TLR2 or TLR9-mediated signalling but not TLR4-mediated signalling. Using HEK cell-line transfected with the respective TLR, we observed that recognition of *E. faecalis* and subsequent NF- κ B activation was dependent on TLR2 or TLR9 but independent of TLR4. It was expected that TLR2 may be involved in recognition of *E. faecalis*, which expresses LTA, as TLR2 recognizes LTA that can

be found on Gram-positive bacteria (Takeuchi et al., 1999). In addition, we also predictable that TLR9 would be involved in the recognition of *E. faecalis* as it has been shown that heat killed *E. faecalis* (which presumably releases CpG DNA from the lysed cells) can induce IL-12 production (Inoue, Nagino, Hoshino, & Ushida, 2011). Surprisingly, in the presence of agonist for specific TLRs, *E. faecalis* suppression or prevention of NF- κ B signalling was dependent on TLR4 but independent of TLR2 and TLR9. Although NF- κ B activation is a common response of TLR2 and TLR4 binding to PAMPs, the signalling of TLR2 and TLR4 are not equivalent due to the differences in secretion of chemokines and cytokines that are induced (Re & Strominger, 2001). A TLR4 agonist is able to promote the production of T_H1-inducing cytokines and chemokines whereas TLR2 agonist can induce Treg, T_H1 or T_H2 response (Agrawal et al., 2003; Dillon et al., 2004; Dillon et al., 2006; Manicassamy et al., 2009; Re & Strominger, 2001; Redecke et al., 2004; Sieling et al., 2003). Although the exact mechanism of how TLR2 can regulate these myriads of responses is not clear, this may be due to differential responses triggered by different ligands or the co-receptors, such as TLR1 and TLR2. A possible explanation for why we only see NF- κ B activation that is similar to the LTA or ODN only positive controls during *E. faecalis* infections at MOI 100 in the presence of TLR agonists might be because of competitive binding of TLR agonists or *E. faecalis* PAMPs to the TLRs. The binding affinity of LTA and ODN to the respective TLR might be greater than that of *E. faecalis* PAMPs, leading to lower NF- κ B activation as compared to *E. faecalis* alone in the absence of TLR agonists. A plausible reason for TLR4 being the sole TLR necessary among the ones tested for *E. faecalis* NF- κ B modulatory activity might be that the interferon regulatory factor 3 (IRF3)-dependent pathway but not the interferon regulatory factor 7 (IRF7)-dependent pathway is involved in this immunomodulatory activity, since IRF3 is absent in TLR2

and TLR9 induction pathway (Kawasaki & Kawai, 2014; Takeda & Akira, 2004). In fact, overexpression of IRF3 can downregulate NF- κ B signalling in hepatocytes of mice thus suggesting that IRF3 might negatively regulate NF- κ B activity (Iwanaszko & Kimmel, 2015; Wang, Zhang, et al., 2014). Therefore, our results suggest that *E. faecalis* may capitalize IRF3-dependent pathway to modulate NF- κ B activity although further investigation is required.

Regulation of immune responses to *E. faecalis* can be examined closely by looking at the cytokine and chemokine profiles of macrophages. Strikingly, our data showed that *E. faecalis* at a high MOI of 100 resulted in a global suppression of both pro- and anti-inflammatory cytokines as well as chemokines. Chemokines such as CCL2, CXCL1, CCL11 are involved in the regulation of migration and infiltration of monocytes and macrophages, neutrophils and eosinophils respectively (Carr et al., 1994; Chintakuntlawar & Chodosh, 2009; Menzies-Gow et al., 2002). Neutrophils are essential in the host immune response not only during fungal and extracellular bacterial infection but also intracellular bacterial infection such as by *L. monocytogenes*, eliciting their functions through phagocytosis, production of reactive oxygen and nitrogen species, neutrophil extracellular trap formation and production of pro-inflammatory cytokines (Mócsai, 2013; Nauseef & Borregaard, 2014; Witter, Okunnu, & Berg, 2016). Therefore, our results suggest that during *E. faecalis* infection at high dosage, the recruitment and activation of innate immune cells especially macrophages and neutrophils to the site of infection may be impaired. This was similarly observed in a CAUTI model, where there was significant increase in the proportion of non-activated to activated macrophages in *E. faecalis* infected mice compared to uninfected but catheterized control (Guiton et al., 2013). This lack of immune cell infiltration and activation may lead to weaker innate immune response and thus less efficient clearance

of *E. faecalis* leading to its survival and persistent infection within the host. The downregulation of these chemokines may also lead to less efficient recruitment and activation of dendritic cells and might result in less robust adaptive immune response since dendritic cells are major APCs that activate T and B cells (Jenkins et al., 2001). A RNA expression profiling on whole bladders 24 hours post catheterization and polymicrobial infection was previously done, and revealed that within the top 50 differentially regulated genes between the mono-infected and co-infected groups, genes specific for dendritic cells (DC), macrophages (MF), and monocytes (MO) were over-represented and showed decreased mRNA levels in co-infected animals, suggesting a reduced infiltration or activation of these cells in the bladder following-co-infection as compared to mono-infection (Tien et al., 2017). Furthermore, the downregulated cytokines play an important role in the activation and regulation of T_H1 , T_H2 and T_H17 responses. T_H1 , T_H2 and T_H17 are subsets derived from $CD4^+$ T cells that play a crucial role in regulating effective immune response during an infection by pathogens. Different strains of *E. faecalis* can modulate T cell differentiation in a myriad of ways. For example, oral administration of lysed *E. faecalis* strain FK-23 can suppress the expression of IL-6 and $TGF\beta$ mRNA resulting in the attenuation of T_H17 cell development and suppression of the asthmatic response in mice (Zhang et al., 2012). In addition, oral administration of heat-killed *E. faecalis* EF-2001 leads to the attenuation of various inflammatory cytokines, such as T_H1 , T_H2 , T_H17 and T_H22 cytokines, in the atopic eczema mice model suggesting that it can inhibit the T_H1 , T_H2 , T_H17 and T_H22 response thereby reducing inflammation (Choi, Iwasa, Han, Kim, Tang, Han, et al., 2016). On the other hand, in various biological models, implantation of live *E. faecalis* CECT7121 and its survival in gastrointestinal tracts can skew the cytokine profile toward the T_H1 phenotype (Castro et al., 2012; Castro et al., 2010; Molina et al., 2015).

Furthermore, IL-6, IL-10 and IL-12 cytokines production can also be induced by cell wall extracts of *E. faecalis* CECT7121 in human peripheral blood mononuclear cells (PBMCs) (Sparo, Delpech, Batistelli, & Basualdo, 2014). The modulation of immune response by *E. faecalis* does not appear to be strain specific. However, the difference in the response elicited may suggest that different *E. faecalis* strains may possess different bacterial factor(s) to modulate the host immune system. Hence, more work is necessary to study in depth the immune modulation capability of *E. faecalis*. Taken together, our results suggest that *E. faecalis* can affect the macrophage response and therefore may be able to modulate the T cell response to help in its survival and persistent infection within the host. This immunomodulatory capability of *E. faecalis* can possibly promote the survival of co-infecting uropathogens resulting in more severe infection. This may further help to explain the frequency at which *E. faecalis* and *E. coli* are often co-isolated from polymicrobial urinary tract infection.

In conclusion, we have shown that *E. faecalis* was able to modulate NF- κ B activity in a dose-dependent manner dependent of TLR4 recognition but independent on TLR2 and TLR9 recognition. In addition, *E. faecalis* still possesses immunomodulatory functions even when co-infected with *E. coli*, which is known to activate NF- κ B. Finally, multiplex analysis of cytokines revealed a global downregulation of cytokines, chemokines and growth factors during *E. faecalis* infection at a higher dose. All of these suggest that the presence of a higher bacterial density would help in promoting their survival and therefore lead to a persistent infection by circumventing the host immune response. Given the prevalence of and widespread *E. faecalis* infection, further investigation into how *E. faecalis* can modulate the host immune response pathway is required. Hence, we aim to identify the virulence factors that are involved in immunomodulation during *E. faecalis* infection in **Chapter 3**.

2.4 Materials and methods

2.4.1 Bacterial growth conditions and strains

Bacteria strains that were used in this study are as shown in **Table 2.1**. *E. faecalis* strains were grown in Brain Heart Infusion (BHI) (Becton, Dickinson, USA) broth or agar at 37°C overnight. *E. coli* strains were grown statically at 37°C overnight in Luria Bertani (LB) (Difco, France). Both *E. faecalis* and *E. coli* strains were streaked from 25% glycerol stock and incubated on the respective agar plates overnight at 37°C. The antibiotics rifampicin and fusidic acid were supplemented at a concentration of 25 mg/L to select for *E. faecalis* OG1RF as and when necessary. Single isolated colonies of each strain were inoculated into liquid culture and grown overnight statically at 37°C for 18-20 hours for all experiments. Overnight bacteria culture of *E. faecalis* were centrifuged at 6,000 g for 5 minutes and washed once with 1X PBS. Normalization to an optical density (OD) of 0.7 was done for *E. faecalis* (equivalent to 2×10^8 CFU/mL) and 0.4 (equivalent to 2×10^8 CFU/mL) for *E. coli*.

Table 2.1| Bacterial Strains used in this study

Strains	Relevant characteristic(s) ^b	References or source
Strains		
<i>Enterococcus faecalis</i>		
OG1RF	Fus ^r , Rif ^r , wild-type strain	(Dunny, Brown, & Clewell, 1978)
V583	Vancomycin resistance strain	(Paulsen et al., 2003)
<i>Escherichia. coli</i>		
MG1655	<i>E. coli</i> K-12 laboratory strain	(Blattner et al., 1997; Browning et al., 2013)
UTI89	Uropathogenic strain (UPEC)	(Chen, Hung, et al., 2006; Mulvey, Schilling, & Hultgren, 2001)

2.4.2 Cell culture

RAW-Blue cells derived from mouse RAW-Blue macrophages (Invivogen™, USA) were used in this study. They contain a plasmid encoding secreted embryonic alkaline phosphatase (SEAP) reporter that is under the transcriptional control of an activator protein 1 (AP-1)/NF-κB inducible promoter. RAW-blue cells and HEK cell lines (**Table 2.2**) were cultured in T75/T175 flask as adherent monolayers (Nunc, Thermo scientific, Singapore) in DMEM with 10% heat inactivated FBS supplemented with 200 µg/mL Zeocin™ (Invivogen™, USA) and 10µg/mL Blasticidin (Invivogen™, USA), when necessary, at 37°C with 5% CO₂.

Table 2.2 Cell lines used in this study		
Cell lines	Relevant characteristic(s) ^b	Source
RAW-Blue	Mouse monocyte macrophages established from a tumor induced by Abelson murine leukaemia virus. Contain AP-1/NF- κ B-induced SEAP reporter plasmid. Zeocin.	Invivogen™, USA
HEK-Blue™ mTLR2	HEK 293 cells with HEK 293 cells co-transfected with AP-1/NF- κ B-induced SEAP reporter plasmid and mTLR2 plasmid. Zeocin and Blasticidin.	Invivogen™, USA
HEK-Blue™ mTLR4	HEK 293 cells co-transfected with AP-1/NF- κ B-induced SEAP reporter plasmid and mTLR4 plasmid. Zeocin and Blasticidin.	Invivogen™, USA
HEK-Blue™ mTLR9	HEK 293 cells co-transfected with AP-1/NF- κ B-induced SEAP reporter plasmid and mTLR9 plasmid. Zeocin and Blasticidin.	Invivogen™, USA

2.4.3 RAW-Blue macrophage infection

RAW-Blue cells were seeded into 96 well, 24 well, 12 well and 6 well microtiter plates (Nunc, Thermo Fisher Scientific, Waltham, MA) with cell density of 1×10^5 , 2×10^5 , 5×10^5 or 1.5×10^6 cells/well respectively in antibiotic free media (DMEM with 10% heat inactivated FBS) and incubated overnight at 37°C with 5% CO₂. Following overnight incubation, the cells were washed once with PBS and fresh media was added. The SEAP reporter assay was established by empirically defining the minimal agonist (lipopolysaccharide (LPS) or lipoteichoic acid (Dreyfus et al.)) concentration that induced the maximum SEAP activity in the absence of cell death. Cells were stimulated using LPS EB ultrapure purified from *E. coli* O111:B4 (Sigma-Aldrich, St Louis, MO) (100 ng/mL) or LTA derived/purified from *Staphylococcus aureus* (Sigma-Aldrich, St Louis, MO) (100 ng/mL) as positive controls, or media alone as a negative control. RAW-Blue cells were infected with *E. faecalis* (at MOI of 100:1, 50:1, 25:1, 10:1 and 1:1) for 6 hours with or without TLR agonists. Overnight bacterial cultures were centrifuged and resuspended in cell culture media. For infection experiments, live bacterial cultures were diluted to achieve the desired multiplicity of infection with macrophages (MOI). Alternatively, bacteria were heat-killed (80°C for 1 hour) prior to addition to macrophage cultures. For co-infection experiments, RAW-Blue cells were simultaneously infected with *E. coli* K12 strain MG 1655 (1:1 MOI) or *E. coli* UTI89 (MOI of 0.125:1) and *E. faecalis* OG1RF (MOIs of 100:1, 50:1, 25:1, 10:1 and 1:1). Heat-killed and UV-killed bacteria were verified by the absence of viable bacteria when plated on BHI agar.

For intracellular bacteria assay, RAW-Blue cells were infected with *E. faecalis* at various MOIs for 3 hours. After 3 hours, the cell culture media was removed, the cells

were washed once with PBS and fresh media with gentamicin-penicillin G (500 µg/mL) (Sigma-Aldrich, St Louis, MO) was added. The cells were then allowed to incubate either for 1 hour or overnight at 37°C with 5% CO₂. Subsequently, supernatant was taken and plated to ensure that there are no viable extracellular bacteria present. Cells were then washed once with PBS and 1 mL of PBS with 1% Triton X-100 (Sigma-Aldrich, St Louis, MO) was added to lyse the macrophages, followed by CFU enumeration.

2.4.4 NF-κB reporter assay

RAW-Blue cells secrete SEAP into the supernatant upon NF-κB activation which serve as an indication of NF-κB activation upon quantification. QUANTI-Blue™ (Invivogen™, USA), a colorimetric enzyme medium, indicates the presence of SEAP through changing of medium colour from pink to purple-blue. Post-infection, 20 µL of supernatant was collected and added to 180 µL of QUANTI-Blue reagent and incubated at 37°C overnight. SEAP levels were quantified at 640 nm using a microplate reader (Tecan infinite M200 Pro, Switzerland). LPS-stimulation serves as positive control where NF-κB activity is considered high where media control (-) is considered as low NF-κB activity. SEAP readings in between that of LPS and media control is considered as medium activity. All experiments were performed in triplicates.

2.4.5 Cell viability assay

Simultaneously with supernatant collection for SEAP determination, culture supernatants were collected from each well to measure lactate dehydrogenase (LDH) release, using an LDH cytotoxicity assay (Clontech, Takara, Japan) according to manufacturer's instructions. Background LDH activity was determined using mock (PBS) treated RAW-Blue cells. Maximal LDH activity was determined by lysing cells

with 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO). Each condition was carried out in triplicate. Percentage cytotoxicity was calculated as follows:

$$\frac{\text{sample absorbance} - \text{background absorbance}}{\text{maximal absorbance} - \text{background absorbance}} \times 100.$$

2.4.6 Cytokine Luminex® xMAP® analysis

Supernatants were collected from RAW-Blue cells 6 hours post-infection and stored at -80°C until assessment by the Bio-Plex Pro™ mouse cytokine 23-plex assay kit (Bio-Rad Laboratories), according to manufacturer's recommendations (Benita et al., 2010). All samples were assessed using the same kit lot and at the same time to avoid inter-assay variability.

2.4.7 Statistical Analysis

Statistical analysis was performed utilizing GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) for pooled data from multiple experiments. One-way ANOVA (Analysis of Variance) were used to compare between media and the different conditions. Statistical significance for the multiplex immunoassay was determined using the Kruskal-Wallis statistical analysis. Unless otherwise stated, values represented means ± SEM derived from at least 3 independent experiments and/or 3 technical replicates. */# indicate P-value < 0.05, **/## indicate P-value < 0.01, ***/### indicating P-value < 0.001 and ****/#### represents P-value < 0.0001.

2.5 Authors contribution

I design the study of this project, performed, and analysed the *in vitro* experiments listed in this chapter. All the experiments are done by me and assisted by Iwan Ng Jian Xiong. Cytokine Luminex® xMAP® was performed by me with the help

of Kelvin Chong Kian Long. The heat map and PCA plot for the Cytokine Luminex® xMAP® analysis were generated by Kelvin Chong Kian Long.

Chapter 3: Lactate dehydrogenase mediates *Enterococcus faecalis* suppression of macrophage immune response

3.1 Introduction

Enterococcus faecalis is a commensal bacteria and is a leading cause of nosocomial infections including endocarditis, wound infections and urinary tract infections. The malleable genome of *E. faecalis* allows it to acquire resistance to new antibiotics via horizontal gene transfer, and it is also intrinsically tolerant to some classes of antibiotics such as β -lactams and aminoglycosides (Hollenbeck & Rice, 2012). Therefore, due to its increasing antibiotic resistance, *E. faecalis* infection can be arduous to treat (Hidron et al., 2008). Further compounding this problem, *E. faecalis* may be able to evade innate immune response since it is able to survive in macrophages for prolonged period of time (Gentry-Weeks et al., 1999; Sabatino et al., 2015; Zou & Shankar, 2016). Furthermore, we have shown that *E. faecalis* may enhance the growth of non-pathogenic *E. coli* by potentially modulating the immune response during polymicrobial CAUTI (Tien et al., 2017). Therefore, the ability of *E. faecalis* to modulate the immune response may have synergistic effect on other microbe species during polymicrobial infection leading to worse disease outcomes or disease severity. Although various studies have investigated *E. faecalis* antibiotic resistance mechanisms and transmission, virulence factors that are involved in innate immune response evasion have not been well characterized.

To efficaciously establish an infection, *E. faecalis* has evolved mechanisms that function to subvert the host immune response at various stages, allowing colonization and subsequent persistence. Several *E. faecalis* virulence factors, including aggregation substance (AS), capsular polysaccharides, gelatinase, enterococcal leucine rich A (ElrA)

and TcpF (TIR domain-containing protein in *E. faecalis*) have been shown to modulate immunity during infection (Nunez et al., 2018; Park et al., 2007; Park et al., 2008; Thurlow, Thomas, Fleming, et al., 2009; Zou et al., 2014). These virulence factors generally play a role in promoting survival of *E. faecalis* in macrophages (Sava, Heikens, & Huebner, 2010). AS, a cell surface protein encoded by a sex pheromone plasmid, promotes phagocytosis and internalization into macrophages and neutrophils via interaction with complement receptor type 3 (Rakita et al., 1999; Sussmuth et al., 2000). While this may seem counterinitative, internalized AS-expressing *E. faecalis* are more resistant to superoxide killing, leading to increased survival in macrophages and neutrophils due to possible inhibition of respiratory burst (Rakita et al., 1999; Sussmuth et al., 2000). Interestingly, the expression of AS in *E. faecalis* strains that lacked AS did not lead to increased colonization in the UTI model suggesting that AS does not contribute to upper or lower urinary tract colonization and infection (Johnson et al., 2004). Capsular polysaccharides, a surface-exposed carbohydrate, plays a major role in virulence of many microbes by aiding in avoiding opsonisation and clearance by the host innate immunity (Graveline, Segura, Radzioch, & Gottschalk, 2007; Peterson et al., 1978; Rubens, Heggen, Haft, & Wessels, 1993; Wessels, Moses, Goldberg, & DiCesare, 1991). Notably, of the different serotype of capsules produced by *E. faecalis*, only two different serotypes (C and D) of capsular polysaccharides aids in immune evasion and pathogenesis (Hancock & Gilmore, 2002; Hancock, Shepard, & Gilmore, 2003; Hufnagel et al., 2004; Thurlow, Thomas, Fleming, et al., 2009; Thurlow, Thomas, & Hancock, 2009). Capsular polysaccharides aid in masking bound C3 and, hence, attenuate C3-opsonophagocytosis and decrease TNF α production (Thurlow, Thomas, Fleming, et al., 2009). Furthermore, encapsulation also helps to mask *E. faecalis* surface PAMPs, such as LTA, from pathogen recognition receptors and therefore attenuates

detection by immune cells such as macrophages leading to reduced inflammation and bacterial clearance (Thurlow, Thomas, Fleming, et al., 2009). The enterococcal polysaccharide antigen (*epa*) locus, consisting of a cluster of genes involved in the biosynthesis of polysaccharides, regulates biofilm formation, resistance to killing by polymorphonuclear leukocytes (PMNs) as well as pathogenicity of mouse peritonitis and UTI model (Singh, Lewis, & Murray, 2009; Teng, Jacques-Palaz, Weinstock, & Murray, 2002).

