Insights into the interactions of enterococcus faecalis with protozoan predators

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF

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MASTER OF SCIENCE IN BIOLOGICAL SCIENCES

2018

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Contents

Abstract

Enterococcus faecalis is a commensal gut bacterium that is also frequently isolated from aquatic environments. Heterotrophic protists, commonly referred to as protozoa, are bacterivorous and are a major mortality factor for bacterial populations in aquatic environments. In order to survive, bacterial prey have evolved mechanisms for evasion of predators or for resistance to predation by these protozoa. In this study, *E. faecalis*, when co-cultured with *Tetrahymena pyriformis*, a ciliated protozoan, was found to resist digestion after being consumed by the ciliate. Live bacteria were found to be encased in expelled food vacuoles (EFVs) that provided protection against antibiotic treatment. EFVs were also released into the environment spontaneously regardless of nutrient availability.

To determine the genetic and phenotypic effects of strong predation pressure on *E. faecalis*, it was co-cultured with the amoeba, *Acanthamoeba castellanii,* continuously over 30 days. Every 3 days, intracellular *E. faecalis* were collected from *A. castellanii* cells and inoculated into a fresh co-culture with the amoeba. Genomic DNA was extracted from the acclimated and non-acclimated isolates and sequenced to identify genetic changes that occurred during acclimation. As biofilm formation is one of the mechanisms which bacteria use to evade predation, it was hypothesised that exposure to *A. castellanii* would augment *E. faecalis* biofilm formation. Interestingly, the data showed that biofilm formation was reduced in acclimated strains as compared to non-acclimated strains. However, this reduced biofilm biomass phenotype was not sustained through subculturing, suggesting that this phenotype is not stable.

Chapter 1 : Introduction

1.1) *Enterococcus faecalis*

Enterococcus faecalis is a Gram-positive diplococcus bacterium that commonly inhabits the human and animal gastrointestinal (GI) tract. However, *E. faecalis* is also an opportunistic invasive pathogen that causes infections with high morbidity and mortality rates. Common infections include bacteraemia, wound infections, endocarditis and urinary tract infections (UTI) (Fisher and Phillips 2009, Agudelo Higuita and Huycke 2014, Ceci et al. 2015). *E. faecalis* is an important agent of nosocomial infections, accounting for up to 90% of enterococcal isolates from patients (Gordon et al. 1992, Lewis and Zervos 1990, Patterson et al. 1995, Ruoff et al. 1990). Enterococcal infections are particularly hard to treat due to their ability to associate with other microbes at infection sites, creating polymicrobial infections (Murray 1990) and their ability to form biofilms. In such situations, combinatorial antibiotic treatment is usually required to achieve synergistic bactericidal effects (Agudelo Higuita and Huycke 2014). Furthermore, enterococci are intrinsically resistant to antibiotics such as penicillins and cephalosporins (Murray 2000) and can acquire other antibiotic resistance genes (Chow 2000). The emergence of multi-drug resistant enterococcal strains (Agudelo Higuita and Huycke 2014, Antony 1998) further hampers treatment efforts, and resistance to vancomycin, a last-resort antibiotic (Dziri et al. 2016, Murray 2000), has caused increasing concern worldwide.

E. faecalis possesses many different factors, both secreted and cell wall associated, that aid in colonisation of different niches, including colonisation of a host. Extensively studied factors include a gelatinase, cytolysin, enterococcus surface protein (Esp), aggregation substance (AS) and endocarditis-and biofilm-associated pili (Ebp). A secreted serine gelatinase can cause degradation of host tissues and promote biofilm formation (Arias and Murray 2012). A

cytolysin is a secreted bacteriotoxin that lyses erythrocytes and some human white blood cells (Arias and Murray 2012, Coburn and Gilmore 2003). Esp and Ebp are both important in formation of *E. faecalis* biofilms (Tendolkar et al. 2004, Toledo-Arana et al. 2001, Singh, Nallapareddy, and Murray 2007) while AS is involved in promoting adhesion, phagocytosis by human macrophage and intracellular survival (Sussmuth et al. 2000, Kayaoglu and Orstavik 2004).

E. faecalis can survive in a variety of environments, including soil, plants and fresh and marine waters. Due to the abundance of enterococci in human and animal faeces, it has been frequently used as an indicator of faecal pollution in recreational waters worldwide (Byappanahalli et al. 2012, Boehm and Sassoubre 2014). Furthermore, *E. faecalis* has high tolerance to environmental insults and is documented to survive in extreme temperatures, high salt concentrations and extreme pH (Fisher and Phillips 2009, Bradley and Fraise 1996). Furthermore, when exposed to sub-lethal stresses, *E. faecalis* becomes more resistant to normally lethal concentrations of bile salts, sodium dodecyl sulphate (SDS) and tolerant to high salt, acidity and alkaline conditions (Flahaut et al. 1996, Rince et al. 2003). *E. faecalis* is also able to enter the viable but non-culturable (VNBC) state when induced chemically (Lleo et al. 2001). Other than being resilient in the environment, *E. faecalis* is able to persist in mouse peritoneal macrophages for extended periods of time (Gentry-Weeks et al. 1999). *E. faecalis* faces oxidative stress when internalised by macrophages, and AS-expressing *E. faecalis* can suppress respiratory bursts (Sussmuth et al. 2000) and the *hypR* gene regulates the oxidative stress response (Verneuil et al. 2004), resulting in intracellular survival. *E. faecalis* can also evade the host innate immune system by persisting within macrophages. pH sensitive dyes and immunofluorescent labelling experiments showed that phagosomes containing *E. faecalis* did not acidify and may be due to the lack of lysosome fusion to the phagosomes (Zou and Shankar 2016). Due to these reasons, treatment for enterococcal

infections are difficult and complicated due to its resilience to both extracellular and intracellular environments.

1.2) Control of bacteria population in the environment

Bacterial biomass in the environment may be controlled by bottom-up and top-down regulation. Bottom-up regulation refers to the effects of the competition for all resources, including limited growth substances such as organic carbon sources, other organic or inorganic sources of nitrogen and phosphorous, and mineral nutrients (such as ammonium, phosphate and silicate) (Cuevas et al. 2011) on bacterial growth within the microbial community (Pace and Cole 1994, Storesund et al. 2015). Other physicochemical resources such as temperature, dissolved oxygen and chlorophyll *a* may also affect population structure (Berdjeb, Ghiglione, and Jacquet 2011). Top-down control refers to the predation by bacteriophages and protozoa (Servais, Billen, and Rego 1985, Proctor, Okubo, and Fuhrman 1993, Weinbauer and Hofle 1998). Most commonly encountered predators are protozoa.

In the environment, predation by protozoa is a major cause of bacterial mortality (Matz and Kjelleberg 2005) and control of environmental bacteria populations (Weekers et al. 1993). Protozoa are heterotrophic protists which are free-living unicellular eukaryotes (Adl et al. 2005). They are ubiquitous in the environment and are commonly found in soil and water sources. Some protozoa are heterotrophic and consume bacteria as food.

There are three morphological groups in protozoa, namely, flagellates, amoeba and ciliates. Flagellates are organisms that are distinguished by the presence of one of more flagella (Figure 1-2 a). Flagella are used mainly for motility to propel flagellates through the environment (Linck 2015). Some flagellates are photosynthetic and take up the role of

primary producers. Most flagellates, however, are heterotrophic feeders and are regarded as principal bacterial consumers in aquatic environments (Patterson 2001).

Amoeba are characterised by their distinctive cellular surface projections called pseudopodia (Figure 1-2 b). Cytoplasmic streaming across surfaces are achieved by contraction and extension of the pseudopodia (Anderson and Rogerson 2011). Amoeba are heterotrophic and engulf bacteria using the pseudopodia. Amoeba can be further classified to two groups, namely the naked amoeba and testate amoeba. Naked amoeba are characterised by lack of cell surface covering while testate amoeba is encased in organic or mineralised shell (Anderson 2010).

Ciliates are morphologically characterised by a decoration of hair-like appendages called the cilia (Figure 1-2 c). Interestingly, they possess two kinds of nuclei, a macronucleus and one or more micronuclei. The macronucleus controls vegetative growth and functions, and does not pass down its genetic material. The micronucleus, on the other hand, carries the germline of the cell (Prescott 1994). Another interesting feature is that ciliates can undergo asexual reproduction or sexual conjugation (Anderson 2010). Ciliates' motility is attributed by coordinated motions of cilia that propels the cell forward. Somatic cilia near to the oral apparatus may also assist in prey capture (Lynn 2008). Ciliates are known heterotrophs and they prey on bacteria and other smaller protozoa. In turn, they are preyed upon by larger microorganisms such as zooplankton and fish (Anderson 2010).

Figure 1-1: Different groups of protozoa. (a) flagellate, (b) naked amoeba and (c) ciliate. Figure is adapted from (Anderson 2010).

1.2.1) Use of protozoa as model organisms

In this study, two model protozoa were utilised, *Tetrahymena pyriformis* and *Acanthamoeba castellanii*. *T. pyriformis* is a ciliate that is commonly isolated from fresh water sources. As the genus name (*Tetrahymena*) implies, it has four (tetra-) distinct membrane-like structures (hymen) which make up its oral apparatus, forming a buccal cavity that food enters (Figure 1-3 A). One of the four membranes is the undulating membrane (Figure 1-3 A, UMC) which acts to sweep food particles (mainly bacteria) into its cytosol (Elliott 1959). Once food enters the buccal cavity, a food vacuole will form and envelopes the food particles. As the newly formed food vacuole moves into the cytosol, its contents acidify, with the pH changing from neutral to a value of 4.0 to 3.5 by 1 h post ingestion (Nilsson 1977). Like phagocytic cells of the immune system, once the food vacuole is formed and acidified, hydrolytic enzymes will be activated to digest the phagosome contents. In *Tetrahymena*, undigested remnants will be expelled out of *Tetrahymena* via the cytoproct (Figure 1B), a specialised structure that is

located at the posterior end of the cell (Allen and Wolf 1979).