In addition, gelatinase facilitates innate immune evasion by interacting with the complement system, by degrading the anaphylatoxin C5a, to reduce opsonization and to decrease neutrophil recruitment (Park et al., 2007; Park et al., 2008; Thurlow et al., 2010). Gelatinase also increases the bacterial burden in a rabbit model of endocarditis with reduced phagocyte infiltration to site of infection (Thurlow et al., 2010). More recently, ElrA, a surface virulence factor that is a member of the internalin family, is important in the avoidance of the detection and migration of macrophages towards *E. faecalis* (Nunez et al., 2018). Furthermore, ElrA can promote virulence of *E. faecalis* as demonstrated in mouse intraperitoneal model where infecting mice with an *elrA* deletion mutant leads to decreased CFU in both the liver and spleen (Brinster et al., 2007; Nunez et al., 2018). TcpF is an *E. faecalis* TIR domain-containing protein and interferes with Toll-like receptor (TLR)-MyD88 interactions, which also depend on MyD88 TIR domain-mediated interactions. As a result, *E. faecalis* TcpF expression results in decreased NF- κ B p65 translocation in RAW macrophages (Kraemer et al., 2014; Zou et al., 2014). TcpF is present in *E. faecalis* V583 and is enriched in UTI isolates but is absent in OG1RF (Kraemer et al., 2014; Zou et al., 2014). Since we observed NF- κ B modulation by both *E. faecalis* OG1RF and V583, TcpF is unlikely to be the factor

mediating high-level NF- κ B suppression in macrophages in our studies. In addition, both capsular polysaccharides and ElrA is absent in *E. faecalis* OG1RF. Therefore, these data suggest that the *E. faecalis* factor(s) involved in immune suppression of macrophages is an uncharacterised virulence factor.

Collectively, while these studies are important in advancing the field on Enterococcal immunomodulation, the lack of evidence showing that a particular virulence factor is essential for macrophage suppression suggests that there might be other undiscovered factor(s) behind this phenomenon of immune modulation by *E. faecalis*. A growing number of studies have shown that secreted virulence factors, such as type III secretion systems in *E. coli* and *Shigella* to downregulate NF- κ B pathway, can be important for subverting the innate immune response (Ashida et al., 2010; McGuire & Arthur, 2015; Okuda et al., 2005; Vossenkämper, Macdonald, & Marchès, 2011). We therefore, decided to investigate whether secreted bacterial factor(s) may be involved in immune suppression during *E. faecalis* infection using the *in vitro* model established in **Chapter 2**. We hypothesized that secreted bacterial factor(s) may be involved in immune suppression because we only observed suppression during high MOI infection and when *E. faecalis* is actively replicating. Using a systematic approach, we tested for immune suppression using *E. faecalis* supernatants obtained under various conditions. In addition, since macrophage suppression can only be observed at high dosage infection and the bacterial factor(s) involved may be secreted, we hypothesized that bacterial factor(s) that are density related and are secreted are important for the immunomodulatory capability of *E. faecalis*. Based on this hypothesis, we tested bacterial factor(s) that are important for quorum sensing as well as known secreted *E. faecalis* factor(s).

Collectively, our results demonstrated the importance of secreted Enterococcal factors in immune evasion. Our results, coupled with results from others, suggest that the surface attached virulence factors being responsible for majority of the immune escape, might not be necessarily true (Johannessen, Askarian, Sangvik, & Sollid, 2013; Lambertz et al., 2012; Sharma et al., 2017). In this Chapter, we show that *E. faecalis* supernatant can suppress NF- κ B activation in a dose-dependent manner similar to infection using live bacteria. Furthermore, we show that lactate dehydrogenase (LDH) is important for the suppression of NF- κ B activation of macrophages during *E. faecalis* infection *in vitro*. Finally, we show that a *ldh* mutant is less able to promote *E. coli* survival during mixed species wound infection. Our results showed that other pathways of immune modulation such as immunomodulatory potential of bacteria metabolic by-products may exist and that we should look beyond the classical virulence factors described in the literature. In addition, elevated serum lactate level in patients is likely multifactorial due to various conditions including tissue hypoperfusion, mitochondrial dysfunction and anaerobic metabolic shift, and is often associated with bacterial infection, lactic acidosis and in more serious cases sepsis leading to increase mortality rate in clinical settings (Brook, 1981; del Portal et al., 2010; Khater, Salah-Eldeen, Khater, & Saleh, 2016). Metabolic reprogramming of host cells to glycolytic metabolism, a hallmark of cancer cells, can also occurs during infection by bacteria such as *M. tuberculosis* and *Legionella pneumophila* leading to increased lactate production (Escoll & Buchrieser, 2018). In addition, accumulating evidence suggests that enterococci are capable of initiating sepsis and septic shock despite being inherently less pathogenic as compared to other Gram-positive bacteria such as *S. aureus* and *S. pneumoniae* (Linden, 2003). Thus, this raises the question whether lactate production during *E. faecalis* infection not only thwarts the immune response but may also increase

the severity of the infection especially in hospitalised, immunocompromised, at risk patient due to additional lactate production by *E. faecalis* apart from host-derived lactate. Therefore, our findings are important for understanding enterococcal infection and the plausible treatment options for *E. faecalis* by targeting lactic acid production.

3.2 Results

3.2.1 Secreted factor(s) present in supernatant may be required for modulation of NF- κ B activity

In **Chapter 2**, we demonstrated that *E. faecalis* needs to be alive for immunomodulation of NF- κ B activity to occur. This led us to hypothesize that the bacterial factor(s) involved in immunomodulation might be secreted out into the extracellular milieu. To test our hypothesis, we first collected and prepared filtered, cell-free supernatants from *in vitro* infections or supernatants from *E. faecalis* grown in the absence of macrophages to determine whether NF- κ B activation was due to cytokines secreted by RAW-Blue macrophages during infection. The filtered bacterial-free infection supernatants were subsequently used to treat uninfected macrophages for 6 hours, without *E. faecalis*, in the presence or absence of LPS. Our results showed a similar trend of dose-dependent NF- κ B suppression in the presence of cell-free infection supernatants as that of the initial infection using live *E. faecalis* cultures (**Figure 3.1A**). In addition, OG1RF supernatants, collected from bacterial cultures grown in absence of macrophages, weakly activated NF- κ B and did not suppress LPS-mediated induction of NF- κ B activity, except at MOI 100 (**Figure 3.1B**). LDH released into the cultural supernatants was also monitored simultaneously to ensure that RAW-Blue cells remained viable when incubated with supernatants, and we observed no increase in LDH release in any of the conditions tested (**Figure 3.1E and F**). Furthermore, the suppressing secreted bacterial factor is of size less than 10 kDa, heat and UV stable as heat and UV treatment did affect supernatant ability to suppress LPS-induced NF- κ B activation (**Figure 3.1C and D**). Therefore, our results suggest that when secreted *E. faecalis* factor(s) accumulates to a higher concentration at high MOI, this leads to suppression of NF- κ B activity.

Figure 3.1 Secreted bacterial factor(s) is involved in immunomodulation ability of *E. faecalis*.

Mouse RAW-Blue macrophages were incubated with different supernatants with or without concurrent treatment with LPS (100 ng/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and cytotoxicity (LDH activity). NF-κB-driven SEAP reporter activity were measured for RAW-blue macrophages incubated with (A) infection supernatant, (B) OG1RF supernatant, (C) heat or UV treated OG1RF supernatant and (D) size filtered OG1RF supernatant. LDH activity of RAW-Blue macrophages incubated with (E) infection supernatant and (F) OG1RF supernatant. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where * $P < 0.05$ and **** $P < 0.0001$ as compared to media alone (-) controls.

3.2.2 Bacterial factor(s) involved in quorum sensing and virulence are not required for modulation of NF- κ B activity

Since we observed that a secreted factor is involved in *E. faecalis* immunomodulatory activities of macrophages (**Figure 3.1**) and is a dose-dependent effect (**Chapter 2**), we hypothesized that the immunomodulation might be dependent on enterococcal density. Since bacteria density-dependent phenotypes is often controlled by quorum sensing systems, we decided to examine the *fsr* system, a two-component signal transduction system, which is comprised of four genes, *fsrA*, *fsrB*, *fsrC* and *fsrD* (Qin et al., 2001). FsrC histidine kinase senses the accumulation of the biosynthesis-activating pheromone peptide, encoded by *fsrBD*, that accumulates in the extracellular space leading to activation of the FsrA response regulator and downstream gene transcription (Del Papa & Perego, 2011; Qin et al., 2001). Fsr proteins are necessary for the secretion of gelatinase, encoded by *gelE*, which helps in evading the complement system of immune response as well as virulence in rabbit model of endocarditis (Hancock & Perego, 2004; Park et al., 2008; Qin et al., 2000, 2001; Thurlow et al., 2010). In addition to the testing the *fsr* system in immune suppression, we also examined the multiple peptide resistance factor (MprF) which is involved in evading killing by phagocyte-associated antimicrobial peptides in *E. faecalis* and is implicated in virulence factor secretion in *L. monocytogenes* (Bao et al., 2012; Kandaswamy et al., 2013; Zemansky et al., 2009).

To investigate the role of these factor(s) in macrophage immune modulation, we exposed macrophages to *E. faecalis* mutants for each factor simultaneously, together with LPS, using the same experimental setup described **Chapter 2**. We observed a dose dependent suppression of NF- κ B for all mutants tested, similar to that of the parental

OG1RF strain without significantly impacting viability of RAW-Blue macrophages (Figure 3.2). Thus, these results suggest that the *fsr* system, gelatinase and MprF is not required for macrophage suppression capability of *E. faecalis* and that other uncharacterised secreted bacterial factor(s) may be involved in this immunomodulation.

Figure 3.2 *E. faecalis gelE*, *mprF1/2* and *fsrAC* are not involved in NF-κB immune suppression in mouse macrophages.

Mouse RAW-Blue macrophages were infected with live *E. faecalis* mutant strains concurrently with LPS (100 ng/ml) at the specified MOI for 6 hours prior to measurement of (A) NF-κB-driven SEAP reporter activity and (B) LDH activity of RAW-Blue macrophages. $\Delta\Delta mprF1/2$ is an *E. faecalis* strain that is deleted for both paralogues of *mprF*. In NF-κB-driven SEAP reporter assays, exposure to media alone (-) represents background NF-κB reporter activity and stimulation with LPS represents the positive control for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed and error bars represent standard error of the mean (SEM). Statistical analysis was performed using the one-way ANOVA test followed with post-hoc Tukey's multiple comparison test between all conditions where ** $P < 0.01$ and **** $P < 0.0001$ as compared to media alone (-) controls; and where # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ among all of the MOIs as compared to MOI 100.

3.2.3 Acidification is important for *E. faecalis* immune modulation activity

One observation that we made during *E. faecalis* infection of macrophages is that the supernatant was more acidic at high MOIs as compared to the lower MOIs. Metabolic acidosis is commonly seen in critical illness, including liver failure, cancer, seizures and kidney disease and can have profound influence on the host's immune system often leading to suppression of the immune system (Kellum, Song, & Li, 2004b; Lardner, 2001). Furthermore, acidification of the cell culture media of RAW 264.7 cells to a pH lower than pH 7 by hydrochloric acid (HCl) or lactic acid can reduce the LPS-induced NF- κ B DNA binding leading to reduced nitric oxide formation (Douvdevani et al., 1995; Kellum et al., 2004b).

To elucidate the effect of acidification on immune modulation activity, we first acidified the cell culture media using HCl and phosphoric acid (H_3PO_4) to pH 6.4, filter sterilized it, and used it for incubation with macrophages. HCl, a primary digestant and disinfectant, is frequently produced by the stomach while phosphoric acid can be derived from soda intake (Davies, 1951). We observed that acidified cell culture medium alone is able to suppress LPS-induced NF- κ B activation without significantly affecting the viability of RAW-Blue cells (**Figure 3.3A and D**). To further verify that acidification is important, we either neutralised the bacterial-free OG1RF supernatants (collected from *E. faecalis* grown at various MOIs in cell culture medium in the absence of macrophages for 6 hours) using Tris to pH 7.8 (which corresponds to the pH readings observed for MOI 1), or acidified the bacterial-free OG1RF supernatants using HCl to pH 6.4 (which corresponds to the readings observed for MOI 100). We observed that acidified bacteria-free supernatants were able to suppress NF- κ B activation at all MOIs even in the presence of LPS stimulation with no cell cytotoxicity (**Figure 3.3B and E**).

On the other hand, we observed that the neutralised supernatants were no longer able to suppress LPS-induced NF- κ B activation even at high MOI 100 (**Figure 3.3C and F**). Therefore, these results suggest that *E. faecalis* factor(s) that contribute to acidification of the environment might be essential for the immunomodulatory capabilities of *E. faecalis*. Taken together, our results suggest that acidification is important for the immune modulation by *E. faecalis* observed in macrophages.

Figure 3.3 Acidification is important for immunomodulation by *E. faecalis*.

Mouse RAW-Blue macrophages were incubated with acidified cell culture media, or acidified or neutralised bacterial-free supernatant alone, or treated concurrently with LPS (100 ng/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and cytotoxicity (LDH activity). NF-κB-driven SEAP reporter activity were measured for RAW-blue macrophages incubated with (A) acidified media, (B) acidified bacterial supernatant and (C) neutralised bacterial supernatant. LDH activity is measure for RAW-blue macrophages incubated with (D) acidified media, (E) acidified bacterial supernatant and (F) neutralised supernatant. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where ****P<0.0001 as compared to media alone (-) controls.

3.2.4 Lactate alone is sufficient for immune modulation activity

A possible mechanism of cell culture media acidification of by *E. faecalis* is by the production of lactic acid since *E. faecalis* is a lactic acid-producing species. It is important to note that, due to the low pK_a of lactic acid ($pK_a = 3.86$), lactic acid is deprotonated to lactate at all physiological pH. Furthermore, lactic acidosis, is important for suppression of inflammatory responses in *in vitro* and *in vivo* particularly in a tumour environment (Bidani, Wang, Saggi, & Heming, 1998; Damgaci et al., 2018; Dietl et al., 2010; Pilon-Thomas et al., 2016).

We first set out to quantify the level of lactate present in the bacteria-free supernatants using blood gas analysis. We determined that the concentration of lactate in infected cell culture media at MOI 100 is significantly higher as compared to the lower MOIs (**Figure 3.4A**). To elucidate whether lactic acid is sufficient to suppress LPS-induced NF- κ B activation, we incubated RAW-Blue macrophages with cell culture media supplemented with lactic acid at increasing concentrations. We observed that lactic acid concentrations higher than 40 mM suppressed LPS-induced NF- κ B activation (**Figure 3.4B and C**). Therefore, these data demonstrate that lactate alone is sufficient for suppression of LPS-induced macrophage activation.

Figure 3.4 Lactate is sufficient to induce NF-κB suppression of macrophages.

(A) Lactate present in bacterial-free supernatant was quantified via blood gas analysis. Mouse RAW-Blue macrophages were incubated with cell culture media supplemented with lactic acid alone, or treated concurrently with LPS (100 ng/mL) at the specified MOI for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and cytotoxicity (LDH activity). (B) NF-κB-driven SEAP reporter activity and (C) LDH activity of RAW-Blue macrophages incubated with supernatant. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ as compared to media alone (-) controls; and where ### $P < 0.001$ and #### $P < 0.0001$ among all of the MOIs.

3.2.5 Lactate dehydrogenase plays a role in the immune modulation of NF- κ B activity

In *E. faecalis*, LDH is involved in homolactic fermentation, which is important for catalysing the conversion of pyruvate to L-lactate which generates NAD⁺ during glycolysis (Brown & Wittenberger, 1971; Wittenberger & Angelo, 1970). There are two *ldh* genes in *E. faecalis*, *ldh1* and *ldh2*, where *ldh1* plays a major role in lactate production as compared to *ldh2* (Jönsson et al., 2009).

In order to test the role of LDH in the immunomodulation process, we first created deletion mutants by knocking out either copy of *ldh* as well creating a *ldh* double mutant. Growth curves in brain heart infusion (BHI) and cell culture medium were performed to check the mutants for any defects in growth. We observed no significant growth defects for any *ldh* mutant compared to the parental strain OG1RF when grown in BHI or cell culture medium (**Figure 3.5A and B**). In addition, *ldh1* and *ldh1/2* mutants grow much better in BHI media as compared to parental strain plateauing at OD₆₀₀ 0.8 instead of 0.6 during stationary phase (**Figure 3.5A**). Furthermore, when grown in cell culture media, *ldh1* and *ldh1/2* mutants also grow to a higher OD as compared to parental strain (**Figure 3.5B**). Importantly, the growth of all *E. faecalis* strains at 6 hours is similar in the cell culture medium and in BHI media, therefore, there is unlikely any growth defects for *ldh* mutants present. To elucidate the role of LDH during *E. faecalis* infection, we infected RAW-Blue macrophages with either the parental strain or the *ldh* mutants with or without LPS co-stimulation. We observed that Δ *ldh1* and Δ *ldh1* Δ *ldh2* mutants were unable to suppress LPS-induced NF- κ B activation as compared to parental strain OG1RF (**Figure 3.5C and D**). Δ *ldh2* is still able to suppress LPS-induced NF- κ B activation, likely because *ldh2* plays a lesser role in lactate production. In addition, we also used a primary cell line to confirm the immune

suppression phenotypes were not specific to immortalized cell lines. We infected murine bone marrow derived macrophages (BMDMs) with the parental strain or *ldh* mutants at MOI 100 in the presence of LPS for 6 hours and measured the production of pro-inflammatory cytokines IL-6. We observed that BMDMs infected with $\Delta ldh1$ and $\Delta ldh1\Delta ldh2$ mutants secrete significantly higher amounts of IL-6 as compared to the parental strain OG1RF (**Figure 3.5E**). Furthermore, to confirm that *ldh1* alone was necessary for the immunomodulatory capability of *E. faecalis*, we also infected either RAW-Blue macrophages or BMDM with $\Delta ldh1$ mutant with complementation of *ldh1* gene. We observed that *ldh1* complementation restored suppression of NF- κ B activation to levels similar to that of the parental strain OG1RF, both resulting in reduced levels of IL-6 production (**Figure 3.5C and E**). Taken together, this is the first study that demonstrate that LDH activity is necessary for immunomodulation of macrophages by *E. faecalis*.