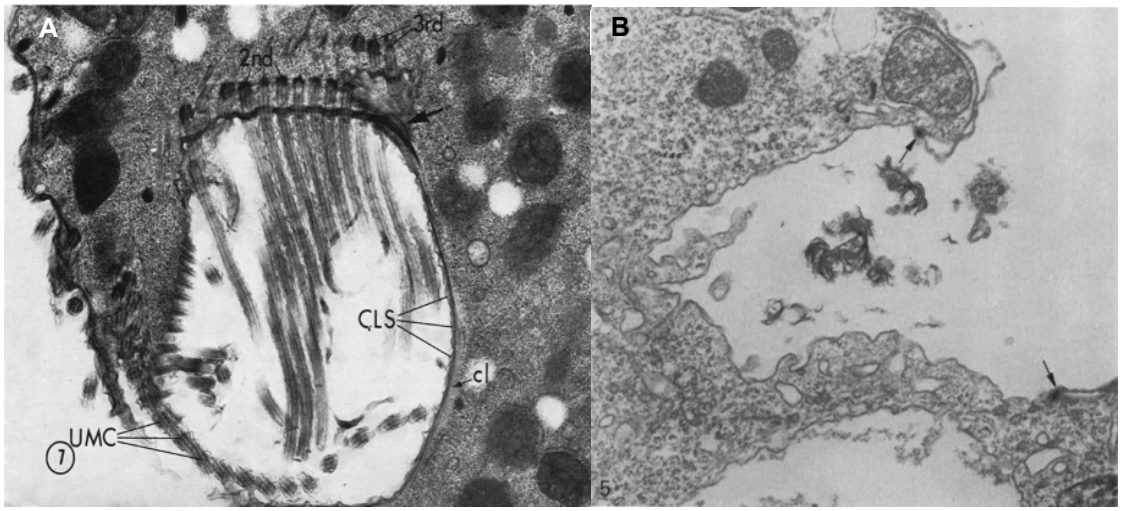

Figure 1-2: Transmission electron micrographs of *Tetrahymena* . (A) Cross section of *Tetrahymena* shows buccal cavity and two out of three membrane-like structures (2nd and 3rd). The forth, undulating membrane (UMC) acts to sweep food inwards (Sattler and Staehelin 1976). (B) Cross section of the cell shows cytoproct opening (area between arrows) (Allen and Wolf 1979).

Acanthamoeba castellanii is a unicellular eukaryote that is usually isolated from soil or water sources. *A. castellanii* has been used as a model organism for cell motility studies, where movement is mediated by F-actin (Pollard et al. 1970). In the presence of cytochalasin, which blocks actin polymerisation, there is a dose-dependent reduction in the uptake of bacteria. At high concentrations of cytochalasin D (100 μM), there was no bacterial uptake at all. In the same study, the authors concluded that tyrosine kinase pathways, Rho GTPases and phosphatidylinositol-3 kinase activity affects phagocytosis (Alsam et al. 2005). Although bacteria can be phagocytosed, some bacteria like pathogenic *Legionella pneumophila* can survive intracellularly after ingested by *Acanthamoeba*. *L. pneumophila* targets and utilises the same mechanisms for invasion and intracellular survival in both amoeba and macrophages (Molmeret et al. 2005). Therefore, knowledge about how virulence traits of pathogenic bacteria are shaped by evolutionary forces arise, since free living amoeba and macrophages are evolutionary distinct.

1.3) Adaptations and defence against protozoa

There are different ways in which bacteria can defend against predation by protozoa (Matz and Kjelleberg 2005). For extracellular pathogens, their main objective is to avoid ingestion and they can achieve this by altering morphological characteristics, increasing swimming speed, changing surface structures, forming microcolonies or secreting toxins.

Heterotrophic protozoan predators are usually size selective and vulnerability of bacteria to protist grazing are categorised, based on cell size, into four classes: weakly grazed (< 0.4 μm), grazing vulnerable (0.4 to 1.6 μm), grazing suppressed (1.6 μm to 2.4 μm) and grazing resistant (Hahn and Hofle 2001, Pernthaler et al. 1996). To evade predation, bacteria cells can alter their morphology to a larger and bulkier form that are not within an edible size range of protists. For example, in the absence of grazers, *Flectobacillus* spp., a morphologically plastic bacteria, mostly have a size distribution of 4 to 7 μm. The proportion of *Flectobacillus* spp. having a filamentous cell chain of ~15 μm increased to 80% of the whole population after grazing by *Ochromonas* spp. The formation of such filamentous chains reduced *Ochromonas* spp. grazing as they were beyond the edible size range of < 7 μm (Corno and Jürgens 2006, Hahn, Moore, and Höfle 1999).