Figure 3.5 LDH is essential for NF- κ B suppression of macrophages by *E. faecalis*.

Growth curve for *E. faecalis* strains grown overnight in either (A) BHI or (B) cell culture media supplemented with 10% FBS. Mouse RAW-Blue macrophages were infected with *ldh1*, *ldh2*, *ldh1/2* mutants alone, or treated concurrently with LPS (100 ng/mL) at MOI100 for 6 hours prior to measurement of (C) NF- κ B-driven SEAP reporter activity and (D) cytotoxicity (LDH activity). (E) BMDM were infected with *ldh1*, *ldh2* mutants and *ldh1* complemented strains for 6 hours prior to measurement of IL-6 production. NF- κ B-driven SEAP reporter assays and IL-6 ELISA: exposure to media alone (-) represented background NF- κ B reporter activity and stimulation with LPS represented positive controls for respectively marker. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where ** P <0.01, *** P <0.001 and **** P <0.001 among all of the MOIs as compared to LPS (+) control for NF- κ B reporter activity or media alone (-) control for IL-6 production; and where # P <0.05 and ### P <0.001 among all of the MOIs. For IL-6 production, ** P <0.01, *** P <0.001 and **** P <0.001 for different conditions as compared to media (-) control.

3.2.6 Other mutants that affect acidification also affect immunomodulation

Since we have previously demonstrated that acidification of the cell culture media is important for immune suppression of macrophages, we wondered if other bacterial factor(s) that affects acidification of environment can also affect immune suppression of macrophages by *E. faecalis*. To test this, another student in the lab utilized a forward genetics approach to screen for *E. faecalis* mutants that fails to suppress LPS-induced NF- κ B activation using an *E. faecalis* transposon library of ~15,000 mutants (Kristich et al., 2008). I subsequently validated two of the transposon mutants, OG1RF_11160::Tn and OG1RF_11161::Tn which are annotated as a thioesterase and a desert hedgehog (DHH) family protein respectively, that also affect acidification of media and hence fails to suppress NF- κ B activation. OG1RF_11160::Tn and OG1RF_11161::Tn are encoded within the same operon with OG1RF_11160::Tn being directly upstream of OG1RF_11161::Tn. I first verified that the transposon mutants do not have any significant defects in growth kinetics as compared to wildtype in brain-heart infusion (BHI) as well as in cell culture media (**Figure 3.6A**). I observed that both OG1RF_11160::Tn and OG1RF_11161::Tn failed to suppress LPS-induced NF- κ B activation as compared to wildtype OG1RF (**Figure 3.6B and C**). Therefore, this suggests that the thioesterase and DHH family protein play a role in immunomodulatory capability of *E. faecalis* and further supports that the immune suppression ability as a result of acidification of environment by *E. faecalis* is multifactorial.

Figure 3.6 Thioesterase and DHH family proteins plays a role in immunomodulation of macrophages by *E. faecalis*.

(A) Growth curve for *E. faecalis* strains grown overnight in either BHI or cell culture media. Mouse RAW-Blue macrophages were infected with wildtype *E. faecalis*, OG1RF_11160::Tn and OG1RF_11161::Tn mutants alone, or treated concurrently with LPS (100 ng/mL) at MOI 100 for 6 hours prior to measurement of NF-κB-driven SEAP reporter activity and cytotoxicity (LDH activity). (B) NF-κB-driven SEAP reporter activity, (C) LDH activity of RAW-Blue macrophages incubated with supernatant. NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where ** $P < 0.01$ and **** $P < 0.001$ among all of the MOIs as compared to LPS (+) control; and ### $P < 0.001$ among all of the wildtype OG1RF.

3.2.7 Lactate production is reduced in *E. faecalis* mutants that fails to suppress macrophage response

To verify that all of the *E. faecalis* mutants that exhibited reduced acidification as compared to wildtype OG1RF, we performed a time course for changes in pH of the *E. faecalis* mutants when grown in cell culture medium. We observed that all *E. faecalis* mutants acidified the medium less than wildtype OG1RF at 6 hours (**Figure 3.7A**). At 24 hours, only $\Delta ldh1\Delta ldh2$ mutant displayed a significantly lower pH of 5.5 as compared to wildtype and transposon mutants that reaches pH 4.5. Next, since we saw a difference in pH level at 6 hours, we hypothesised that the lack of acidification was due to reduced lactate production. Consistent with our hypothesis, we observed a significant reduction in lactate in the $\Delta ldh1$ and $\Delta ldh1\Delta ldh2$ mutants relative to wildtype (**Figure 3.7B**). On the other hand, lactate production was not affected for $\Delta ldh2$ and was comparable to that of wildtype OG1RF. Although LDH1 plays a major role in lactate production, we still observed approximately 15% of the lactate being produced by parental strain which further decreased to 4% in $\Delta ldh1\Delta ldh2$ as compared to wildtype. This suggests that *ldh2* is not redundant for lactate production and may only play a more significant role in lactate production in the absence of *ldh1*. Finally, we observed a significant reduction of lactate production for transposon mutants as compared to wildtype OG1RF (**Figure 3.7B**). Therefore, our collective results suggest that lactate secretion plays a role in NF- κ B suppression in macrophages.

Figure 3.7 The ability to produce lactate contributes to macrophage suppression by *E. faecalis*.

(A) pH for *E. faecalis* strains grown in cell culture media measured over a time course. Statistical analysis was performed using unpaired T-test for difference in pH level between $\Delta ldh1\Delta ldh2$ as compared to wildtype OG1RF at 24h. (B) Lactate concentration was measured using 6 hpi bacterial free supernatant. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where **** $P < 0.0001$ among all of the different *E. faecalis* strains as compared to wildtype OG1RF.

3.2.8 $\Delta ldh1\Delta ldh2$, OG1RF_11160::Tn and OG1RF_11161::Tn mutants are less able to survive extracellularly

Release of cytokines, inducible NO-synthase-mediated NO production and killing of pathogens are all responses that arise upon NF- κ B activation (Hayden et al., 2006). We speculated that since $\Delta ldh1\Delta ldh2$, Tn::OG1RF_11160 and Tn::OG1RF_11161 mutants are

unable to suppress NF- κ B activation, the mutants may also survive less well in macrophages. To test this, we infected 10^6 RAW-Blue macrophages with various *E. faecalis* strains at MOI 100 for 6 hours, collected the supernatant first for enumeration of extracellular *E. faecalis* before killing the extracellular *E. faecalis* using gentamicin and penicillin to enumerate the intracellular population. After gentamicin and penicillin treatment, the supernatant was first collected and plated to ensure that all extracellular bacteria were dead (data not shown). Surprisingly, we only observed a significant reduction in extracellular survival of *E. faecalis* mutants but not the intracellular survival of *E. faecalis* within the macrophages (**Figure 3.8**). Thus, this suggests that ability of *E. faecalis* to modulate NF- κ B activation in macrophages may be important for extracellular survival.

Figure 3.8 *E. faecalis* mutants have reduced extracellular survival capability during infection in macrophages.

Mouse RAW-Blue macrophages were infected with wildtype *E. faecalis*, *ldh1/2* mutant, Tn::OG1RF_11160 and Tn::OG1RF_11161 transposon mutants alone at MOI100 for 6 hours prior to measurement before CFU was enumerated. (A) Intracellular CFU and (B) extracellular CFU was enumerated after 6 hpi with *E. faecalis* strains. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where **** $P < 0.001$ among all of the different *E. faecalis* strains as compared to wildtype OG1RF.

3.2.9 *ldh* double mutant is less able to promote *E. coli* survival during mixed species wound infection

E. faecalis can be commonly isolated from wound infections together with *E. coli*, *P. aeruginosa* and *S. aureus* (Citron et al., 2007; Dowd et al., 2008; Gjodsbol et al., 2006). In addition, our lab has previously shown that, in mouse wound infection model, co-infection with *E. faecalis* promotes *E. coli* virulence and colonisation (Keogh et al., 2016). We hypothesized that this could be, at least in part, due to the ability of *E. faecalis* to suppress the immune response and thus enable *E. coli* to grow better. Therefore, we tested whether the $\Delta ldh1\Delta ldh2$ mutant would fail to enable better *E. coli* growth since $\Delta ldh1\Delta ldh2$ mutant fails to suppress macrophage activation. To elucidate the effect of *ldh1/2* deletion during polymicrobial infection, we used a modified mouse wound excision model established by our lab (Chong et al., 2017; Keogh et al., 2016). We observed similar *E. faecalis* titres in both single and mixed infection regardless of the presence or absence of *E. coli* UTI89 (**Figure 3.9A**). In addition, we also saw an augmentation of *E. coli* growth during polymicrobial infection with wildtype *E. faecalis* OG1RF (**Figure 3.9B**). However, this augmentation of *E. coli* growth in polymicrobial infection was abolished when $\Delta ldh1\Delta ldh2$ mutant was used (**Figure 3.9B**). Therefore, these data suggest that LDH-mediated immune suppression by *E. faecalis* creates an environment that favours *E. coli* growth during infection.

Figure 3.9 *E. faecalis* $ldh1/2$ mutant are less able to augment *E. coli* growth during polymicrobial wound infection.

Using a wound model, female C57BL/6NTac mice were infected with 10² CFU/wound *E. coli* UTI89 or 10⁶ CFU each of *E. faecalis* OG1RF or $ldh1/2$ mutant in single-species control or mixed species infections. 24 hpi bacteria enumeration from both single control and mixed-species infection for (A) *E. faecalis* and (B) *E. coli*. Data were combined from 2 independent experiments; individual values were graphed, and error bars represent standard deviation (SD). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where * P < 0.05 and ** P < 0.01 among the different conditions.

3.3 Discussion

E. faecalis is among the top five most commonly isolated microbial species responsible for all hospital acquired infections including CAUTI, central line-associated bloodstream infection and surgical site infection (Hidron et al., 2008; Weiner et al., 2016). As proposed by the Red Queen hypothesis, bacteria and the host immune system are in a constant evolutionary arms race for the survival of their species (Johannessen et al., 2013; Muraille, 2013; Reddick & Alto, 2014). Hence, bacteria have evolved many different strategies in order to evade or subvert the immune response and successfully establish an infection despite the myriad immune responses to protect the host against invading pathogens (Johannessen et al., 2013; Reddick & Alto, 2014). Various studies suggested an immunomodulatory potential of *E. faecalis* due to their ability to survive for prolonged period within immune cells such as macrophages (Gentry-Weeks et al., 1999; Sabatino et al., 2015; Wang, Hibberd, et al., 2014; Wang et al., 2008; Zou et al., 2014; Zou & Shankar, 2014; Zou & Shankar, 2016). Although TcpF and ElrA are virulence factors implicated in immune suppression and anti-phagocytic activity within macrophages, these genes are absent in the *E. faecalis* strain OG1RF used in this study. Furthermore, our lab has shown that 16 out of 49 clinical wound isolates were able to suppress NF- κ B activity in macrophages while 9 strains were hyperinflammatory (Kelvin Chong, personal communication). Importantly, in that analysis, there was no correlation between immune modulatory phenotypes with presence or absence of known *E. faecalis* virulence genes. Thus, these results indicate the importance of understanding *E. faecalis* factor(s) which are involved in macrophage suppression phenotype as seen in **Chapter 2**. Hence, in this chapter, we aimed to elucidate virulence factor(s) that are important for macrophage suppression.

Consistent with our results in **Chapter 2**, we have shown that a secreted factor is involved in mediating high level NF- κ B suppression in macrophages. Therefore, we first examined *E. faecalis* factors known to be involved in immune evasion and quorum sensing system that regulates virulence gene expression at high bacterial densities, since immune suppression can only be observed at high dosage infection (Bao et al., 2012; Kraemer et al., 2014; Park et al., 2008; Sussmuth et al., 2000). *fsr* quorum sensing system is important for regulation of known secreted factors such as gelatinase and SprE (Hancock & Perego, 2004; Qin et al., 2000, 2001). Our results suggest that each of these known *E. faecalis* secreted factors, such as gelatinase which is positively regulated by *fsr* quorum sensing system, may not be involved in macrophage suppression during *E. faecalis* infection. This would suggest that although gelatinase plays a role in virulence and colonization *in vivo* in a mice model of endocarditis through possible immunomodulation effects, they are not linked to the ability of *E. faecalis* to modulate NF- κ B responses in macrophages (Bao et al., 2012; Park et al., 2007; Park et al., 2008; Thurlow et al., 2010). *E. faecalis* possess several secretion machineries to transport proteins involved in virulence across the cell membrane, where the secretion (Sec) system, which involves both sortases and SecA, is the most well characterised (Kline et al., 2009; Mitra, Afonina, & Kline, 2016). In addition, the genome also contains genes for type IV secretion system, which is involved in horizontal plasmid transfer during conjugation, and type VII secretion system (Das, Ghosh, & Mande, 2016; Goessweiner-Mohr, Arends, Keller, & Grohmann, 2013). Furthermore, the secreted immunomodulatory *E. faecalis* factor(s) is heat and UV stable and is of low molecular weight of lesser than 10 kDa. Therefore, these results suggest that the secreted *E. faecalis* factor(s) is unlikely to be protein in nature unless it is a heat stable protein. However, since the bacterial factor is lesser than 10 kDa whereas most *E. faecalis* heat-shock protein is at least 60 kDa or heavier (Boutibonnes et al., 1993), this further indicates that the secreted bacterial factor is not protein in nature. Furthermore, metabolic by

products may also be released into the extracellular milieu. Importantly, since suppression of LPS-induced NF- κ B activation can only be observed at MOI 100, this suggests that the secreted factor(s) needs to accumulate in the supernatant to a certain concentration. This would further support our observation that *E. faecalis* suppression of macrophage response is dose-dependent since sufficient accumulation of immunomodulatory bacterial factor(s) is essential and thus inhibitory activity is only notable at high MOI. Finally, since we have shown that acidification of the bacterial-free supernatant is important, and studies have shown that metabolic by products secreted by *E. faecalis* can acidify the environment, we postulated that metabolic products present in the bacteria supernatant were important for immunomodulatory capability of *E. faecalis*.

Of the metabolites known to be secreted by *E. faecalis*, acidification of environment is predominantly due to lactate production in pyruvate dissimilation during the final stage of sugar metabolism (Mehmeti et al., 2012; Ramsey et al., 2014; Snoep et al., 1991). Hence, we hypothesized that lactate secreted by *E. faecalis* plays an important role in the immunomodulatory capabilities of *E. faecalis*. If *E. faecalis* produced lactic acid is indeed important, this would support our previous observation that *E. faecalis* suppression of macrophage response is dose-dependent since there will only be more lactate accumulation at MOI 100. We first showed that lactate is sufficient for suppression of NF- κ B activation. This is consistent with reports showing that lactic acid, and not HCl which increase NF- κ B DNA binding, is able to significantly inhibit LPS-induced NF- κ B activation at pH 7 in RAW 264.7 macrophages (Kellum, Song, & Li, 2004a). In addition, extracellular pH of 7, achieved by adding HCl, inhibits pro-inflammatory cytokine secretion by J774 macrophages by increasing the binding of anti-inflammatory p50-p50 homodimer (Gerry & Leake, 2014). Hence, these suggests that extracellular pH is likely to have marked effects on NF- κ B activation.

Furthermore, lactate has been shown to play an essential role in tumour microenvironments resulting in tissue acidosis and often leading to suppression of NF- κ B activity and subsequent inhibition of immune responses (Colegio et al., 2014; Dietl et al., 2010; Husain, Huang, Seth, & Sukhatme, 2013; Morrot et al., 2018; Mu et al., 2018; Romero-Garcia, Moreno-Altamirano, Prado-Garcia, & Sánchez-García, 2016). Importantly, lactate acidosis is associated with higher mortality rates, at 56% of all patients with metabolic acidosis, as compared to those with no metabolic acidosis in intensive care unit (ICU) patients including patients with hepatic failure, heart failure, cancer and general surgery (Gunnerson, Saul, He, & Kellum, 2006). Interestingly, in wounds, lactate can be considered a double-edged sword where low lactate concentrations can help in promoting angiogenesis and accelerates superficial wound healing due to superoxide radical generation (Ghani et al., 2004; Porporato et al., 2012). By contrast, especially in chronic or recalcitrant wounds, elevated lactate concentration can exacerbate wounds by increasing susceptibility to bacterial infection and impairing fibroblasts growth at above 7 mM, whereas lactate concentration is around 1 to 3 mM in normal tissue (Hunt, Hopf, & Hussain, 2000; Loffler et al., 2011). Taken together, consistent with our results, lactate plays an imperative role in immune suppression of macrophages instead of just being a metabolic end-product produced by *E. faecalis*. Future investigation regarding the lactate concentration in mice wound exudate, in serum or site of infection is required in order to better understand the role of lactate during *E. faecalis* infection.

Using genetic manipulation of *E. faecalis*, we also showed that LDH, the enzyme responsible for lactate production, is necessary to suppress LPS-induced NF- κ B activation and promotes the ability of *E. coli* to cause infection in wounds when both species are present. Indeed, LDH has been shown to be essential for stress resistance by helping in maintaining the redox balance of *E. faecalis* (Rana et al., 2013). As a result, LDH deficient *E. faecalis* was less

able to colonize host organs in systemic infection murine model as compared to wildtype (Rana et al., 2013). Similarly, LDH is also important for virulence of *S. aureus* as *ldh* mutants are less virulent, with the double *ldh* knockout mutant being virtually avirulent, in a murine sepsis model (Richardson, Libby, & Fang, 2008). Lactate production, catalysed by NO-inducible L-lactate dehydrogenase, is important for redox homeostasis of *S. aureus* to resist antimicrobial radical nitric oxide produced by the host and hence, tightly linked to its virulence (Richardson et al., 2008). Furthermore, other lactic acid bacteria (LAB) such as *Lactobacillus* have been shown to suppress LPS-induced NF- κ B activation leading to decrease secretion of pro-inflammatory cytokines in human myometrial cells as well as suppressed polyethylene-induced NF- κ B in RAW 246.7 macrophages (Esvaran & Conway, 2018; Kim, Shynlova, & Lye, 2019). This suggests that lactate-induced suppression may be a widespread immunomodulatory phenomenon used by bacteria, particularly LAB, but with the caveat that fermentation needs to be occurring *in vivo*. Interestingly, besides LDH, other metabolic enzyme also affects *E. faecalis* pathogenicity. Using *Galleria mellonella* larvae infection model, citrate fermenting deficient *E. faecalis* was less virulent and did not result in mortality as compared to wildtype strain (Martino, Perez, Magni, & Blancato, 2018). Furthermore, citrate fermentation promotes *E. faecalis* growth in blood, urine and *G. mellonella* suggesting that citrate fermentation plays an important role in *E. faecalis* pathogenicity (Martino et al., 2018). In addition to the potential role of *E. faecalis* metabolites in virulence, costless metabolite exchange can also occur during cross-feeding interactions between different species of bacteria within the environment to promote synergistic interspecies interaction (D'Souza et al., 2018; Pacheco, Moel, & Segrè, 2019). For example, L-ornithine produced by *E. faecalis* cues *E. coli* upregulation of genes involved in biosynthesis of the enterobactin siderophore leading to increased biofilm growth both *in vitro* and *in vivo* during polymicrobial infections in iron limiting conditions. Therefore, future experiments are required to understand the possible benefit or negative impact of lactate

on other bacteria species during polymicrobial infection. Overall, our results suggest that, in addition to being an essential enzyme during sugar metabolism, LDH can also play a role in suppressing the immune system which could potentially impact the fitness of *E. faecalis* during infections. Moreover, this is the first report that decreased virulence in LDH-deficient *E. faecalis* strain is due to lack of ability to suppress the immune system.