Increasing swimming speed or motility is another adaptive trait bacteria can employ to evade ingestion by protists. Survival of motile bacteria is contributed by three factors, contact, capture and handling. For fast swimming bacteria (swimming speeds > 30 μm s^{-1}), although they have a higher chance of encountering predators, ingestion rates remained low due to the inefficient handling and capturing these motile bacteria (Matz and Jürgens 2005). As such, bacterial motility is an effective mechanism to evade and prevent ingestion by protozoan predators.

Some protozoa rely on receptor specific interactions for uptake of bacteria. For example, *Naegleria gruberi* and *Acanthamoeba* spp. have different uptake efficiency of *S. enterica* based on the O-antigen expressed (Wildschutte et al. 2004). This suggests that modifying the epitope of cell surface ligands can prevent host-specific recognition and is a method to evade from predators.

Formation of microcolonies or biofilms is another effective adaptive trait bacteria use to defend against predation. Unlike planktonic cells that can move freely in a medium, biofilm associated cells are firmly anchored onto surfaces (Donlan 2002). In the environment, because they are not able to evade predators by motility or morphological changes, biofilm associated cells are expected to be exposed to intense predation pressure. Despite this, biofilms persist, suggesting antipredator mechanisms have been developed. Indeed, the aggregation of bacteria cells, held together by extracellular polymeric substance (EPS), can reach beyond a size that protozoan predators can feed on (Matz and Kjelleberg 2005, Hahn, Moore, and Hofle 2000, Matz 2007). Formation of grazing resistant *Pseudomonas* spp. MWH1 microcolonies were favoured in the presence of heterotrophic flagellates, while a decline in microcolonies was demonstrated when flagellates were removed (Hahn, Moore, and Hofle 2000). This suggests that formation of microcolonies is a form of antipredator response. However, grazing resistance of biofilms is dependent on feeding types of protozoa. For example, *Tetrahymena* spp. and *Acanthamoeba polyphaga* were effective in clearing mature *Pseudomonas* spp. biofilms where they were shown to reduce biomass reduction by 70% and 100% respectively (Weitere et al. 2005). Therefore, biofilm formation is an effective defence strategy against grazers, but not against certain ciliates and amoeba.

Quorum sensing (QS) genes such as *rhlR* and *lasR* were shown to be important for production and secretion of toxic antipredator compounds in mature *Pseudomonas*

aeruginosa biofilms. For example, flagellate *Rhynchomonas nasuta* were killed when exposed to *P. aeruginosa* wild type PAO1 mature biofilm but survived in biofilms of *rhlR*/*lasR* mutants (Matz, Bergfeld, et al. 2004). Another study demonstrated that supernatants collected from wildtype *Vibrio cholerae* had higher inhibitory effect on flagellate *R. nasuta* than QS mutant *hapR*, showing secretion of antipredator compounds were dependant on QS.

There are other bacteria species that secrete antipredator compounds without QS systems. For example, during interactions with *Naegleria americana*, genes for putrescine catalysis was upregulated in *Pseudomonas fluorescens*. Putrescine induced encystment of *N. americana* and negatively affected cyst viability (Song et al. 2015). *Janthinobacterium lividum* and *Chromobacterium violaceum* were shown to produce highly toxic antipredator chemical, violacein, and adversely affected the survival of nanoflagellates (Matz, Deines, et al. 2004).

For intracellular pathogens, one of the post-ingestion defence mechanism is to resist digestion in the phagosome. When ingested, bacteria are packaged into food vacuoles that become acidified and loaded with degradative enzymes. Undigested bacteria can stay within the protozoa without multiplication (Figure 1-1 A), or some bacteria such as *Legionella pneumophila*, which utilise the Dot/Icm Type IV secretion system to bypass the default phagosome maturation pathway and multiply within these specialised compartments (Hilbi, Segal, and Shuman 2001, Qiu and Luo 2014, Abu Kwaik et al. 1998). Multiplication within protozoan hosts may eventually lead to cell lysis and dissemination of the bacteria. Some protozoan hosts, *Acanthamoeba* spp. for instance, encyst to survive in harsh environmental conditions (Marciano-Cabral and Cabral 2003). Ingested bacteria can survive in the cysts or cysts walls. For example, *L. pneumophila* were protected by *A. polyphaga* cyst from at least

50 mg L^{-1} of free chlorine (Kilvington and Price 1990). Lastly, undigested bacteria can be egested out of the protozoa in the form of vesicles or faecal pellets.

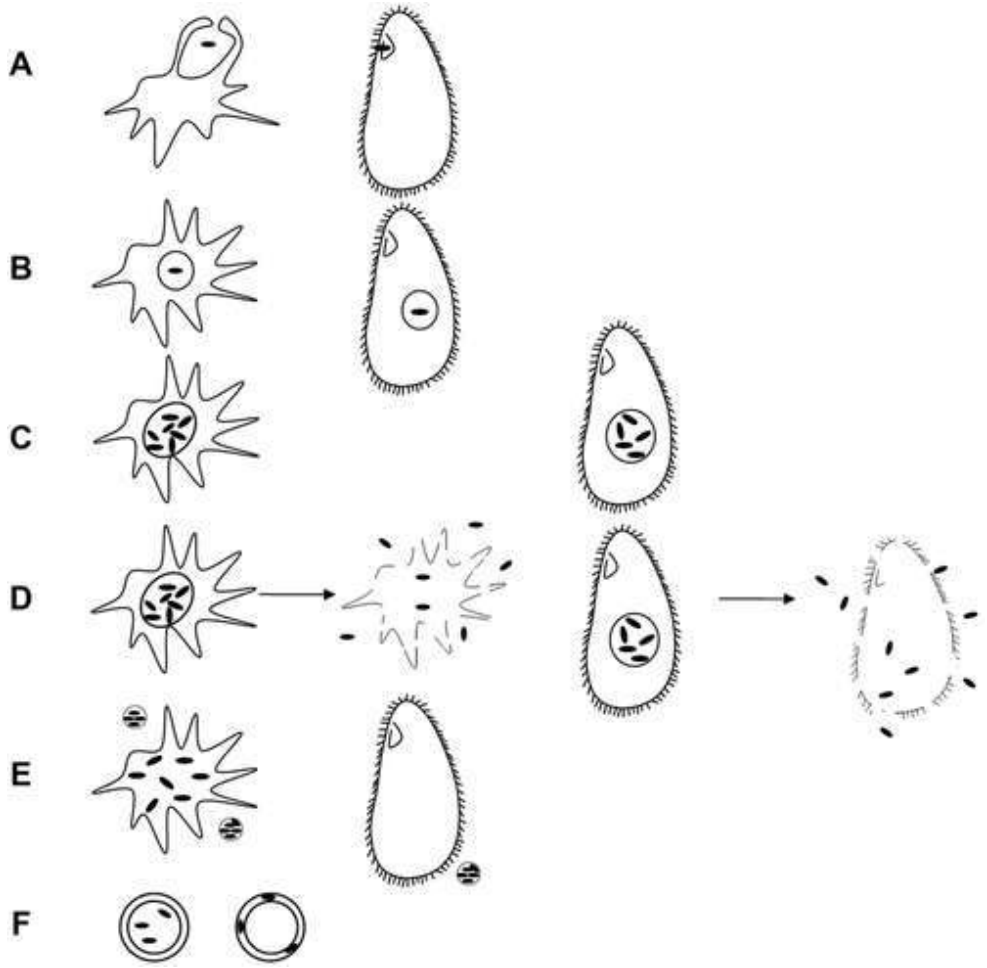

Figure 1-3: Different fate of bacteria post-ingestion by protozoa. (A) Bacterial uptake by protozoa. (B) Intracellular survival without replication. (C) Replication of bacteria within cellular compartment. (D) Replication of bacteria leading to rupture and lysis of protozoan hosts. (E) Egestion of bacteria via vesicles or fecal pellets. (F) Bacterial survival within cysts or cyst walls. (J.M. et al. 2014)

Certain pathogens, including *L. pneumophila*, *Salmonella enterica*, some strains of *Escherichia coli* and *Campylobacter jejuni* survive ingestion and are packaged into membrane bound vacuoles that are expelled from protists (J.M. et al. 2014). These vacuoles are also termed expelled food vacuoles (EFVs), vesicles, pellets, and multi-laminar bodies (MLBs). As early as 1980, *L. pneumophilia* was hypothesised as being transmissible from the

environment to humans by inhalation of vesicles containing live bacteria (Rowbotham 1980). EFVs containing *S. enterica* produced by *Tetrahymena* was shown to protect the encased bacteria against chemical stresses. *S. enterica* within EFVs can withstand calcium hypochlorite treatment (2 ppm) while planktonic bacteria surrounding the EFVs became non-viable (Brandl et al. 2005). In separate studies, *C. jejuni* and *L. pneumophila* were also shown to be packaged into EFVs and released into the culture media by *T. pyriformis* and *A. castellanii* respectively. These EFVs were subjected to nutrient-poor medium for extended periods of time (up to 60 h for *C. jejuni* and 6 months for *L. pneumophila*) and live bacteria cells from the EFVs could still be recovered, suggesting EFVs protect encased bacteria against starvation (Trigui et al. 2016, Bouyer et al. 2007). *L. pneumophila* released from EFVs incubated for 90 days in Osterhout's buffer were found to be more infectious than stationary-phase forms in human pneumocytes, indicating possible clinical relevance (Koubar et al. 2011). Taken altogether, due to the increased protection of bacteria in the EFVs against environmental stresses such as biocides and starvation, it has been suggested that EFVs provide a method of dissemination of pathogenic bacteria into the environment, possibly tainting food produce and causing human infections. Studies on clinical isolates of diarrheagenic pathotypes of *E. coli*, *H. pylori* and *S. enterica* support this hypothesis (Gourabathini et al. 2008, Smith et al. 2012). It has also been suggested that the presence of *L. pneumophila* in EFVs supported growth and survival of *T. pyriformis*, indicating a possible role for EFVs acting as a food stockpile for protozoa (Hojo et al. 2012). Currently, there are no reports on the interactions between protozoa and enterococci, resulting in a research gap on the fate of *E. faecalis* post-ingestion by protozoa.