Next, since we know that acidification plays an important role in immunomodulation of macrophages, we further validated two transposon mutants, annotated as thioesterase and a DHH family protein, that were identified by a fellow lab member during a library screen as mutants that both failed to acidify the growth media medium and failed to suppress NF- κ B activity. Thioesterase plays an important role in the synthesis of fatty acids by hydrolysing thioester bonds between a fatty acyl moiety and fatty acyl acyl carrier proteins (ACPs) (Cantu, Chen, & Reilly, 2010). Thioesterase has been implicated in maintenance of bacterial physiology such as cell envelope synthesis and remodelling and may play a role in pathogenicity in *Mycoplasma tuberculosis* and *E. faecalis* (Santucci et al., 2018; Zhu, Zou, Cao, & Cronan, 2019). Considering that the DHH family protein lies immediately downstream of thioesterase within the same operon and that that transposon insertion in thioesterase might confer polar effects on the transcription of the DHH family protein a, it is plausible that DHH family might play a more important role in regulation of NF- κ B. Genetic complementation studies that are underway will address this possibility in a definitive way. DHH family proteins can function as phosphodiesterases to cleave bis-(3'-5')-cyclic dimeric adenosine monophosphate (c-di-AMP) into phosphoadenylyl adenosine (pApA) and subsequently adenosine monophosphate (AMP). In *S. pneumoniae*, phosphodiesterase, a member of the DHH family proteins, plays a role in cell wall integrity maintenance by regulating lipid homeostasis, controlling growth and cell size, biofilm formation, drug resistance as well as

virulence in mouse pneumonia model (Bai et al., 2013; Banerjee et al., 2010; Corrigan et al., 2011; Corrigan et al., 2013; Griffiths & O'Neill, 2012; Holland et al., 2008; Kuipers et al., 2016). A plausible reason for why we see higher NF- κ B activity during macrophage infection with DHH transposon mutant would be that c-di-AMP, a putative secondary signalling molecule produced exclusively by bacteria, is known to trigger innate immunity via stimulator of interferon genes (STING) or DDX41 recognition leading to NF- κ B activation and type 1 IFN response (Barker et al., 2013; Bowie, 2012; Burdette et al., 2011; Jin et al., 2011; Parvatiyar et al., 2012; Sauer et al., 2011; Woodward, Iavarone, & Portnoy, 2010). A transposon insertion in DHH family protein would result in the accumulation of c-di-AMP as no functional phosphodiesterase is present. The accumulation of c-di-AMP, recognised by STING, then results in downstream NF- κ B activation and triggers the inflammatory response by macrophages (Barker et al., 2013; Bowie, 2012; Burdette et al., 2011; Woodward et al., 2010). Therefore, the ability of the DHH family protein transposon mutant to trigger NF- κ B activation may outweigh the suppression of NF- κ B activity. In addition, both transposon mutants also showed reduced lactate production as compared to wildtype strain. Hence, the reduction in lactate production coupled with increased c-di-AMP production results in greater NF- κ B activation even in the absence of TLR-agonist LPS.

We observed that lactate production for $\Delta ldh2$ is not reduced which is consistent with literature for *E. faecalis* strain V583, which reported that LDH2 plays a minor role in lactate production relative to LDH1 (Jönsson et al., 2009; Rana et al., 2013). Lactate production was reduced but still observed in the *ldh1* mutant which is further decreased in the double *ldh1/2* deletion mutant, further supporting that although LDH2 plays a minor role in lactate production, it is not silent as has been reported for *L. lactis* and in *E. faecalis* strain V583 (Gaspar et al., 2007; Jönsson et al., 2009). Since NAD⁺ regeneration via lactate production in *ldh1* mutants is

absent, NAD⁺ is regenerated via mixed acid fermentation which leads to production of end products such as formate, ethanol and acetoin which help in maintaining intracellular pH (Hugenholtz, Perdon, & Abee, 1993; Jönsson et al., 2009; Mehmeti et al., 2012; Tsau, Guffanti, & Montville, 1992). Metabolic products of mixed acid fermentation may also result in acidification of environment, although not to the magnitude of lactate, which could explain why we still observed some form of acidification in the *ldh1* mutants. The residual lactate production observed in the *ldh1/2* mutant may be due to lactate produced in other metabolic pathways (Ferain, Schanck, & Delcour, 1996; Leclercq & Courvalin, 1997). Regardless, we have shown that the *ldh1* mutant indeed exhibits reduced lactate production and this decrease in lactate leads to failure in suppressing LPS-induced NF-κB.

Finally, to validate our results in an *in vivo* setting, we used a polymicrobial wound infection model previously established by our lab (Keogh et al., 2016). We hypothesized that since *ldh* mutants are less immune suppressive, they may be less able to promote *E. coli* colonization during mixed species wound infection. We observed that during co-infection of *E. coli* and *ldh1/2* mutant, there was an absence of augmentation compared to that observed during *E. faecalis* wildtype co-infection. This result suggests that since the *ldh1/2* mutant is less able to suppress macrophage response, that the inflammatory response may be increased and thus promoting *E. coli* clearance. Alternatively, since it was proposed that lactate production by *E. faecalis* promotes enterobactin production of *E. coli* may promote growth of *E. coli*, *ldh1/2* mutant produce minimal lactate and hence may be less able to promote *E. coli* growth (Keogh et al., 2016; Miethke & Marahiel, 2007). Further investigation is necessary to understand whether the lack of *E. coli* augmentation is due to immune suppression or lack of lactate production in polymicrobial infection with *ldh1/2* mutants. Interestingly, we do not see a decrease in colonization of *ldh1/2* mutant during single species wound infection as compared

to wildtype. There are two reasons, which are not mutually exclusive, which could explain the lack of decreased *E. faecalis* colonization. Firstly, our *in vitro* setting only includes macrophages whereas the wound environment is more complex and involves not just immune cells, including macrophages, neutrophils and dendritic cells, but also keratinocytes. Therefore, other bacterial factor(s) may still interact with host cells to promote colonization of the *ldh* mutant. Alternatively, since *E. faecalis* can prevent phagolysosome formation and subsequent autophagy within host cells, they may evade the immune response by hiding within host cells such as in keratinocytes (Zou & Shankar, 2016). Another probable reason could be that the NF- κ B suppression exhibited by *E. faecalis* is multifactorial. Therefore, by knocking out only *ldh*, other bacterial factor(s) are still able to suppress NF- κ B activation. In addition, we have shown that lack of NF- κ B suppression as compared to wildtype strain can only be observed during co-infection with the TLR agonist LPS. Hence, since the TLR agonist which induces inflammation is not added during *in vivo* setting, the difference in virulence between wildtype and *ldh* mutant may not be obvious during single species *E. faecalis* infection. Finally, since lactate has been implicated in wound healing, it would be interesting to investigate whether there is a difference in wound healing between wildtype *E. faecalis* and *ldh1/2* mutant. Nonetheless, we have shown that our *in vitro* assays are parallel to *in vivo* infection setting in terms of the testing for mutants involved in immune suppression and can therefore serve as a model for further verification of *E. faecalis* bacterial factor(s) involved in immune suppression.

In summary, our work suggests secreted *E. faecalis* factors or metabolites that contributes to acidification of the local environment play an essential role in immunomodulatory capabilities of *E. faecalis*. Furthermore, we have shown that LDH plays an essential role in modulating the macrophage response due to the decreased in lactate production. Moreover, using transposon mutants, we demonstrated that thioesterase and DHH

family protein can help to regulate macrophage suppression. Finally, we confirmed a role for LDH in *E. faecalis* virulence in an *in vivo* setting where *ldh1/2* mutant fails to augment *E. coli* virulence and the *in vivo* model can be used future validation of *E. faecalis* mutants that fail to suppress immune response. Given that *E. faecalis* infections are common and often difficult to eradicate due to antibiotic tolerance and resistant, understanding the contribution of bacterial factors to *E. faecalis* pathogenicity is crucial and may provide an alternative immunomodulatory approach for eliminating these recalcitrant infections. Moving forward in **Chapter 4**, we will elucidate the immune pathways in macrophages by which *E. faecalis* can suppress as well as how LDH might affect the immunosuppression of macrophages.

3.4 Materials and methods

3.4.1 Bacterial growth conditions and strains

Bacteria strains that were used in this study are as shown in **Table 3.** *E. faecalis* strains were grown in Brain Heart Infusion (BHI) (Becton, Dickinson, USA) broth or agar at 37°C overnight. *E. coli* strains were grown statically at 37°C overnight in Luria Bertani (LB) (Difco, France). Both *E. faecalis* and *E. coli* strains were streaked from 25% glycerol stock and incubated on the respective agar plates overnight at 37°C. The antibiotics rifampicin and fusidic acid at were supplemented at a concentration of 25 mg/L to select for *E. faecalis* OG1RF as and when necessary. Single isolated colonies of each strains were inoculated into liquid culture and grown overnight statically at 37°C for 18-20 hours for all experiments. Overnight bacteria culture of *E. faecalis* were centrifuged at 6,000 *g* for 5 minutes and washed once with 1X PBS. Normalization to an optical density (OD) of 0.7 was done for *E. faecalis* (equivalent to 2×10^8 CFU/mL) and 0.4 (equivalent to 2×10^8 CFU/mL) for *E. coli*.

Table 3.1| Bacterial Strains used in this study

Strains	Relevant characteristic(s) ^b	References or source
Strains		
<i>Enterococcus faecalis</i>		
OG1RF	Fus ^r , Rif ^r , wild-type strain	(Dunny et al., 1978)
$\Delta gelE$	<i>gelE</i> knockout strain	Lab strain
$\Delta\Delta mprF1/2$	<i>mprF1</i> and <i>mprF2</i> knockout strain	(Kandaswamy et al., 2013)
Tn:: <i>fsrA</i>	Transposon insertion in <i>fsrA</i>	(Kristich et al., 2008)
Tn:: <i>fsrC</i>	Transposon insertion in <i>fsrC</i>	(Kristich et al., 2008)
$\Delta ldh1$	<i>ldh1</i> knockout strain	Lab strain
$\Delta ldh2$	<i>ldh2</i> knockout strain	Lab strain
$\Delta ldh1\Delta ldh2$	<i>ldh1</i> and <i>ldh2</i> double knockout strain	Lab strain
$\Delta ldh1_pGCP123ldh1$	<i>ldh1</i> knockout strain complemented back with <i>ldh1</i> . Plasmid maintained by kanamycin selection (50 µg/mL).	Lab strain
Tn::OG1RF_11160	Transposon insertion in OG1RF_11160. Annotated as thioesterase.	(Kristich et al., 2008)
Tn::OG1RF_11161	Transposon insertion in OG1RF_11161. Annotated as DHH family protein.	(Kristich et al., 2008)
<i>Escherichia. coli</i>		
UTI89	Uropathogenic strain (UPEC)	(Chen, Hung, et al., 2006; Mulvey et al., 2001)

3.4.2 Genetic manipulations

To create deletion mutants, we used a thermosensitive shuttle plasmid pCGP213 which was isolated using the Monarch® Plasmid Miniprep Kit (New England BioLabs Inc., USA) following the manufacturer's instruction (Paulsen et al., 2003). OG1RF genomic DNA is isolated using Wizard® Genomic DNA Purification Kit (Promega, USA) and Phusion DNA polymerase (Thermo Fisher Scientific, Singapore) was used to amplify approximately 1 kb upstream and downstream of the gene of interest in accordance to manufacturer's protocol. Primers used in the study are listed in **Table 3.** Subsequently, the amplified products were sewn together using the primers dLDH1_For, dLDH1_Rev, dLDH2_For and dLDH2_Rev. *ldh1* and *ldh2* constructs were then subcloned individually into pGCP213 using In-Fusion® HD Cloning Kit using (Clontech, Takara, Japan) following manufacturer's instruction. Deletion constructs were then transformed into *E. coli* and correct transformants were selected at 37°C under erythromycin (Sigma-Aldrich, St Louis, MO) pressure 500 µg/mL (MP, France). The amplified plasmids were purified from *E. coli* using the Monarch® Plasmid Miniprep Kit. The plasmids were then transformed into OG1RF using electroporation, and the correct transformants were selected at 30°C under erythromycin pressure 25 µg/mL. Chromosomal integrations were selected by passaging 37°C in the absence of erythromycin. The loss of *ldh1* or *ldh2* is then verified by PCR using primer pairs LDH1_F, LDH1_R, LDH2_F and LDH2_R. $\Delta ldh1\Delta ldh2$ was created by subcloning the pGCP213-*ldh1* deletion construct into $\Delta ldh2$. All deletion constructs were subsequently confirmed using whole genomic sequence to ensure that only the gene of interest is deleted in the absence of any spontaneous mutation.

Gene	Oligo Name	Sequence 5' to 3'
<i>ldh1</i>	dLDH1_For	CATGCTCGAGCGGCCATTATATGTTGGCACATCCAGG
	dLDH1_Rev	CAGTGTGCTGGAATTAATTTAACGCACTCACTCGAT
	dLDH1_SewF	AAGCGGAGTAACAATAGTGGGTAGCTCTTACATGTGCG TAAT
	dLDH1_SewR	ATTACGCACATGTAAGAGCTACCCACTATTGTTACTCCG CTT
	LDH1_F	GAAGAATAAATTAACCATTGCTTCTTCATC
	LDH1_R	CTTATCTCACGCATTAGCATTTAC
<i>ldh2</i>	dLDH2_For	CATGCTCGAGCGGCCGTCGTTTACTCTGTCCATCATTT
	dLDH2_Rev	CAGTGTGCTGGAATTTTTTCATTGTCCAGACCGAT
	dLDH2_SewF	ATAATCGCGTTGTGTACTTCACACGAACTTTTATAGGTGT GGTT
	dLDH2_SewR	AACCACACCTAAAAAGTTCGTGTGAAGTACACAACGCG ATTAT
	LDH2_F	GAGGCAATTACTAAAATTCCATCAAAAC
	LDH2_R	CAATTGACTTATTAGATGGTGTGTCTT

Table 3.2| Primers for *ldh* deletion construction

3.4.3 Growth kinetics and pH curve

Bacterial cultures were grown as described above and overnight cultures were normalized to obtain a final concentration of 10^3 cells/mL before inoculating at 1:25 ratio into 200 μ L of either BHI or DMEM supplemented with 10% FBS. Subsequently, growth rates were measured by recording OD 600 over 20 hours at 37°C using a microplate reader (Tecan infinite M200 Pro, Switzerland).

For pH measurements, bacterial cultures were grown and normalized to 3.4×10^7 cells/mL (assuming MOI 100 for 10^6 macrophages) in DMEM supplemented with 10% FBS.

The pH was then measured at 1 hour interval for up to 6 hours, and subsequently measured at 8 hours and 24 hours using a digital pH meter (Sartorius, Germany).

3.4.4 Cell culture

RAW-Blue cells derived from mouse RAW 264.7 macrophages (Invivogen™, USA) were used in this study. They contain a plasmid encoding secreted embryonic alkaline phosphatase (SEAP) reporter that is under the transcriptional control of an activator protein 1 (AP-1)/NF- κ B inducible promoter. RAW-blue cells were cultured in T75/T175 flask as adherent monolayers (Nunc, Thermo scientific, Singapore) in DMEM with 10% heat inactivated FBS supplemented with 200 μ g/mL Zeocin™ (Gibco, Thermo Fisher Scientific, Singapore), when necessary, at 37°C with 5% CO₂.

3.4.5 Bone marrow derived macrophages isolation and generation

BMDMs were harvested according to previously published protocol (Weischenfeldt & Porse, 2008). Briefly, femurs were harvested from 6 to 10 weeks old C57BL/6J mice (InVivos Pte. Ltd., Lim Chu Kang, Singapore). Following euthanasia, the legs were first sprayed with 70% ethanol before dissection and removing the skin and muscles. The bone was then placed in 70% ethanol and transferred into a sterile flow hood. Subsequently, the bone was cut using scissor and bone marrow was flushed out with cell culture media (containing DMEM with 10% FBS and supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco, Thermo Fisher Scientific, Singapore)) using a 26G needle into a sterile falcon tube (Becton, Dickinson and Company, Franklin Lakes, NJ). Subsequently, cell suspension was centrifuged at 500 G for 5 mins and the supernatant was removed. The cell pellet was then resuspended in 1X RBC Lysis Buffer (eBioscience™, Thermo Fisher Scientific, Singapore) for lysis of erythrocytes. After 5 mins, PBS was added to inactivate the lysis buffer before centrifuging the cell suspension. Supernatant was discarded, and cell pellet was resuspended in cell culture media

supplemented with 100 ng/mL macrophage colony-stimulating factor (M-CSF) (Biolegend, San Diego, USA). Cells were adjusted to 2×10^6 cells/mL and seeded into T₁₇₅ flask (Nunc, Thermo scientific, China) and incubated at 37°C with 5% CO₂. After 2-3 days, cells were washed, and fresh cell culture media supplemented with 100 ng/mL M-CSF was added. Macrophages are fully differentiated after 6 to 7 days and ready for experimental use.

3.4.6 RAW-Blue macrophage and BMDM infection

RAW-Blue cells and BMDM were seeded into 96 well, 24 well, 12 well and 6 well microtiter plates (Nunc, Thermo Fisher Scientific, Singapore) with cell density of 1×10^5 , 2×10^5 , 5×10^5 or 1×10^6 cells/well respectively in antibiotic free media (DMEM with 10% heat inactivated FBS) and incubated overnight at 37°C with 5% CO₂ when necessary. Following overnight incubation, the cells were washed once with PBS and fresh media was added. The SEAP reporter assay was established by empirically defining the minimal agonist (lipopolysaccharide (LPS)) concentration that induced the maximum SEAP activity in the absence of cell death. Cells were stimulated using LPS purified from *E. coli* O111:B4 (Sigma-Aldrich, St Louis, MO) (100 ng/ml) as positive control, or media alone as a negative control. RAW-Blue cells were infected with *E. faecalis* (at MOI of 100:1, 50:1, 25:1, 10:1 and 1:1) for 6 hours with or without TLR agonists.

3.4.7 Collection of bacteria cell-free culture supernatants

Bacteria were grown in cell culture media for 6 hours, and bacteria-free culture supernatants were collected after centrifugation (6,000 g) followed by filtration (using a 0.2 µm syringe filter). Alternatively, supernatants were collected after infecting macrophages with bacteria at various MOIs and then filtered by using a 0.2 µm syringe filter. Sterility of bacteria-free culture supernatants were verified by the absence of viable bacteria when plated on BHI agar.

3.4.8 NF-κB reporter assay

RAW-Blue cells secrete SEAP into the supernatant upon NF-κB activation which serve as an indication of NF-κB activation upon quantification. QUANTI-Blue™ (Invivogen™, USA), a colorimetric enzyme medium, indicates the presence of SEAP through changing of medium colour from pink to purple-blue. Post-infection, 20 µL of supernatant was collected and added to 180 µL of QUANTI-Blue reagent and incubated at 37°C overnight. SEAP levels were quantified at 640 nm using a microplate reader (Tecan infinite M200 Pro, Switzerland). LPS-stimulation serves as positive control where NF-κB activity is considered high where media control (-) is considered as low NF-κB activity. SEAP readings in between that of LPS and media control is considered as medium activity. All experiments were performed in triplicates.

3.4.9 Cell viability assay

Simultaneously with supernatant collection for SEAP determination, culture supernatants were collected from each well to measure lactate dehydrogenase (LDH) release, using an LDH cytotoxicity assay (Clontech, Takara, Japan) according to manufacturer's instructions. Background LDH activity was determined using mock (PBS) treated RAW-Blue cells. Maximal LDH activity was determined by lysing cells with 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO). Each condition was carried out in triplicate. Percentage cytotoxicity was calculated as follows: $(\text{sample absorbance} - \text{background absorbance}) / (\text{maximal absorbance} - \text{background absorbance}) \times 100$.

3.4.10 IL-6 quantification

Supernatants were collected from RAW-Blue cells 6 hours post-infection and stored at -80°C until assessment by the ELISA MAX™ Deluxe Set Mouse IL-6 assay kit (Biolegend,

San Diego, USA), according to manufacturer's recommendations. All samples were assessed using the same kit lot and at the same time to avoid inter-assay variability.

3.4.11 Lactate concentration measurement

Bacteria were grown in DMEM supplemented with 10% FBS for 6 hours in the absence of macrophages before collecting the supernatant and storing it at -80°C until assessment by the L-Lactate Assay Kit (Abcam, Cambridge, UK), according to manufacturer's recommendations. All samples were assessed using the same kit lot and at the same time to avoid inter-assay variability. Alternatively, blood gas analysis using abl90 flex series analyser (Radiometer Medical, Singapore) to quantify lactate concentration.

3.4.12 Mouse model of polymicrobial wound infection

Bacterial cultures, prepared as described above, were used to infect wounds in 7 to 8 weeks old, 20 to 23 g female C57BL/6J mice (InVivos Pte. Ltd., Lim Chu Kang, Singapore). Wounds were prepared as previously described (Keogh et al., 2016). Briefly, mice were anesthetized with isoflurane (3%) and the dorsal hair was trimmed. After trimming, remaining fine hairs were removed by first applying Nair™ Cream (Church and Dwight Co, Charles Ewing Boulevard, USA) and shaving using a scalpel. Next, the skin was first disinfected using 70% ethanol and a 6 mm biopsy punch (Integra Miltex, New York, USA) was used to create a full-thickness wound and inoculated with bacteria subsequently. Inoculum volumes of 10 µl, containing a bacterial suspension of either single or polymicrobial species prepared in PBS: (i) 10² CFU of *E. coli* strain UTI89 with 10⁶ CFU *E. faecalis* OG1RF, and (ii) 10² CFU of *E. coli* strain UTI89 with 10⁶ CFU *E. faecalis* Δ ldh1 Δ ldh2. Single species controls (10² CFU of *E. coli* strain UTI89, 10⁶ CFU *E. faecalis* OG1RF, or 10⁶ CFU *E. faecalis* Δ ldh1 Δ ldh2) were performed alongside polymicrobial infections. The wound site was then sealed with a sterile transparent dressing (Tegaderm™ 3M, St Paul Minnesota, USA). After 24 hours, animals were

euthanized by carbon dioxide inhalation and cervical dislocation and a 1 cm by 1 cm squared piece of skin surrounding the wound was excised and homogenized in 1 mL PBS for CFU enumeration by serial dilution on MacConkey agar (Becton, Dickinson and Company, Franklin Lakes, NJ) or BHI agar supplemented with 10 µg/mL colistin (Sigma-Aldrich, St Louis, MO) and 10 µg/mL nalidixic acid (BHI-CNA)(Sigma-Aldrich, St Louis, MO) to isolate *E. coli* or *E. faecalis*, respectively. All studies and protocols were approved by the Nanyang Technological University Institutional Care and Use Committee (IACUC NTU).

3.4.13 Statistical Analysis

Statistical analysis was performed utilizing GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) for pooled data from multiple experiments. One-way ANOVA (Analysis of Variance) were used to compare between media and the different conditions. Statistical significance for multiplex immunoassay was determined using kruskal-wallis statistical analysis. Unless otherwise stated, values represented means $\pm$ SEM derived from at least 3 independent experiments and/or 3 technical replicates. */# indicate P-value < 0.05, **/## indicate P-value < 0.01, ***/### indicating P-value < 0.001 and ****/#### represents P-value < 0.0001.

3.5 Authors contribution

I design the study of this project, performed, and analysed the *in vitro* experiments listed in this chapter. I generated the $\Delta ldh2$ and $\Delta ldh1\Delta ldh2$ mutants while $\Delta ldh1$ and $\Delta ldh1_pGCP123ldh1$ were made by Casandra Tan Ai Zhu. In addition, Kao Hsien Neng performed the unbiased library screen for *E. faecalis* mutants that are unable to suppress LPS-induced NF- κ B activation. I performed the *in vitro* experiments using transposon mutants identified from the screen. Finally, *in vivo* experiment was performed and analysed by me, assisted by Casandra Tan Ai Zhu.

Chapter 4: *Enterococcus faecalis* immunomodulation in macrophages is dependent on I κ B α , ERK and p65

4.1 Introduction

Enterococcus faecalis infections are recalcitrant to treatment due to their ability to resist innate host defences, as well as their intrinsic and acquired antibiotic resistance (Hidron et al., 2008). Despite various studies investigating *E. faecalis* antibiotic resistance mechanisms and virulence factors, mechanistic studies exploring how enterococci can subvert the host immune response are lacking (Giridhara Upadhyaya, Ravikumar, & Umapathy, 2009; Hollenbeck & Rice, 2012). We showed in **Chapter 2** that *E. faecalis* infection results in NF- κ B suppression of macrophages in a dose-dependent manner leading to global downregulation of both pro- and anti-inflammatory cytokines and chemokines. In **Chapter 3**, we demonstrated that bacterial-derived LDH plays an important role in the immunomodulatory function of *E. faecalis*. However, the mechanism behind LDH-mediated suppression of macrophages remains unelucidated. In this chapter, we aim to identify host pathways that are affected by *E. faecalis*-derived LDH.

The innate immune response is the first line of defence in the host, which subsequently transits to the adaptive immune response (Janeway et al., 2001; Machado, Araújo, Carvalho, & Carvalho, 2004). Innate immune cells are induced upon binding of PAMPs to PRRs such as TLRs leading to downstream activation of NF- κ B and inflammatory responses (**Figure 4.1**)(Takeuchi & Akira, 2010). In addition, TAK1 activation can also lead to activation of the MAPK pathway upon PPR activation, both leading to activation of immune response (Pearson et al., 2001). Hence, to avoid immune recognition and activation to promote their lifestyle in the host, bacteria need to produce a variety of virulence factors that interact with multiple host

proteins and signalling pathways to modulate immune response such as cytokine production and phagocytosis.

Many molecular mechanisms by which bacteria can inhibit host immune responses have been elucidated over the past decades. Interestingly, not only are bacteria able to subvert antimicrobial peptides and avoid detection by PPRs, but certain bacteria are able to target intracellular signal transduction cascades of the host at different levels (Johannessen et al., 2013; Reddick & Alto, 2014). For example, *S. typhimurium* AvrA, a secreted protein that is a member of acetyltransferases family, can deubiquitinate I κ B α to prevent degradation of I κ B α and thus inhibit downstream NF- κ B activation (Ye et al., 2007). Besides inhibiting the NF- κ B pathways, bacteria can target the MAPK pathway to subvert immune surveillance (Krachler, Woolery, & Orth, 2011). As an example, metalloprotease LF secreted by *B. anthracis* can inhibit activation of p38 by cleaving between the N-terminal and the catalytic domain, which is necessary for binding and phosphorylation of downstream MAPKs such as p38, of upstream MAPKKs leading to lysis of macrophages due to ATP release and opening of connexin-43 channel resulting in downstream inflammasome activation (Ali et al., 2011; Park et al., 2002). Therefore, bacterial effector molecules may target multiple immune pathways to impede the immune response.

Bacteria can also target the phosphatidylinositol 3-kinase (PI3K)/Akt (also known as protein kinase B, PKB) signalling pathway which can regulate the host inflammatory response (**Figure 4.1**) (Hawkins & Stephens, 2015). PI3K/Akt signalling pathways, which are highly conserved in mammalian cells, are one of the key regulators of a myriad of host cell signal transductions including autophagy, oxidative burst, metabolism, proliferation, differentiation, cell survival, endocytosis, and inflammatory response (Engelman, Luo, & Cantley, 2006; Song, Ouyang, & Bao, 2005). Activation of PI3K occurs upon binding of regulatory subunits or

adaptor molecules to the receptors which convert its catalytic domain to phosphatidylinositol (3,4,5)-triphosphate (PIP₃) (Hemmings & Restuccia, 2012). Subsequently, Akt binds to PIP₃ and 3-phosphoinositide-dependent protein kinase 1 (PDK1) leading to phosphorylation and activation of Akt which regulates multiple cellular function via regulating other pathways (Hemmings & Restuccia, 2012). It is hardly surprising that bacteria have also evolved mechanisms to target PI3K/Akt pathway to prevent immune activation. For example, *Francisella tularensis* is able to downregulate the expression of TLR2, PI3K p85 α and Akt leading to downregulation of host PI3K/Akt signalling pathway leading to the suppression of the immune response (Butchar et al., 2008). This allows *Francisella* to colonize and cause infection without eliciting an immune response. Altogether, bacteria can target different immune signalling pathways to thwart the innate immune response in order to establish an infection.

Figure 4.1 NF-κB, PI3K/Akt and AP-1 signalling pathway and crosstalk.

NF-κB canonical pathway is shown on the left, where upon ligand binding, TLR undergoes conformation changes resulting in MyD88 activation and subsequent downstream cell signalling. Phosphorylated TAK1 can activate the AP-1 pathway by directly phosphorylating p38, Erk and JNK. In addition to activation via TAK1, AP-1 pathway can be indirectly regulated by PI3K and Akt. Phosphorylated Akt can also activate IKK complex in the NF-κB signalling pathway leading to NF-κB activation. NF-κB and AP-1 are in turns capable of directly modulating each other's activity since AP-1 expression is regulated by NF-κB and vice versa. Finally, AP-1 can also indirectly regulate NF-κB activation by inducing expression of E2 factor (E2F), a group of activating or repressing transcription factors involved in the regulation of the cell cycle and RNA synthesis. E2F-1, a key transcriptional regulatory of the cell cycle transition from G1 to S-phase, interacts with p65 leading to downregulation of NF-κB genes, such as IκBα, resulting in increased activation of NF-κB activation (Ankers et al., 2016). During S-phase, E2F-4 binds to NF-κB resulting in suppression of NF-κB activation (Ankers et al., 2016).

Regardless of the strategies adopted by pathogenic microbes in subverting the innate immune system, there is a dearth of understanding of enterococcal immunomodulation from the host perspective (Rahman & McFadden, 2011; Reddick & Alto, 2014). Understanding the complex molecular battle between the host and enterococci affords propitious countermeasures

against these evasion strategies that could tip the scales in favour of the host. Considering that we showed that the immunomodulation of NF- κ B activation during *E. faecalis* infection is not entirely dependent on presence of TLRs (**Chapter 2**), we hypothesized that *E. faecalis* could be interacting with components within NF- κ B signalling pathway downstream of TLRs. Furthermore, there is increasing evidence of microbes can simultaneously inhibit multiple signalling pathways in host (McGuire & Arthur, 2015; Reddick & Alto, 2014). Hence, we postulated that *E. faecalis* may target multiple immune signalling pathways to modulate macrophages response.

In this Chapter, we showed that *E. faecalis* inhibits macrophage activation through suppressing I κ B α and ERK level, and preventing p65 phosphorylation. Furthermore, preliminary data suggests that *E. faecalis* may activate the hypoxia pathway, particularly HIF1 α , to promote NF- κ B suppression. Based on transcriptional analysis, *E. faecalis* can prevent polarisation of macrophages toward both M1 and M2 phenotype. Understanding the immune pathway that *E. faecalis* regulates in macrophages will not only increase our understanding of *E. faecalis* pathogenesis and provide new and exciting directions for future research, but also uncovers novel therapeutics targets for treatment and prevention of recalcitrant *E. faecalis* infection.

4.2 Results

4.2.1 *E. faecalis* prevents phosphorylation of NF- κ B p65 subunit at high infection doses

We have shown in **Chapter 2** and **Chapter 3** that *E. faecalis* prevents macrophage activation in a dose-dependent manner, by quantifying SEAP secreted into the supernatant upon NF- κ B activation. Firstly, we hypothesized that the decreased NF- κ B-induced SEAP secretion by macrophages into the supernatant during *E. faecalis* infection is due to decreased p65 phosphorylation. NF- κ B, consisting of p50 and p65 subunit, is sequestered in the cytosol by I κ B α to prevent its activation via phosphorylation in un-stimulated macrophages. Upon induction by signals triggered by invading pathogens, I κ B α is phosphorylated and targeted for proteasomal degradation. The unbound p65 subunit of NF- κ B then undergoes phosphorylation by IKK complex at Ser 536 and translocate to the nucleus to activate gene transcription (Lawrence, 2009; Moynagh, 2005a; Oeckinghaus, Hayden, & Ghosh, 2011).

To investigate whether *E. faecalis* infection interferes with phosphorylation of NF- κ B in macrophages, we performed western blot analysis for unphosphorylated (total NF- κ B) and phosphorylated (activated NF- κ B) p65 subunit of NF- κ B for *E. faecalis* infection at various MOIs in the presence or absence of LPS and quantified the level of each protein band after normalizing each to respective GAPDH blot. We observed that *E. faecalis* infection can lead a slight decrease in the overall levels of p65 at MOI 100 as compared to LPS, but it is not significant (**Figure 4.2A and 4.2B**). However, phosphorylated p65 is significantly reduced at MOI 100 as compared to the LPS control or compared to MOI 10 with or without LPS stimulation (**Figure 4.2C**). Furthermore, the ratio of phosphorylated p65 to total p65 is significantly lower at MOI 100 as compared to MOI 10 with or without LPS stimulation (**Figure 4.2D**). Taken together, these results suggest that *E. faecalis* can prevent p65 phosphorylation.

Figure 4.2 *E. faecalis* infection prevents phosphorylation of NF- κ B p65 subunit at high dosage. Western blot performed on protein extracted from RAW-Blue macrophages infected with *E. faecalis* OG1RF at the indicated MOIs (100, 10, 1) with or without LPS (100ng/mL) for 6 hours and probed with antibodies raised against (A) p65, phospho-p65 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). M represents uninfected macrophages. GAPDH was used as an internal control for loading control. Quantification of band intensity for (B) p65 (C) phospho-p65 (D) ratio of phospho-p65 to total p65 after normalising to the respective GAPDH blot. Data shown are compilation of at least eight independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis test where * $P < 0.05$, ** $P < 0.01$ and ns (non-significant) $P > 0.05$ among the different condition indicated.

4.2.2 *E. faecalis* infection modulates I κ B α levels at high MOI

Bacteria have various mechanisms to interfere with almost every single step of the NF- κ B pathway to thwart the host response (Johannessen, Askarian, Sangvik, & Sollid, 2013). We proposed that *E. faecalis* could target at certain steps of the NF- κ B pathway to prevent NF- κ B phosphorylation and activation in macrophages. To elucidate which step of the NF- κ B signalling pathway *E. faecalis* modulates, western blot analysis was performed for MyD88, TAK1, IKK complex and I κ B α which are present in the canonical pathway (**Figure 4.1**). We

observed that the abundance of MyD88 is slightly lower in MOI 100 as compared to MOI 10 in the presence or absence of LPS although the decrease is not statistically significant (**Figure 4.3A & 4B**). Furthermore, the level of MyD88 is only significantly lower in MOI 100 with LPS stimulation as compared to LPS alone. This suggests that *E. faecalis* may not strongly affect levels of MyD88 but more experiment repeats, as well as probing for phosphorylated, MyD88 will be required to better understand this observation. In addition, we observed an overall reduction of levels of phosphorylated TAK1 at MOI 100 compared to LPS control without significant changes in total TAK1 protein (**Figure 4.3C & 4.3D**). However, since there are no significant changes in the ratio of phosphorylated TAK1 to total TAK1, the modulatory effect of *E. faecalis* on TAK1 is not conclusive and further experiment will be required to verify this. Moving on, we observed that there is no significant change in total IKK α and IKK β levels at MOI 100 as compared to LPS with or without LPS stimulation although we observed a significant reduction in levels of IKK β at MOI 100 in the presence of LPS stimulation as compared to media control can be observed (**Figure 4.3E & 4.3F**). Whereas, the phosphorylated IKK α/β is decreased for MOI 100 as compared to MOI 10 (**Figure 4.3G**) suggesting that *E. faecalis* can modulate the activation of IKK complex at high MOI although not strongly since the decrease is absent with LPS co-stimulation. Lastly, we observed a reduction in both total I κ B α and phosphorylated I κ B α at MOI 100 as compared to LPS control regardless in the presence or absence of LPS stimulation although there are no significant changes in the ratio of phosphorylated I κ B α to total I κ B α level (**Figure 4.3I, 4.3J and 4.3K**). The decreased in total I κ B α at MOI 100 may be a downstream or secondary effect due to NF- κ B regulation of I κ B α and a decreased in phosphorylated I κ B α leads to overall decrease in phosphorylated p65. Despite this, our results suggest that *E. faecalis* modulates I κ B α level during infection at high MOI.

Figure 4.3 *E. faecalis* can modulate the canonical NF- κ B signalling pathway.

Western blot performed on protein extracted from RAW-Blue macrophages infected with *E. faecalis* OG1RF at the indicated MOIs (100, 10, 1) with or without LPS (100ng/mL) for 6 hours and probed with antibodies raised against (A) MyD88, TAK1, phospho-TAK1, IKK α , IKK β , phospho-IKK α/β , I κ B α , phospho-I κ B α and GAPDH. M represents uninfected macrophages. GAPDH was used as an internal control for loading control. Quantification of band intensity for (B) MyD88 (C) TAK1 (D) phospho-TAK1 (E) ratio of phospho-TAK1 to total TAK1 (F) IKK α (G) IKK β (H) phospho-IKK α/β (I) I κ B α (J) phospho-I κ B α and (K) ratio of phospho-I κ B α to total I κ B α after normalising to the respective GAPDH protein levels. Data shown are compilation of at least four independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis test where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns (non-significant) P>0.05 among the different condition indicated.

4.2.3 *E. faecalis* regulates ERK levels but does not modulate PI3K pathway

Bacterial pathogens are able to manipulate the MAPK pathway to subvert the host immune response to establish an infection (Krachler et al., 2011). TLRs can also activate the MAPK pathway via TAK1 leading to AP-1 activation (**Figure 4.1**) (Pearson et al., 2001), we speculated that the downstream AP-1 pathway may also be inactivated. To examine whether *E. faecalis* can also interfere with the MAPK pathway, western blot analysis was performed by tagging ERK, JNK, p38 and AP-1 (consisting of c-Jun and c-Fos). In contrast to the decrease in phosphorylated p65 levels during *E. faecalis* infection, we do not observe significant changes in either the levels of phosphorylated c-Jun or c-Fos at MOI 100 as compared to lower MOIs, or in the ratio of phosphorylated c-Jun and c-Fos to total c-Jun and c-Fos. These results suggest that *E. faecalis* does not interfere with AP-1 activation (**Figure 4.4A and 4.4E to 4.4I**). In addition, we observed similar protein levels for both JNK and p38 under all conditions, whereas the abundance of ERK was significantly reduced at MOI 100 as compared to MOI 10 regardless of the presence or absence of LPS stimulation (**Figure 4.4A to 4.4D**). In addition, we also did not observe any significant changes in levels of phosphorylated p38 (**data not shown**). These data suggest that the decrease in levels of ERK protein does not impact downstream AP-1 activity. Taken together, these data suggest that macrophage suppression induced by *E. faecalis* is most likely not due to modulating the AP-1 pathway.