1.4) Shaping of virulence traits by protozoa

Infections in human hosts are sometimes an evolutionary dead-end for some pathogens, as pathogens may cause disease in human hosts but are not capable of transmission to other

hosts. This may be due to eventual death of hosts, for example, pneumonia, otitis media and sepsis caused by *S. pneumoniae* though usually result in high mortality rates, but are non-contagious conditions (Brown, Cornforth, and Mideo 2012). In diseases caused by contact with non-human sources (such as zoonotic pathogens such as *C. jejuni*, *S. enterica* and *Borrelia burgdorferi*), or with environmental sources (such as *L. pneumophilia*), humans do not partake or play a negligible role in the transmission of these pathogens (Adiba et al. 2010, Sokurenko, Gomulkiewicz, and Dykhuizen 2006), and are thus a dead-end. Hence, to understand what evolutionary forces drive these pathogens to develop virulence traits that target human host systems but are of no or little value in onward transmission, the hypothesis of "co-incidental evolution" of virulence factors was formed.

The "co-incidental evolution" hypothesis states that virulence is a consequence of adaptation to other ecological niches outside the host and results in pathogenicity in the host, whereby defence mechanisms that allows the pathogen to survive predation can also cause harm or morbidity in a human host (Adiba et al. 2010). In that study, it was found that virulent *E. coli* strains were more resistant to amoeba grazing than avirulent strains (Adiba et al. 2010), suggesting a connection between ecology and pathogenicity. This phenomenon is not limited only to *E. coli*. Other studies reported *L. pneumophilia* could replicate intracellularly in amoeba and macrophages, even though the hosts are evolutionary distinct (Hilbi, Segal, and Shuman 2001, Molmeret et al. 2005). Using the Dot/Icm Type IV secretion system, *L. pneumophila* can establish intracellular survival in both human cells and protozoan hosts (Segal and Shuman 1999), showing common survival mechanisms within these two hosts. Comparative genomics studies have also shown both environmental and clinical *Chlamydia* possess orthologous genes for ADP/ATP translocases, a gene that is only found in obligate intracellular bacteria (Schmitz-Esser et al. 2004). This suggests the mechanisms for intracellular survival could be evolved from a common ancestor 700 million years ago,

preceding the evolution of higher eukaryotes (Horn et al. 2004, Schmitz-Esser et al. 2004). As such, intracellular survival could be evolved from the interaction with primitive unicellular eukaryotes (present day protozoa) (Strassmann and Shu 2017) and predation by protozoan grazers exert selective pressure on bacteria, leading it to acquire and keep adaptations that were once essential for survival, which now evolved to be a disease causing virulence trait (Amaro et al. 2015). Taken together, it was suggested that, in a non-clinical environment, protozoa can act as an evolutionary "training grounds" or "biological gym" for the evolution of virulence in pathogenic bacteria (Molmeret et al. 2005, Harb, Gao, and Abu Kwaik 2000, Harald 2007, Greub and Raoult 2004).

Thesis aims and organisation:

Protozoan predation in the environment is a major factor for bacterial mortality in the environment. To defend themselves from feeding protozoa, bacteria have developed various strategies to either evade capture or survive within the predator. As *Enterococcus* spp. are prevalent in the environment, they routinely encounter protozoa. Enterococcal grazing rates were shown to be 0.02 h^{-1} using typical dilution methods (Boehm, Keymer, and Shellenbarger 2005). In another study, enterococcal DNA was labelled with radioactive thymidine and by monitoring loss of radioactivity, mortality rates due to grazing were determined to be 0.01 to 0.03 h^{-1} (Menon, Billen, and Servais 2003). However, no studies so far described the interaction between protozoa and *E. faecalis*. Hence this thesis focuses on short-term (up to 24 h) and long-term (30 days) interactions between two model protozoa (*T. pyriformis* and *A. castellanii*). The aims of each chapter are:

Chapter 2: To determine fate of *E. faecalis* after ingestion by ciliate *T. pyriformis*. *E. faecalis* was demonstrated to survive intracellularly in phagocytes. Preliminary data showed that *E. faecalis* were packaged into EFVs. Therefore, this chapter will examine the form and function of these EFVs.