The PI3K pathway plays a role in cell metabolism, growth, proliferation, and survival, through the activation of ERK pathway (Hemmings & Restuccia, 2012). We postulated that downregulation of PI3K can reduce the abundance of ERK. To investigate if the reduced ERK levels is due to reduced PI3K, we performed western blot tagging for PI3K and Akt, which lies downstream of PI3K. We observed that there are no significant changes in the level of phosphorylated PI3K at MOI 100 relative to MOI 10, although a significant increase can be

observed in MOI 1 with LPS stimulation as compared to the media control (**Figure 4.5A and 4.5B**). This is not surprising since both LPS and *E. faecalis* infection at low doses activate macrophages. Furthermore, we observed a non-significant increase in phosphorylated Akt levels, as well as in the ratio of phosphorylated Akt to Akt, at MOI 100 relative to MOI 10 with or without LPS stimulation (**Figure 4.5C and 4.5D**). Coupled with our observation that AP-1 phosphorylated level is not significantly affected during *E. faecalis* infection, our results suggests that the PI3K/Akt pathway does not play a major role in macrophage suppression by *E. faecalis*.

A **B** **C** **D** **E** **F** **G** **H** **I** **J**

Figure 4.4 *E. faecalis* reduces ERK levels but does not modulates AP-1.

Western blot performed on protein extracted from RAW-Blue macrophages infected with *E. faecalis* OG1RF at the indicated MOIs (100, 10, 1) with or without LPS (100ng/mL) for 6 hours and probed with antibodies raised against (A) p38, JNK, ERK, c-Jun, phospho c-Jun, c-Fos, phospho c-Fos and GAPDH. M represents uninfected macrophages. GAPDH was used as an internal control for loading control. Quantification of band intensity for (B) p38 (C) JNK (D) ERK (E) c-Jun (F) phospho-c-Jun (G) ratio of phospho-c-Jun to total c-Jun (H) c-Fos (I) phospho-c-Fos (J) ratio of phospho-c-Fos to total c-Fos after normalising to the respective GAPDH blot. Data shown are compilation of at least four independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis test where **P<0.01, ***P<0.001, ****P<0.0001 and ns (non-significant) P>0.05 among the different condition indicated.

Figure 4.5 *E. faecalis* modulation of macrophage suppression is independent of PI3K/Akt pathway
 Western blot performed on protein extracted from RAW-Blue macrophages infected with *E. faecalis* OG1RF at the indicated MOIs (100, 10, 1) with or without LPS (100ng/mL) for 6 hours and probed with antibodies raised against (A) phospho-PI3K, Akt, phospho-Akt and GAPDH. M represents uninfected macrophages. GAPDH was used as an internal control for loading control. Quantification of band intensity for (B) phospho-PI3K (C) Akt (D) phospho-Akt and (E) ratio of phospho-Akt to total Akt after normalising to the respective GAPDH blot. Data shown are compilation of at least three independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis test where * $P < 0.05$ and ns (non-significant) $P > 0.05$ among the different condition indicated.

4.2.4 *E. faecalis* suppresses immune response via activation of Hif-1 α pathway

E. faecalis LDH is required for the suppression of macrophage responses (**Chapter 3**). We proposed that another possible pathway by which *E. faecalis* could prevent NF- κ B activation would be through the Hif-1 α signalling pathway. Lactate is able to stabilise hypoxia-inducible factor-1 α (Hif-1 α) leading to induction of arginase-1 (Arg1) and vascular endothelial growth factor (VEGF), which can suppress NF- κ B activation, production of which results in skewing of macrophages towards a M2 phenotype (Colegio et al., 2014). We further hypothesized that *E. faecalis* could stabilised Hif-1 α , possibly due

to lactate produce by *E. faecalis* resulting in downstream downregulation of NF- κ B. To elucidate whether Hif-1 α plays a role in *E. faecalis* modulation of macrophage response, we performed western blot analysis specific for Hif-1 α . Consistent with our hypothesis, we observed significantly increased levels of Hif-1 α at MOI 100 as compared to media control in the absence or presence of LPS stimulation (**Figure 4.6A and B**). In addition, a significant increase in Hif-1 α levels can also be observed for MOI 100 as compared to LPS control (**Figure 4.6B**). The difference in band size of Hif-1 α at different MOIs may be due to the different post-translational modification of Hif-1 α including acetylation, hydroxylation, phosphorylation and ubiquitination, although the exact reason for different modification at different MOI is unknown (Brahimi-Horn, Mazure, & Pouyssegur, 2005). Furthermore, when LW6, a Hif-1 α inhibitor which increases proteasomal degradation of Hif-1 α (Lee et al., 2010), was used to pre-treat macrophages prior to *E. faecalis* infection at MOI 100, we observed a significant increase in NF- κ B activation as compared to *E. faecalis* infected macrophages without LW6 treatment (**Figure 4.6C**). Treatment of macrophages with LW6 prior to *E. faecalis* infection did not affect the viability of macrophages (**Figure 4.6D**). Taken together, our preliminary results suggest that the ability of *E. faecalis* to suppress the NF- κ B response in macrophages might involve the upregulation of Hif-1 α pathway although future experiment is required to verify this.

Figure 4.6 *E. faecalis* suppresses immune response via activation of Hif-1α pathway.

Western blot performed on protein extracted from RAW-Blue macrophages infected with *E. faecalis* OG1RF at the indicated MOIs (100, 10, 1) with or without LPS (100ng/mL) for 6 hours and probed with antibodies raised against (A) Hif-1α and GAPDH. M represents uninfected macrophages. GAPDH was used as an internal control for loading control. Quantification of band intensity for (B) Hif-1α after normalising to the respective GAPDH blot. Data shown are compilation of five independent experiments; mean values were graphed, and error bars represent the standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Kruskal-Wallis test where * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ among the different condition indicated. Mouse RAW-Blue macrophages were infected with *E. faecalis* at MOI 100, with or without overnight LW6 treatment at various concentration, for 6 hours prior to measurement of (C) NF-κB-driven SEAP reporter activity and (D) cytotoxicity (LDH activity). NF-κB-driven SEAP reporter assays: exposure to media alone (-) represented background NF-κB reporter activity and stimulation with LPS represented positive controls for reporter activity. LDH assays: Triton-X treatment served as a positive control (+) for cell death. Data were combined from 3 independent experiments; mean values were graphed, and error bars represent standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test where * $P < 0.05$, ** $P < 0.01$ and **** $P < 0.001$ among all of the various conditions stated.

4.2.5 Transcriptional analysis of macrophages infected with *E. faecalis*

To further investigate the host mechanisms underlying *E. faecalis* infection in macrophages, we performed transcriptomic analysis of macrophages infected with *E. faecalis* at various MOIs in the presence or absence of LPS using RNA sequencing. The transcription profiles were first analysed by plotting principle component analysis (PCA). PCA revealed different clustering when macrophages were infected with *E. faecalis* at different MOIs as compared to the media and LPS control (**Figure 4.7**). Furthermore, *E. faecalis* was able to shift the transcriptional profile closer to that when macrophages infected with bacteria alone rather than LPS control in the presence of LPS stimulation, consistent with our findings that *E. faecalis* is able to suppress LPS-induced activation of macrophages (**Figure 4.7**).

Figure 4.7 Distinct transcriptional profile for macrophages infected with *E. faecalis* at increasing MOIs. Principal component analysis (PCA) comparing the transcriptional profile of macrophages infected with *E. faecalis* at the respective MOIs in the presence or absence of LPS. Each individual condition was represented with different colours. Each dot represents a sample at specific condition. Data were combined from three independent experiment with two technical replicates each.

4.2.5.1 High MOI of *E. faecalis* prevents polarisation of macrophages towards M1 and M2 phenotypes

In **Chapter 2**, we showed that *E. faecalis* infection leads to a global downregulation of pro- and anti-inflammatory cytokines and chemokines. Coupled with the previous observation that *E. faecalis* infection at high dosage leads to increased Hif-1 α levels, we hypothesized that *E. faecalis* can prevent polarisation of macrophages towards the M1 phenotype, potentially skewing macrophages towards the M2 phenotype. Polarisation of macrophages towards the M1 phenotype typically promotes an inflammatory response whereas M2 macrophage polarization promotes anti-inflammatory response (Martinez & Gordon, 2014; Murray et al., 2014). To investigate whether *E. faecalis* affects macrophage polarisation, we looked for changes in expression of genes encoding macrophage polarisation markers in our transcriptomic data. We observed that 47.9% of M1 markers were downregulated, 22.9% were upregulated, and 29.2% were unchanged after *E. faecalis* infection at MOI 100 in the presence of LPS stimulation as compared to MOI 10 in the presence of LPS (**Figure 4.8**). Interestingly, for M2 markers, we observed only 17.9% upregulation, 46.4% downregulation and 35.7% unchanged for *E. faecalis* infection at MOI 100 with LPS stimulation as compared to MOI 10 with LPS stimulation simultaneously (**Figure 4.8**). Therefore, based on transcriptional data this suggests that *E. faecalis* infection can prevent skewing of macrophages towards both M1 and M2 phenotype. Furthermore, based on our previous results where we observed increased in Hif-1 α levels (**Figure 4.6**) and decreased in overall pro-inflammatory cytokines (**Figure 2.8**), this further supports that *E. faecalis* is able to prevent macrophage polarisation towards M1 phenotype.

Figure 4.8 *E. faecalis* infection suppresses M1 macrophages polarisation.

Heat map of M1 and M2 gene expression in macrophages infected *E. faecalis* at respective MOIs for 6 hours. Data were plotted as log₂ fold change (log₂FC). Data were combined from three independent experiment in which two technical replicates were averaged in each.

4.3 Discussion

Bacteria have evolved a variety of tactics to subvert or avoid the immune response in order to establish an infection (Johannessen et al., 2013; Reddick & Alto, 2014). Most studies about the molecular mechanisms of *E. faecalis* infection are associated with the signalling pathways that stimulate host inflammation, which is largely observed at lower MOIs (Wang, Hibberd, et al., 2014; Zou & Shankar, 2015). Despite multiple studies, including ours, having hinted at the immunomodulatory potential of *E. faecalis*, the exact mechanism by which *E. faecalis* does so has not been fully characterised (Tien et al., 2017; Wang et al., 2008; Zou et al., 2014). Therefore, in this chapter, we aimed to elucidate the molecular details of how exactly does *E. faecalis* modulate the macrophage immune signalling pathway.

Given that bacteria are not only able to avoid recognition and phagocytosis by immune cells, but can also directly modulate the intracellular signalling pathways, we investigated if *E. faecalis* could also modulate the immune signalling pathways to inhibit immune response. We showed that *E. faecalis* infection at high dosage may interfere with the abundance of I κ B α . A possible mechanism in which *E. faecalis* can modulate I κ B α level would be to promote the ubiquitination of I κ B α . For example, LegK1, a *Legionella pneumophila* eukaryotic-like Ser/Thr kinase, can directly phosphorylate I κ B α on Ser-32 and Ser-36 leading to I κ B α activation and subsequent degradation (Ge et al., 2009). Interestingly, one would expect an increase in activation of NF- κ B pathway if there is a decreased in I κ B α abundance since I κ B α binds NF- κ B to sequester it in the cytoplasm, but we still observed suppression of NF- κ B activity during *E. faecalis* infection at high dosage. Indeed, there are more bacterial factors known to prevent the ubiquitination of I κ B α in

order to prevent NF- κ B activation instead of promoting I κ B α activation (Johannessen et al., 2013). Since we observed suppression of NF- κ B activity even when the sequestering protein I κ B α abundance level is lowered, this may suggest that *E. faecalis* ability to prevent NF- κ B activation has a more important effect than affecting I κ B α abundance level. Non-locus of enterocyte effacement (LEE) encoded effector C (NleC), a type III secretion protease, from *E. coli* can directly cleave p65 subunit of NF- κ B to decrease the total nuclear entry of active p65 (Baruch et al., 2011; Mühlen, Ruchaud-Sparagano, & Kenny, 2011; Pearson et al., 2011; Yen et al., 2010). Furthermore, vibrio outer protein S (VopS) from *Vibrio parahaemolyticus* can directly bind to p65 to prevent DNA binding activity of NF- κ B leading to its suppression (Bhattacharjee et al., 2006). Therefore, it is possible that *E. faecalis* is able to prevent NF- κ B activation by promoting degradation of p65 since we observed a significant decreased in the abundance of phosphorylated p65 subunit of NF- κ B. Future experiments are required to probe for ubiquitin ligases involved in NF- κ B degradation such as elongin BC-cullin 2-suppressor of cytokine signalling-box (EC2S), EC5S and postsynaptic density 65-discs large-zonula occludens 1-abnormal cell lineage 11-islet 1- mechanosensory abnormal 3 domain-containing protein 2 (PDLIM2) to test this possibility. In addition, to investigate whether *E. faecalis* can affect translocation of activated NF- κ B into the nucleus, cell fractionation can also be performed to verify if there is difference in the level of activated NF- κ B in the cytoplasm and nucleus although it is less likely that *E. faecalis* will affect p65 translocation since there is a decrease in phosphorylated p65. Regardless, the NF- κ B reporter assay coupled with western blot analysis strongly support that *E. faecalis* can suppress NF- κ B activation in macrophage during infection at high dose.

Moving on, we have shown that *E. faecalis* can suppress the canonical NF- κ B signalling pathway, particularly by modulating I κ B α abundance and preventing NF- κ B phosphorylation. However, one limitation of our study is that we have only investigated the canonical NF- κ B signalling pathway triggered by binding of PAMPs to TLRs. In fact, other PRRs, such as NLRs, are involved in pathogen recognition aside from TLRs leading to downstream NF- κ B activation to trigger inflammation response. Particularly, NOD1 is involved in the recognition of meso-diaminopimelic acid-containing PGN fragments of mainly Gram-negative bacteria while NOD2 recognises muramyl dipeptide which are found in PGN of nearly all Gram-positive and Gram-negative bacteria (Chamaillard et al., 2003; Girardin, Boneca, Carneiro, et al., 2003; Girardin, Boneca, Viala, et al., 2003; Inohara et al., 2003). Binding NOD1 and NOD2 to their respective ligands initiates the oligomerization of nucleotide-binding oligomerization domain (NBD) and recruitment of receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through a homotypic caspase activation and recruitment domain (CARD)-CARD interactions (Kobayashi et al., 2002; Nembrini et al., 2009; Park et al., 2007). RIPK2 subsequently recruits and activates TAK1 leading to downstream NF- κ B activation (Inohara et al., 2003). Therefore, future experiment to probe for the NOD signalling pathway should also be carried out to investigate the role of other upstream mediators of I κ B α and NF- κ B in addition to MyD88-dependent pathway.

The TLR signalling pathway has been linked to the MAPK signalling pathway through TAK1 leading to AP-1 activation (Pearson et al., 2001). Since bacterial pathogens are able to manipulate the MAPK pathway (Krachler, Woolery, & Orth, 2011) and *E. faecalis* is able to modulate NF- κ B, we hypothesized that *E. faecalis* might also modulate

the MAPK signalling pathway. We showed that *E. faecalis* can modulate ERK levels during infection at high dosage although it does not activate downstream AP-1. Current known bacterial factors that can inhibit ERK are mainly from Gram-negative bacteria (Krachler et al., 2011). Outer shigella protein F (OspF), a phosphothreonine lyase, from *Shigella flexneri* inactivates ERK by dephosphorylation in nucleus of HeLa and Caco-2 cell lines leading to inhibition of pro-inflammatory response (Arbibe et al., 2007). In addition, VopA from *V. parahaemolyticus* is able to inhibit ERK activation by acetylating the lysine in the catalytic loop of ERK to prevent ATP binding (Trosky et al., 2007; Trosky et al., 2004). Therefore, it may be possible that *E. faecalis* employs a similar mechanism to either prevent the phosphorylation of ERK or promote the degradation or cleaving of ERK to regulate ERK levels. However, since we only probed for total ERK in macrophages after *E. faecalis* infection, future experiments are required to examine the level of phosphorylated ERK to elucidate whether *E. faecalis* prevents ERK phosphorylation or promotes ERK degradation. Further studies are also necessary to clearly demonstrate the exact mechanism by which *E. faecalis* can inhibit ERK and the bacterial factor(s) that are involved. Although decreased ERK level does not impact downstream AP-1 activity, it is possible that ERK can regulate NF- κ B activity through mitogen and stress-activated kinase 1 (MSK1). MSK1 can induce NF- κ B activation by phosphorylating Ser276 on p65 subunit of NF- κ B and thus a reduction of ERK activation leads to reduced NF- κ B activation (Reber, Vermeulen, Haegeman, & Frossard, 2009; Vermeulen et al., 2003). Therefore, western blot analysis probing for MSK1 should also be conducted in the future. An ERK inhibitor can also be used during *E. faecalis* infection at MOI 10 to see if ERK inhibition results in NF- κ B suppression similar to that observed during infection at high dose. Alternatively, ERK activator treatment

during *E. faecalis* infection at MOI 100 can also be conducted to investigate whether NF- κ B suppression can be reversed. In addition, inhibition of ERK has also been linked to impaired differentiation of monocytes toward the M2 phenotype in tumor cell proliferation leading to decreased expression of IL-10, CCL17 and CCL18 (Neamatallah, 2019; Zhang et al., 2013). Therefore, the decreased levels of ERK may support our results that *E. faecalis* infection prevents M2 macrophage polarisation based on transcriptional analysis. Taken together, our results suggest that *E. faecalis* might regulate macrophage activation through modulating ERK levels and future experiments is essential to investigate this further.

Next, our results suggest that *E. faecalis* is unlikely to modulate phosphorylation of PI3K/Akt pathway considering that we do not observed significant differences in the abundance of phosphorylated proteins at MOI 100 as compared to MOI 10 in the absence or presence of LPS stimulation. However, despite the lack of statistical significance, we do observe a trend of lower levels of phosphorylated PI3K and Akt at MOI 100 relative to MOI 10. Furthermore, the number of repeats for probing phosphorylated PI3K and Akt is only five times. Therefore, the lack of statistical significance might be possibly due to the low n numbers as compared to other proteins which has 8 repeats. Various bacterial factors have been implicated in either suppression or activation of PI3K within host cells (Butchar et al., 2008; Nakayama et al., 2015). Gingipains (RgpA, RgpB and Kgp) of *Porphyromonas gingivalis* proteolytically cleave PI3K p85 α of epithelial cells resulting in the inactivation of PI3K and downstream signalling pathway leading to destruction of epithelial barrier (Nakayama et al., 2015). YopH of *Yersinia spp.* also disrupts the PI3K signalling pathway by binding to and dephosphorylating the Fyn-binding protein (Fyb) in macrophages, leading to decreased phagocytosis. Taken together, since our observation that *E. faecalis* is

unable to modulate phosphorylation of PI3K and Akt may not be very conclusive, future experimental repeats are imperative to confirm this finding. Alternatively, ELISA can be performed to obtain a more accurate comparison of the differences in levels of PI3K and Akt across the different conditions of interest.

Bacteria, such as *Bartonella henselae*, can interfere with expression of the hypoxia pathway to mediate infection (Volkhard et al., 2005). *B. henselae* can exploit Hif-1 α to increase its pathogenicity by stimulating VEGF expression via Hif-1 α resulting in angiogenesis and vasculoproliferative disorders such as bacillary angiomatosis and bacillary peliosis (Volkhard et al., 2005). Furthermore, Hif-1 α activation during systemic infections is linked to higher mortality in a *S. aureus* peritonitis model (Werth et al., 2010). Similarly, we showed that *E. faecalis* at high infectious dose may increase levels of Hif-1 α in macrophages. In addition, *E. faecalis* appears to be less capable of suppressing NF- κ B activation in macrophages upon treatment with the Hif-1 α inhibitor LW6, which is consistent with *E. faecalis* suppressing the immune response by activating Hif-1 α leading to downstream NF- κ B inhibition although the change is subtle. Indeed, Hif-1 α has been identified to inhibit NF- κ B transcriptional activity in *Drosophila melanogaster* and HeLa cells as depletion of Hif-1 α leads to increased NF- κ B-dependent IL-8 expression (Bandarra et al., 2015). However, since LW6 treatment did not fully alleviate NF- κ B suppression by *E. faecalis*, it is important to note that future experiments are necessary to check whether and to what extent Hif-1 α is degraded after overnight treatment with LW6 by western blot to verify whether Hif-1 α plays a major role in the suppressive effect on NF- κ B by *E. faecalis* at high MOI. Furthermore, *E. faecalis* infection at MOI 10 should also be included as an additional positive control to help in the evaluation of the effect of reversal NF- κ B

suppression after LW6 treatment since we observed maximum NF- κ B stimulation at MOI 10. In addition, future experiments using primary macrophage cell line to test the effect of LW6 treatment on *E. faecalis* infection following LW6 treatment should also be conducted to ensure that the effect of Hif-1 α inhibitor is not limited to RAW 264.7 cell line and also because the LW6 effects may be even more pronounced in primary macrophages. Taken together, despite these limitations, our preliminary results suggest that the ability of *E. faecalis* to suppress the NF- κ B response in macrophages might involve the upregulation of Hif-1 α pathway. Future experiments to investigate whether the increased in Hif-1 α is indeed due to the presence of higher concentrations of lactate during *E. faecalis* infection at high MOI by using *ldh* mutants is also required.

Lactate-induced Hif-1 α activation in tumour microenvironment is associated with promoting polarisation of tumour associated macrophages towards M2 phenotype instead of M1 (Colegio et al., 2014; Leblond et al., 2015; Mu et al., 2018). Given that we observed global downregulation of cytokines and chemokines (**Chapter 2**) and increased level of Hif-1 α during *E. faecalis* infection, we postulated that *E. faecalis* prevents M1 polarisation and may potentially promotes M2 polarisation. However, our transcriptional data coupled with cytokines and chemokines profile in **Chapter 2** demonstrated that macrophages can be inhibited adopting a M1 and M2 phenotype during *E. faecalis* infection at high dosage. A plausible explanation for the small increment in M2 associated markers would be the existence of various subtypes of M2 macrophages and that M1 and M2 classification do not fully take into consideration the plasticity of macrophages where macrophages could be in various subtypes at different timepoints. Future studies with more specific markers to identify the specific subtypes of macrophages polarisation (Martinez & Gordon, 2014;

Röszer, 2015) during *E. faecalis* infection would be necessary to have a better understanding of macrophage polarisation as a result of *E. faecalis* infection and potential impact of macrophage polarisation on infection outcome. Importantly, macrophages are highly plastic and thus, the macrophages may be in intermediate phenotypes. In addition, since our observations are limited to a relatively short infection time of 6 hours, the macrophages could still be in transition towards a M2 phenotype. However, due to the high infectious dose that was used, it would be hard to prolong the infection timepoint to 24 hours as the viability of macrophages would be impacted using our *in vitro* infection model. A possible way to overcome the timepoint issue would be to look at macrophages polarisation during mice wound model *in vivo*. M1 macrophages polarisation are commonly associated with acute infectious disease with microbicidal effect, due to ROS production, on invading pathogens (Shapouri-Moghaddam et al., 2018). *Salmonella* and *Mycobacterium spp* have shown to inhibit macrophage M1 polarisation (Benoit, Desnues, & Mege, 2008). Specifically, the *Salmonella* pathogenicity island 2 (SPI-2) encoding a type III protein secretion system in *S. typhimurium*, helps in preventing NADPH oxidase-dependent killing by macrophages by inhibiting phagosome relocalisation of NADPH oxidase and hence dampens M1 phenotype (Vazquez-Torres et al., 2000). Interestingly, *M. tuberculosis* is able to drive macrophage polarisation to either M1 or M2 phenotype depending on the stages of infection (Huang et al., 2015; Refai, Gritli, Barbouche, & Essafi, 2018). During primo-infection, where there is promotion of granuloma formation, early secreted antigenic target protein 6 (ESAT-6) secreted by *M. tuberculosis* ESAT-6 was able to drive M2 macrophages and non-polarised macrophages towards the M1 phenotype (Refai et al., 2018). However, during the later stages of granuloma formation, macrophage

expression of M1 markers decreases while M2 markers expression increases driving the fully activated M1 macrophages towards M2 phenotype where IL-12 and TNF α secretion is inhibited and increased IL10 production (Refai et al., 2018). In addition, ESAT-6 can interfere with M1 activation via direct binding to TLR2 to activate Akt and inhibiting downstream activation of NF- κ B and IFN-regulatory factors (Pathak et al., 2007). Therefore, understanding polarisation of macrophages during *E. faecalis* infection may provide further insights into the mechanism by which *E. faecalis* subvert the host innate immune response and may provide foundation for alternative therapies based on macrophage polarisation reprogramming. Nonetheless, we report for the first time that *E. faecalis*, a non-classical intracellular pathogen, can inhibit M1 and M2 macrophages polarisation by hindering several immune signalling pathways with the extracellular population playing a more important in this immune suppression.

In summary, our work suggests that *E. faecalis* suppresses the immune response in macrophages by inhibiting the canonical NF- κ B signalling pathways, which is likely achieved through modulating I κ B α and ERK expression, and ultimately preventing p65 phosphorylation (**Figure 4.9**). In addition, preliminary results suggest that *E. faecalis* may also promote Hif-1 α activation in macrophages during infection. Furthermore, *E. faecalis* infection can inhibit M1 and M2 macrophage polarisation thus preventing both anti-inflammatory and inflammatory response. Understanding the virulence and pathogenesis of *E. faecalis* and host immune response may subsequently provide new insights to alternative treatment for these recalcitrant infections. For example, considering the essential role that lactate plays in macrophage suppression, drugs inactivating LDH can be designed as an alternative medicine for treating *E. faecalis* infection. In addition, drugs that

inhibit I κ B α and Hif-1 α , or that stimulate NF- κ B and ERK, may also be helpful in treating *E. faecalis* infection. Moving forward in **Chapter 5**, we will summarise our work as well as provide some future perspectives in this field of work.

Figure 4.9 Immune signalling pathway inhibited in macrophages by *E. faecalis*.

E. faecalis can suppress canonical NF- κ B signalling pathway by possibly regulating ERK and I κ B α levels and p65 subunit phosphorylation. Hif-1 α signalling pathways activation may also be involved in *E. faecalis* infection leading to downstream NF- κ B suppression in macrophages.

4.4 Materials and methods

4.4.1 Bacterial growth conditions and strains

Bacteria strains that were used in this study are as shown in **Table 3.** *E. faecalis* strains were grown in Brain Heart Infusion (BHI) (Becton, Dickinson, USA) broth or agar at 37°C overnight. *E. faecalis* strains were streaked from 25% glycerol stock and incubated on the BHI agar plates overnight at 37°C. The antibiotics rifampicin and fusidic acid at were supplemented at a concentration of 25 mg/L to select for *E. faecalis* OG1RF when necessary. Single-isolated colonies of each strain were inoculated into BHI and grown overnight statically at 37°C for 18-20 hours for all experiments. Overnight bacteria culture of *E. faecalis* were centrifuged at 6,000 g for 5 minutes and washed once with 1X PBS. Normalization to an optical density (OD) of 0.7 was done for *E. faecalis* (equivalent to 2 x 10⁸ CFU/mL).

Table 4.1| Bacterial Strains used in this study

Strains	Relevant characteristic(s) ^b	References or source
Strains		
<i>Enterococcus faecalis</i>		
OG1RF	Fus ^r , Rif ^r , wild-type strain	(Dunny et al., 1978)
$\Delta ldh1\Delta ldh2$	<i>ldh1</i> and <i>ldh2</i> double knockout strain	Lab strain

4.4.2 Cell culture

RAW-Blue cells derived from mouse RAW 264.7 macrophages (Invivogen™, USA) were used in this study. The modified RAW 264.7 macrophages contain a plasmid encoding secreted embryonic alkaline phosphatase (SEAP) reporter that is under the transcriptional control of an activator protein 1 (AP-1)/NF-κB inducible promoter. RAW-blue cells were cultured in T₇₅/T₁₇₅ flask as adherent monolayers (Nunc, Thermo scientific, Singapore) in DMEM high glucose with L-glutamine and 10% heat inactivated FBS (Gibco, Thermo Fisher Scientific, Singapore) supplemented with 200 µg/mL Zeocin™ (Gibco, Thermo Fisher Scientific, Singapore), when necessary, at 37°C with 5% CO₂.

4.4.3 RAW-Blue macrophage infection

RAW-Blue cells and BMDM were seeded into 6 well microtiter plates (Nunc, Thermo Fisher Scientific, Waltham, MA) at 1x 10⁶ cells/well in antibiotic free media (DMEM with 10% heat inactivated FBS) and incubated overnight at 37°C with 5% CO₂. Following overnight incubation, the cells were washed once with PBS (Gibco, Thermo Fisher Scientific, Singapore) and fresh media was added. For HIF-1α inhibitor treatment, RAW-Blue cells were incubated overnight at 37°C with 5% CO₂ with antibiotic free media (DMEM with 10% heat inactivated FBS) supplemented with respective concentration of LW6 (Axon Medchem, Groningen) (Lee et al., 2010). Following overnight incubation, the cells were washed once with PBS (Gibco, Thermo Fisher Scientific, Singapore) and fresh media with LW6 was added.

The SEAP reporter assay was established by empirically defining the minimal agonist (lipopolysaccharide (LPS)) concentration that induced the maximum SEAP activity in the absence of cell death. Cells were stimulated using LPS purified from *E. coli* O111:B4

(Sigma-Aldrich, St Louis, MO) (100 ng/ml) dissolved in cell culture media as positive control, or media alone as a negative control. RAW-Blue cells were infected with *E. faecalis* (at MOI of 100:1, 10:1 and 1:1) for 6 hours with or without TLR stimulation.

4.4.4 NF- κ B reporter assay

RAW-Blue cells secrete SEAP into the supernatant upon NF- κ B activation which serve as an indication of NF- κ B activation upon quantification. QUANTI-Blue™ (Invivogen™, USA), a colorimetric enzyme medium, indicates the presence of SEAP through changing of medium colour from pink to purple-blue. Post-infection, 20 μ L of supernatant was collected and added to 180 μ L of QUANTI-Blue reagent and incubated at 37°C overnight. SEAP levels were quantified at 640 nm using a microplate reader (Tecan infinite M200 Pro, Switzerland). LPS-stimulation serves as positive control where NF- κ B activity is considered high where media control (-) is considered as low NF- κ B activity. SEAP readings in between that of LPS and media control is considered as medium activity. All experiments were performed in triplicates.

4.4.5 Cell viability assay

Simultaneously with supernatant collection for SEAP determination, culture supernatants were collected from each well to measure lactate dehydrogenase (LDH) release, using an LDH cytotoxicity assay (Clontech, Takara, Japan) according to manufacturer's instructions. Background LDH activity was determined using mock (PBS) treated RAW-Blue cells. Maximal LDH activity was determined by lysing cells with 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO). Each condition was carried out in triplicate. Percentage cytotoxicity was calculated as follows: $(\text{sample absorbance} - \text{background absorbance}) / (\text{maximal absorbance} - \text{background absorbance}) \times 100$.

4.4.6 Western blot analysis

Following infection with *E. faecalis* OG1RF as described previously, proteins were harvested using 2x Laemmli buffer (Bio-rad, Hercules, CA) containing 50 mM dithiothreitol (Sigma Aldrich, USA) and denatured at 99°C for 10 minutes. 30 µL of protein samples were loaded and subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using NuPAGE® 4-12% Bis-Tris Gel (Invitrogen™, ThermoFisher Scientific, USA) in 1 x NuPAGE® MOPS SDS Running Buffer (Invitrogen™, ThermoFisher Scientific, USA). The protein samples were transferred to polyvinylidene fluoride (PVDF) membrane using iBlot® Gel Transfer Device (Invitrogen™, ThermoFisher Scientific, USA) as per manufacturer's instructions. The PVDF membrane was then blocked using 5% BSA in Tris-buffered saline (20 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.1% Tween-20 (Sigma Aldrich, USA) at room temperature for 1 hour. Subsequently, primary antibody (**Table 4.2**) was used for probing overnight at 4°C. The membranes were washed with Tris-buffered saline and incubated with 1:3000 diluted anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Invitrogen™, ThermoFisher Scientific, USA) at room temperature for 1 hour. Following which, membranes were washed, and proteins were detected using SuperSignal West Femto® Maximum Sensitivity Substrate (Invitrogen™, ThermoFisher Scientific, USA) and autoradiography films (Kodak, USA). Relative band intensity was measured using ImageJ software.

Table 4.2| Antibodies list

Primary antibodies	Dilution	Source
MyD88 (D80F5) Rabbit mAb	1:2000	Cell Signalling Technology, USA
TAK1 Antibody	1:1000	Cell Signalling Technology, USA
Phospho-TAK1 (Thr184/187)	1:1500	Cell Signalling Technology, USA
IKK α Antibody	1:1000	Cell Signalling Technology, USA
IKK β Antibody	1:1000	Cell Signalling Technology, USA
Phospho-IKK α / β (Ser176/180) (16A6) Rabbit mAb	1:1000	Cell Signalling Technology, USA
I κ B α Antibody	1:1000	Cell Signalling Technology, USA
Phospho-I κ B α (Ser32) (14D4) Rabbit mAb	1:1000	Cell Signalling Technology, USA
NF- κ B p65 (D14E12) XP [®] Rabbit mAb	1:1000	Cell Signalling Technology, USA
Phospho-NF- κ B p65 (Ser536) (93H1) Rabbit mAb	1:1000	Cell Signalling Technology, USA
SAPK/JNK Antibody	1:1000	Cell Signalling Technology, USA
p38 MAPK Antibody	1:1000	Cell Signalling Technology, USA
p44/42 MAPK (Erk1/2) Antibody	1:1000	Cell Signalling Technology, USA
c-Jun (60A8) Rabbit mAb	1:1000	Cell Signalling Technology, USA
Phospho-c-Jun (Ser73) Antibody	1:1000	Cell Signalling Technology, USA
c-Fos (9F6) Rabbit mAb	1:1000	Cell Signalling Technology, USA
Phospho-c-Fos (Ser32) (D82C12) XP [®] Rabbit mAb	1:1000	Cell Signalling Technology, USA
Akt Antibody	1:1000	Cell Signalling Technology, USA

Continued next page

Phospho-Akt (Ser473) (D9E) XP [®] Rabbit mAb	1:1000	Cell Signalling Technology, USA
Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Antibody	1:1000	Cell Signalling Technology, USA
HIF-1 α (D2U3T) Rabbit mAb	1:1000	Cell Signalling Technology, USA
GAPDH (14C10) Rabbit mAb	1:3000	Cell Signalling Technology, USA

4.4.7 RNA extraction and sequencing

After 6 hpi with *E. faecalis* at various MOIs in the presence or absence of LPS, cells were washed once with the tissue culture grade 1X PBS (Gibco, Thermo Fisher Scientific, Singapore) prior to RNA extraction. 1 mL of Trizol[™] (Invitrogen[™], ThermoFisher, USA) per well and was stored at -80°C until RNA extraction using Direct-zol RNA Miniprep Plus kit (Zymo Research, USA) according to manufacturer's protocol. Briefly, 200 μ L of chloroform (Sigma Aldrich, USA) was first added to 1mL of Trizol[™] extracted samples, mixed vigorously for 2 minutes and allowed to stand at room temperature for minimum 10 minutes. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C and the top layer (aqueous phase) was transferred to 500 μ L of ethanol (Fisher Scientific, Canada) before loading into the Zymo-Spin[™] IIICG Column². Subsequently, RNA extraction was performed according to manufacturer's protocol for Direct-zol RNA Miniprep Plus kit. Next, extracted RNA samples were further cleaned and concentrated using RNA Clean & Concentrator[™]-5 (Zymo Research, USA) following the manufacturer's protocol.

RNA samples were first screened using TapeStation (Aligent Technologies, USA) to obtain RIN value and concentration. Qubit analysis was also performed to measure the DNA and RNA concentration using Qubit[™] dsDNA HS Assay Kit (Invitrogen[™], ThermoFisher Scientific, USA) and Qubit[™] RNA BR Assay Kit (Invitrogen[™],

ThermoFisher Scientific, USA), before storage at -80°C until sequencing using an Illumina HiSeq®2500 v.2 (Illumina, Singapore), 150 bp paired-end.

4.4.8 Transcriptomics analysis

Sequencing data are available at NCBI's BioProject (accession no. PRJ-NA335539). RNA sequencing results were analyzed as described in (Rousseau et al., 2016). Briefly, reads were quality checked and adapters trimmed with Bowtie2 option. The mm10 mouse genome was used as reference for tophat-2.0.11.Linux_x86_64 (Kim et al., 2013) and transcriptional read counts obtained using HTSeq-0.6.1 (Anders, Pyl, & Huber, 2015) with default parameters with a non-stranded analysis. Unless otherwise stated, all further analyses were performed in the R statistical computing environment (version 3.3.3) (R Development Core Team, 2013). Differential analysis of macrophages infection with *E. faecalis* OG1RF at various MOIs was performed using the R/Bioconductor package DESeq2, (version 1.40.1) (Love, Huber, & Anders, 2014); using default settings from that package. The NCBI file gene2refseq (downloaded 03/03/2016) was used to convert Refseq to Entrez identifiers for further analysis of Gene Ontology annotations and Immunological Genome Project (ImmGen) data for functional analysis.

4.5 Authors contribution

I designed the study of this project and performed the experiments listed in this chapter. I performed all the experiments assisted by Siti Nurmahsuri Binte Mutaza, Ria Sorayah and Kelvin Chong Kian Long. RNA extraction was performed by me, sequenced by SCELSE sequencing facility and analysis was done by Guangan Hu and Kelvin Chong Kian Long.

Chapter 5: Conclusion

5.1 Summary

Enterococcus faecalis is an opportunistic pathogen that causes a myriad of infections such as CAUTIs, wound infections, and endocarditis which are all often recalcitrant to treatment due to intrinsic and acquired antibiotic resistance, as well as phenotypic antibiotic tolerance of biofilms (Hidron et al., 2008). Although *E. faecalis* virulence factors are relatively well characterised, there have been fewer studies to unravel the interaction of *E. faecalis* and immune system. The overarching aim of this thesis was to understand and characterised the interaction of *E. faecalis* and macrophages. We hypothesised that *E. faecalis* can modulate the immune response by macrophages during infection. To address this, we i) characterised the immunomodulatory interaction of *E. faecalis* and macrophages using an *in vitro* model that we established; ii) identified *E. faecalis* virulence factors involved in macrophage suppression; and iii) determined the macrophage pathways which *E. faecalis* modulates to suppress immune activation. In this concluding Chapter, I will review and summarise my findings and their implications, as well as discuss future perspectives to enhance our understanding of *E. faecalis* interactions with the immune response which could potentially lead to alternative treatments for *E. faecalis* infection.