Chapter 3: To determine genetic and phenotypic changes after long-term acclimation to predation by *A. castellanii*. Long-term co-culture was established by feeding *E. faecalis* to *A. castellanii* for a period of 30 days and sequenced to investigate genetic changes due to acclimation to amoeba.

Chapter 2: Fate of *Enterococcus faecalis* post predation by *Tetrahymena pyriformis*

2.1) Introduction

Protozoan grazing is a major mortality factor for bacteria in natural environments and contributes significantly in the shaping of bacterial communities (Jurgens and Matz 2002). Predation by protozoa exerts a strong selective pressure, resulting in the evolution of anti-predatory mechanisms (Matz and Kjelleberg 2005, Jousset 2012). One such mechanism is the ability of some bacteria to resist digestion after being ingested by protozoa.

Undigested bacteria can replicate in the phagosome of the protist and may eventually lead to cell lysis and release of bacteria into the environment. During nutrient deprivation, some protozoa, such as *Acanthamoeba* spp. may form cysts and previously internalised bacteria can associate with the cyst wall or within the cyst, thereby protecting themselves from the harsh external environment (El-Etr et al. 2009, Kahane et al. 2001, Kilvington and Price 1990). Lastly, several studies had observed the packaging of pathogenic bacteria such as *L. pneumophila*, *S. enterica*, *E. coli*, and *C. jejuni* into vesicles, faecal pellets, or multilaminar bodies (MLB) and expelled out of the cell (Berk et al. 2008, Trigui et al. 2016, Brandl et al. 2005, Smith et al. 2012). These observations were commonly made in ciliates, such as *Tetrahymena* spp. (Berk et al. 2008, Brandl et al. 2005, Gourabathini et al. 2008, Hojo et al. 2012, Smith et al. 2012, Trigui et al. 2016) and various species of amoeba (Berk et al. 1998, Marciano-Cabral and Cabral 2003). These expelled food vacuoles (EFVs) have been suggested to be involved in protecting encased bacteria from external stress and dissemination of bacteria into the environment (Smith et al. 2012, Gourabathini et al. 2008, Brandl et al. 2005).

Here, the resultant post-ingestion fate of *E. faecalis* by a heterotrophic ciliate, *T. pyriformis* was investigated. *E. faecalis* was shown to able to survive intracellularly within food

vacuoles of *T. pyriformis*. Continuous co-culture of *E. faecalis* and *T. pyriformis* resulted in the formation of EFVs that contained live bacteria. The role of EFVs was further examined by investigating the conditions that lead to the release of bacteria from the EFVs. The data suggest that EFVs can lyse and release bacteria when incubated in media with different nutrient levels, suggesting presence of nutrients is not the major trigger for bacterial release. EFVs were shown to confer protection against antibiotics to bacteria encased inside, where live bacteria were a recovered after incubating EFVs in a mixture of gentamycin and penicillin G.

2.2) Materials and Methods

2.2.1) Organisms and culture conditions

E. faecalis OG1RF (ATCC 47077) was grown in BHI broth (Becton Dickinson, USA). It was plated on BHI agar from glycerol stocks that were stored at -80°C before inoculating a single colony in 10 ml BHI broth and growing at 37°C with shaking overnight before every experiment. Bacterial cultures were pelleted by centrifugation at 8000 × *g* and resuspended in 1× M9 salts (MP Biomedicals, USA). The concentration of bacterial cells was then estimated by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Shimadzu). Unless otherwise stated, the concentration of *E. faecalis* was adjusted to $OD_{600\ nm}$ of 0.125 which corresponds to approximately 10^8 cells/ml.

T. pyriformis was maintained at room temperature with shaking at 50 rpm in PYG media (2% peptone, 0.1% yeast extract; Becton Dickinson, USA, and supplemented with 0.1 M glucose; Sigma Aldrich, USA). *T. pyriformis* was passaged every week by transferring 500 µl to a new tissue culture flask containing 10 ml of fresh PYG media. Unless otherwise stated, 10 µl aliquots were mixed with 10 µl of Lugol's solution (Sigma Aldrich, USA) before

enumeration by microscopy using a haemocytometer. *T. pyriformis* density was adjusted to 10^4 cells/ml using 1× M9 at the start of each experiment.

2.2.2) Co-culture assay (*E. faecalis* and *T. pyriformis*)

T. pyriformis (*c.* 3×10^3 cells/ml) and *E. faecalis* (*c.* 3×10^7 cells/ml) were incubated in 1 × M9 and 0.5 × tryptic soy broth (Sigma Aldrich, USA) at room temperature with shaking at 50 rpm. Unless otherwise stated, Hoechst 33342, a cell-permeable nucleic acid dye was used to stain double stranded DNA in the co-culture. To check for bacterial uptake and persistence of *E. faecalis* within *T. pyriformis*, every 4 h, 10 µl of the stained samples was extracted, fixed with 2.5% glutaraldehyde and viewed microscopically (Axio Observer.Z1 Epifluorescence Widefield microscope; Carl Zeiss, Germany). Hoechst 33342 has an excitation/emission spectrum of 346 nm and 460 nm.