We first sought to characterise the interaction of *E. faecalis* with macrophages and to establish an *in vitro* model that would serve as a foundation for the entire thesis. Since there are conflicting reports in NF- κ B activation and suppression by *E. faecalis* possibly due to the difference in cell lines and *E. faecalis* strains used, we first sought to investigate

E. faecalis immunomodulatory activity within macrophages. We resolved previous discrepancies and showed that *E. faecalis* can suppress NF- κ B activity in a dose-dependent manner which leads to global downregulation of anti- and pro-inflammatory cytokines. Importantly, high infection dosage, at MOI 100, is required in order for immunomodulation by *E. faecalis* to be observed. In addition, *E. faecalis* needs to be alive to suppress macrophage activation. Finally, we showed that *E. faecalis* can prevent *E. coli*-mediated immune activation in macrophages during polymicrobial infection *in vitro*. This was later supported in an *in vivo* mouse CAUTI model where *E. faecalis* was associated with the suppression of macrophage activation-associated genes and was able to augment *E. coli* titres in the kidney during polymicrobial infection (Tien et al., 2017). Our results are consistent with other studies, where, in polymicrobial infection, *E. faecalis* is able to promote the growth or virulence of other bacteria such as *E. coli* and *P. aeruginosa* often leading to poorer disease prognosis (Hughes & Winter, 2016; Keogh et al., 2016; Lavigne et al., 2008; Lee et al., 2017; Peters et al., 2012; Tay et al., 2016). Commensal relationships during polymicrobial infection can also arise due to host immune response modulation resulting in augmented bacteria growth. For example, similar to what we observed in our *in vivo* CAUTI polymicrobial infection, uropathogenic *E. coli* is able to colonise bladder better during coinfection with GBS as compared to mono-species UPEC infection due to suppression of leukocyte recruitment by GBS capsular sialic acids leading to decreased inflammation (Kline et al., 2012; Kline et al., 2011). Therefore, our results suggest that *E. faecalis* may be able to suppress the host immune system which could potentially allow less pathogenic *E. coli* strains to colonize better during polymicrobial infection therefore resulting in poorer disease prognosis. Taken together, these observations served as the

foundation for further characterization of *E. faecalis* and macrophage interactions. **Chapter 2**

Although our work in **Chapter 2** underscored *E. faecalis* ability to modulate macrophage response, it did not show which bacterial virulence factor(s) were involved in macrophage suppression. To address this, we used a targeted systematic approach to probe for potential virulence factor(s). We demonstrated that secreted bacterial factors which result in acidification of surrounding environment could be involved in immune suppression of macrophages by *E. faecalis*. Moving on, we reported that, consistent with other studies, lactate is sufficient to suppress LPS-induced NF- κ B activation (Kellum et al., 2004a; Van Hée et al., 2015). Therefore, we hypothesised and proved that LDH, which produces lactate in *E. faecalis* and therefore plays a major role in environment acidification, is imperative for immunomodulatory capabilities of *E. faecalis*. Indeed, LDH has been shown to be important for virulence of *E. faecalis* and LDH-deficient mutants are less able to colonize host organs during systemic infection using murine model due to defects in intrinsic fitness (Rana et al., 2013). We further showed that an LDH-deficient *E. faecalis* mutant was not able to promote *E. coli* growth during polymicrobial wound infection, whereas wild type *E. faecalis* did promote *E. coli* growth. Interestingly, we did not see a reduction of virulence in LDH-deficient mutant as suggested by the previous study but this may be due to a different infection model being used (Rana et al., 2013). Finally, we also reported that other bacterial factors that affect acidification, such as a DHH family protein and thioesterase, can affect the immunomodulatory capabilities of *E. faecalis* and their impact on immunomodulatory can be partly attributed to reduced lactate production. Collectively, our results suggest that LDH plays an essential role in the ability of *E. faecalis*

to suppress macrophage activation. Furthermore, our results also hinted that ability of *E. faecalis* to acidify the environment is multifactorial. Understanding the pathogenesis of *E. faecalis* can provide us new insights into alternative treatments for these recalcitrant infections. **Chapter 3**

Finally, to answer the question of how exactly does *E. faecalis* modulate the macrophage immune response, we performed western blot analysis of the various immune signalling pathways present in macrophages. We demonstrated that *E. faecalis* not only reduced I κ B α and ERK expression, but also prevents p65 phosphorylation leading to suppression of polarisation of macrophages towards inflammatory phenotype. In addition, preliminary data suggests that *E. faecalis* may activate Hif-1 α to suppress macrophage response. Our finding also demonstrated the ability of *E. faecalis* to interfere with M1 polarisation, and possibly M2 polarisation, expanding our current knowledge that such regulation can also be achieved by *E. faecalis*. Taken together, these results explain how *E. faecalis* is able to suppress macrophage response leading to *E. faecalis* persistence during infection. **Chapter 4**

In conclusion, we have addressed our primary goal of this thesis. We have established an *in vitro* model to test for *E. faecalis* virulence factors important in immune suppression and this can be translated into *in vivo* settings. In addition, our findings also serve as the foundation for future investigation of *E. faecalis* interaction with the host immune system which would be discussed in the next section.

5.2 Future perspective

The interaction of *E. faecalis* with the immune system remains largely unexplored, and many virulence factors that are involved in *E. faecalis* virulence (Bao et al., 2012; Hancock & Gilmore, 2002; Kandaswamy et al., 2013; Qin et al., 2000; Rakita et al., 1999; Sussmuth et al., 2000; Thurlow, Thomas, Fleming, et al., 2009; Thurlow, Thomas, & Hancock, 2009; Thurlow et al., 2010; Zou et al., 2014) were nonessential for macrophage suppression in this study. We have shown that LDH is important for macrophage suppression. We postulated that the immune suppression is due to lactate production and lactate eventually leads to suppression of macrophage activation similar to lactate present in cancer microenvironment (Husain et al., 2013; Kellum et al., 2004b; Romero-Garcia et al., 2016). Although we have shown that lactic acid alone is sufficient to prevent LPS-induced NF- κ B activation, we did not show that lactate produced by *E. faecalis* is sufficient for NF- κ B suppression. To test this, lactate fermented by *E. faecalis* can be extracted and purified using various methods including ion exchange resin, precipitation and liquid-liquid extraction before adding it to macrophages (Bishai, De, Adhikari, & Banerjee, 2015; Joglekar et al., 2006; Sun et al., 2006). In addition, if bacterial lactate is indeed important for modulation of macrophage response, it would be interesting to study whether such a phenotype holds true for other lactic acid bacteria (LAB) as well. Considering the possibility that bacterial metabolites from one species may be beneficial for the other species through metabolic cross-feeding, a plausible future direction would be to investigate the beneficial or detrimental effect of lactate of on other bacteria species. Since *E. faecalis* is commonly isolated with *P. aeruginosa*, *S. aureus* and *E. coli* in wounds, the effect of lactate on the growth or biofilm formation of these bacteria species can be

investigated both *in vitro* and *in vivo* (Citron et al., 2007; Dowd et al., 2008; Giacometti et al., 2000; Gjodsbol et al., 2006). If lactate is indeed important for augmentation of *E. coli* growth, this could explain why we observed lower *E. coli* titres during co-infection with LDH-deficient mutant as compared to wildtype *E. faecalis*. Regardless, this would be the first evidence that lactate, an *E. faecalis* metabolite, plays an indispensable role in macrophage response modulation as opposed to being just a metabolic by-product.

Next, while we showed that an LDH-deficient mutant is unable to promote *E. coli* colonization during polymicrobial infection as compared to wildtype OG1RF, there was no reduction in LDH-deficient mutant titres in both mono- and polymicrobial infection. Why is there no difference in colonization of LDH-deficient *E. faecalis* as compared to wildtype although it promotes increased production of pro-inflammatory cytokines IL-6 in *in vitro*? One plausible explanation would be that for our *in vivo* experiment, only a single timepoint at 24 hpi was tested in this thesis. Our lab has previously shown that strong pro-inflammatory cytokines and chemokines can be observed at 24 hpi which began dissipating by 3 days post infection (dpi) in mice wound model (Chong et al., 2017). An increased in MHCII- macrophages in infected wound was only observed in 3 dpi, but not at 24 hours, suggesting a possibility of *E. faecalis* modulating macrophages at a later time point *in vivo* (Chong et al., 2017). Therefore, although LDH-deficient *E. faecalis* may be able to colonize at 24 hpi, they may not be able to persist at 3 dpi if they are unable to modulate the immune response. Hence, a longer time point should be utilized to investigate the difference in persistence of LDH-deficient strain as compared to wildtype OG1RF. Moreover, lactate accumulation in wound has been associated with improved wound healing due to its angiogenic effect and stimulation of collagen deposition (Ghani et al., 2004; Trabold et al.,

2003). Interestingly, lactic acid bacteria, *Lactobacillus reuteri*, also have been used as vector for the delivery and on-site production of CXCL2 for local bioengineering of the wound to promote wound healing (Vågesjö et al., 2018). Therefore, this prompts the question whether there is a difference in wound healing between wildtype strain and LDH-deficient *E. faecalis* during single or mixed species infection. To address this, a 7 dpi should be utilised, and skin samples can be harvested before subjecting to histological examination. The lactate concentration in wound fluid and serum can also be simultaneously quantified to detect any possible correlation between wound healing and lactate accumulation. Finally, histological sectioning, fluorescence *in situ* microscopy (FISH) and scanning electron microscopy could also be used to monitor the difference in microcolonies developed between LDH-deficient strain and wildtype in mono- and mixed-species infection. Therefore, these methods would allow us to uncover more information about *E. faecalis* virulence factor(s) and spatial distribution of the species during single- or polymicrobial wound infection.

Moving on, our results suggested that *E. faecalis* is modulating the macrophage immune response by reducing I κ B α and ERK expression, and preventing p65 phosphorylation leading to downregulation of the downstream signalling pathways. Our findings raise new questions for investigation including what are the *E. faecalis* factor(s) involved in the regulating the activity of I κ B α , ERK and p65? Is LDH-deficient *E. faecalis* unable to regulate I κ B α , ERK and p65 phosphorylation? What are the interactions involved for *E. faecalis* factor(s), namely lactate, to regulate I κ B α , ERK and p65 phosphorylation? To explore this, immunoprecipitation using purified I κ B α , ERK and p65 can be used as bait protein to find out possible interacting bacterial factor(s) if *E. faecalis* is indeed able to

directly regulate these proteins. Furthermore, ERK inhibitors or activation can also be used to verify if ERK is indeed essential for *E. faecalis* ability to suppress macrophage activation. Furthermore, we hypothesised and showed that *E. faecalis* can suppress NF- κ B activation by possibly activating Hif-1 α . Therefore, it would be expected that the LDH-deficient mutant would not be able to activate Hif-1 α and thus unable to suppress LPS-induced NF- κ B activation. In addition, if lactate is indeed important for macrophage suppression, lactate can be supplemented during *E. faecalis* infection using LDH-deficient strains to help restore the macrophage suppression phenotype. Furthermore, RNA interference (RNAi) can be used to investigate how lactate is taken up in macrophages, other than monocarboxylate transporter, and how exactly does it stabilise Hif-1 α . To explore whether lactate can directly interact with I κ B α , ERK, p65 and Hif-1 α , immunoprecipitation from cell lysate can be employed. Finally, another limitation of our study is that we only investigated the role of canonical NF- κ B signalling pathway triggered by binding of PAMPs to TLRs. Future studies should investigate other PPRs, such as NLRs, which are involved in bacterial recognition and also can lead to downstream TAK1 and NF- κ B activation. Understanding how *E. faecalis* is able to suppress macrophage activation and what virulence factor(s) is important in modulation of immune response will provide insights into not only its pathogenicity, but also possible new treatments against *E. faecalis* infections.

Another important outstanding question that we have yet to answer is whether we can observe similar trend of immune suppression in *in vivo* setting as compared to that seen in *in vitro*. We hypothesised that the inability of LDH-deficient mutant to augment *E. coli* growth during polymicrobial infection is because of its inability to modulate the immune

response. To investigate this, we can perform flow cytometry for different immune cell populations, such as activated and inactivated macrophages, dendritic cells and neutrophils, using both early and late time points in mono- or polymicrobial infection. In addition to identifying the populations of immune cells present, multiplex ELISAs can also be performed to elucidate the cytokines and chemokines profile in the different infection settings. In addition, single deletion mutant of LDH1 or LDH2 can also be employed to elucidate whether the lack of *E. coli* growth augmentation is due to decrease in lactate accumulation or immune suppression, since LDH1 plays a more crucial role in lactate production as compared to LDH2. To address specifically whether NF- κ B activity is suppressed in macrophages *in vivo*, besides flow cytometry, histological staining for macrophage markers and NF- κ B or NF- κ B reporter transgenic mice could be used. Fully characterising the immune response during *E. faecalis* mono- or mixed-species wound infection would provide insights into the nature, diagnosis and treatment of these polymicrobial wound infections.

Importantly, characterizing the interaction of the invading pathogen and host not only allow us to have a better understanding of the pathogenesis of infection and potential treatment targets, but can have further implications in the clinical field. Importantly, NF- κ B activation is not only limited to stimulation by pathogen but can also be stimulated by host molecules. Given the key role of NF- κ B in the inflammatory response, it is unsurprising that uncontrollable NF- κ B activation has been associated with septic shock, multiple inflammatory disease, autoimmunity and cancer (Park & Hong, 2016; Sun, Chang, & Jin, 2013; Tak & Firestein, 2001; Xia, Shen, & Verma, 2014). Existing drugs that are used for anti-inflammatory and anti-tumour treatment can be attributed to, at least partially,

their ability to interfere with the IKK-NF- κ B system. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sodium salicylate can inhibit TNF α -induced NF- κ B target genes such as vascular cell adhesion molecule 1 and intracellular adhesion molecule-1, as well as impairing phosphorylation of I κ Bs and subsequent NF- κ B activation (Kopp & Ghosh, 1994; Pierce et al., 1996; Yin, Yamamoto, & Gaynor, 1998). Although the cause-and-effect relationships of NF- κ B activation in these diseases are not fully elucidated, activation of NF- κ B by carcinogens is a common event that drives cancer formation and is associated with resistance to apoptosis in human cells (Bernal-Mizrachi, Lovly, & Ratner, 2006; Chen, Li, et al., 2006; Naugler & Karin, 2008; Rahman et al., 2007; Xia et al., 2014). Given the importance of inhibition of the NF- κ B pathway with therapeutic efficacy of drugs, as well their potential toxicity, more investigation would be required for better understanding of the target specificity. Nonetheless, novel anticancer or anti-inflammatory therapeutics that can inhibit NF- κ B by targeting specifically a particular upstream kinase in the signalling pathways have shown promising results in preclinical trials including SPC-839, a specific IKK β inhibitor developed by Celgene, which can inhibit TNF α production in LPS-challenged rats and reducing paw oedema in rat arthritis model (Karin, Yamamoto, & Wang, 2004; Palanki et al., 2002; Park & Hong, 2016). However, it is worth remembering that, pathogens are much better at exploiting the immune signalling pathways, in part due to evolution, than us. Therefore, we can draw upon experience from these successful pathogens to identify the “druggable” targets in the signalling pathways. Furthermore, it may be possible to exploit the pathogen-derived molecules as both anticancer and anti-inflammatory therapeutic in the future.

The intricate interaction of infecting pathogens and their host immune system determines the pathogenesis of infection. Several discrete stages exist for interaction between a plethora of infectious agents and the multigenic immune system. This series of events for the host includes recognition of invading pathogens, amplification of innate immune response and initiation of adaptive response to kill the pathogens. For the pathogens, this involves inoculation at site of infection, infection of target cells and subsequent replication to propagate. This dissertation has made significant headway towards understanding the interaction between *E. faecalis* and the immune system. Our work has resolved previous conflicts and shown that *E. faecalis* can suppress NF- κ B in a dose-dependent manner even in the presence of TLR-agonist leading to global downregulation of cytokines and chemokines in **Chapter 2**. With this developed *in vitro* model, we could uncover novel *E. faecalis* virulence factors involved in immune modulation. We showed that LDH plays an indispensable role during *E. faecalis* infection in **Chapter 3**. Furthermore, our work suggests that the ability of *E. faecalis* to modulate host immune response can benefit *E. coli* colonization during polymicrobial wound infection *in vivo*. Since *E. faecalis* is commonly isolated from polymicrobial wound infection, our work also raises possibilities that other pathogens can also benefit from the additive effect of immune modulation by *E. faecalis*, leading to non-healing chronic state of infection. Hence, future investigation to understand how *E. faecalis* contributes to polymicrobial infection is imperative despite the complexity in studying these species. Regardless, since *E. faecalis* infections are difficult to treat due to antibiotic tolerance and resistance, a more complete understanding of *E. faecalis* virulence mechanisms will provide insights into better future treatment (Hidron et al.,

2008). Finally, we also revealed probable macrophage immune pathway which *E. faecalis* can modulate, including suppressing I κ B α and ERK level, preventing p65 phosphorylation and possibly activating Hif-1 α , to prevent downstream macrophage activation in **Chapter 4**. In addition, *E. faecalis* can also prevent M1 and M2 macrophage polarisation which may explain why *E. faecalis* infection are often recalcitrant to treatment. Nevertheless, we have established an *in vitro* model which can serve as a platform to test for virulence factors involved in immune modulation which can be validated in *in vivo* wound model.

In conclusion, our work has shown that *E. faecalis* is able to modulate the macrophage response which may ultimately lead to persistent wound or urinary tract infection. Furthermore, we provided preliminary evidence of beneficial interaction of *E. faecalis* with other co-infecting microbial species during *in vivo* infection as well as identified host signalling pathways that *E. faecalis* may interfere with. Our findings may be important for understanding why patients with *Enterococcus spp* infection during bloodstream infection and endocarditis are associated with higher mortality (Falcone et al., 2019; Pericàs et al., 2018). Furthermore, polymicrobial infection are often associated with poorer disease prognosis and is a risk factors for mortality (Ceci et al., 2015; Falcone et al., 2019). Therefore, our findings would potentially aid in understanding Enterococci infection and provides novel alternative treatments strategies or therapeutics to mitigate persistent infection within the host, especially in the event of polymicrobial infection.

5.3 Key Summary

Key findings of the thesis

- *E. faecalis* is able to suppress LPS-induced or *E. coli*-induced NF- κ B activation in a dose-dependent manner.
- *E. faecalis* immunomodulatory capability leads to global downregulation of cytokines and chemokines, and prevents M1 and M2 macrophage skewing, during *in vitro* infection at high dosage.
- Lactic acid, secreted by *E. faecalis*, and the lactate dehydrogenase enzyme responsible for synthesising it is essential for macrophage suppression.
- Lactate dehydrogenase-mediated immune suppression promotes *E. coli* survival after co-infection with *E. faecalis* during polymicrobial wound infection.
- *E. faecalis* prevents macrophage activation by suppressing I κ B α and ERK level, preventing p65 phosphorylation and possibly activating Hif-1 α .

Future directions

- What is the exact mechanism employed by *E. faecalis* to reduce I κ B α and ERK levels, prevent p65 phosphorylation, and activate Hif-1 α ? How is lactate, or other bacterial factor(s), involved in this? Are other signalling pathways apart from canonical NF- κ B being impacted?
- How does the host respond to *E. faecalis* single infection as compared to mixed infection with *E. coli*? What benefit (if any) does *E. faecalis* derived from this immune suppression mechanism during mono- and mixed-infection?

- Will other lactic acid bacteria be able to suppress immune response in a way similar to *E. faecalis* *in vitro* and *in vivo*, and what is the consequence on infections caused by those species?
- Will *E. faecalis* be able to similarly suppress dendritic cells and potentially the adaptive immune response as a consequence of this immune suppression?

Chapter 6: Citations

6.1 Citations

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