2.2.3) Thin-section transmission electron microscopy (TEM).

Co-cultures were prepared as described above. Using a cell-scraper, samples were extracted and resuspended in a 2% paraformaldehyde, 2.5% glutaraldehyde solution (Polysciences, Warrington, PA) in 100 mM PBS (pH 7.4) for 1 h at room temperature. The samples were then embedded in 2% low-melting-point agarose, washed in PBS, and postfixed in 1% osmium tetroxide for 1 h. Samples were rinsed extensively in distilled water (dH_2O) prior to enbloc staining with 1% aqueous uranyl acetate (Ted Pella, Inc., Redding, CA) for 1 h. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella, Inc., Redding, CA). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL, USA), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).

2.2.4) Live-cell imaging

Live cell imaging was performed to determine which conditions were required for lysis of EFVs. EFVs were generated by co-culturing *T. pyriformis* and *E. faecalis* for 24 h. Wells were washed twice with 1 × M9 to remove the ciliates and planktonic bacteria before replacing the wells with 500 µl of fresh BHI, spent BHI or 1 × M9. Fresh BHI represents nutrient rich media. Spent BHI was collected by sterile filtration of overnight culture and represents nutrient depleted media. 1 × M9 represents media with no nutrients. Live-cell imaging was performed on Axio Observer.Z1 Epifluorescence Widefield microscope (Carl Zeiss, Germany) at 25 °C.

2.2.5) Isolation of EFVs

To obtain a pure sample of EFVs, a sterile cell-scraper was used to dislodge settled EFVs and then the mixture was filtered through a nitrocellulose membrane filter (8 µm pore size) (Merck Millipore, Germany) to remove *T. pyriformis*. The membrane filter was washed with 2 ml of 1× PBS (0.8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4) and the filtrate was passed through a 3 µm pore size nitrocellulose membrane filter to separate the EFVs from planktonic bacteria. The membrane filter was washed with 2 ml of 1× PBS and retained EFVs were washed off by vortexing in a 50 ml tube containing 2 ml of 1× PBS. To check for the presence of EFVs, a 10 µl sample was observed under the microscope. EFVs were treated with 1% Triton X-100 and vortexed for 1 min to lyse the EFVs. Samples were serial diluted with 1 × PBS before drop-plating on BHI agar plates for determination of colony forming units (CFU).

2.2.6) Phagosomal tracking in *T. pyriformis*

LysoTracker® Red DND-99 (ThermoFisher Scientific, USA), a cell-permeable red-fluorescent dye that labels acidic organelles, was used to stain for the acidified phagosomes

and/or lysosomes in *T. pyriformis*. LysoTracker® Red DND-99 (final concentration of 225 nM) was added to a 1.5 ml mixture of *T. pyriformis* (10^4 cells/ml) and *E. faecalis* (10^8 cells/ml). The sample was incubated statically in the dark for 30 minutes at room temperature. To immobilise *T. pyriformis*, 8 µl of the stained sample was combined with 8 µl of $NiCl_2$ (final concentration of 2 mM) on a microscope slide and viewed by microscopy (Axio Observer.Z1 Epifluorescence Widefield microscope; Carl Zeiss, Germany). LysoTracker® Red DND-99 has excitation/emission wavelengths of 577/590 nm.

2.2.7) Plasma membrane staining

CellMask™ (ThermoFisher Scientific, USA) was used to stain plasma membranes. Co-cultures in 24-well microplates and were fixed with 2% paraformaldehyde (PFA: 4% dissolved in water and adjusted to pH 7.4 by conc. HCl) and incubated statically at 37°C for 1 h. Fixed samples were viewed under Axio Observer.Z1 Epifluorescence Widefield microscope (Carl Zeiss, Germany). The excitation/emission spectrum for CellMask™ is 646/666 nm.

2.2.8) Microscopic image analysis

Microscopic images were analysed using ImageJ (Schneider, Rasband, and Eliceiri 2012, Schindelin et al. 2012). MicrobeJ plugin (Ducret, Quardokus, and Brun 2016) was used to measure the size distribution of EFVs. Three-dimensional representations of EFVs were generated using ImageJ 3D viewer plugin (Schmid et al. 2010).

2.3) Results

2.3.1) *E. faecalis* persists within *T. pyriformis* post-ingestion

The predator-prey relationship between *E. faecalis* and *T. pyriformis* was examined by co-incubation over 24 h at the ratio of $1_{predator}$:10000_{prey}. After co-culture, the samples were

stained with Hoescht 33342 to label the dsDNA of *T. pyriformis* and *E. faecalis*. The ciliates began to take in the bacteria internalised them upon co-incubation. Individual blue stained cocci within the cell were discernible over 24 h, suggesting that the bacterial cells were not digested during the co-incubation (Figure 2-1).

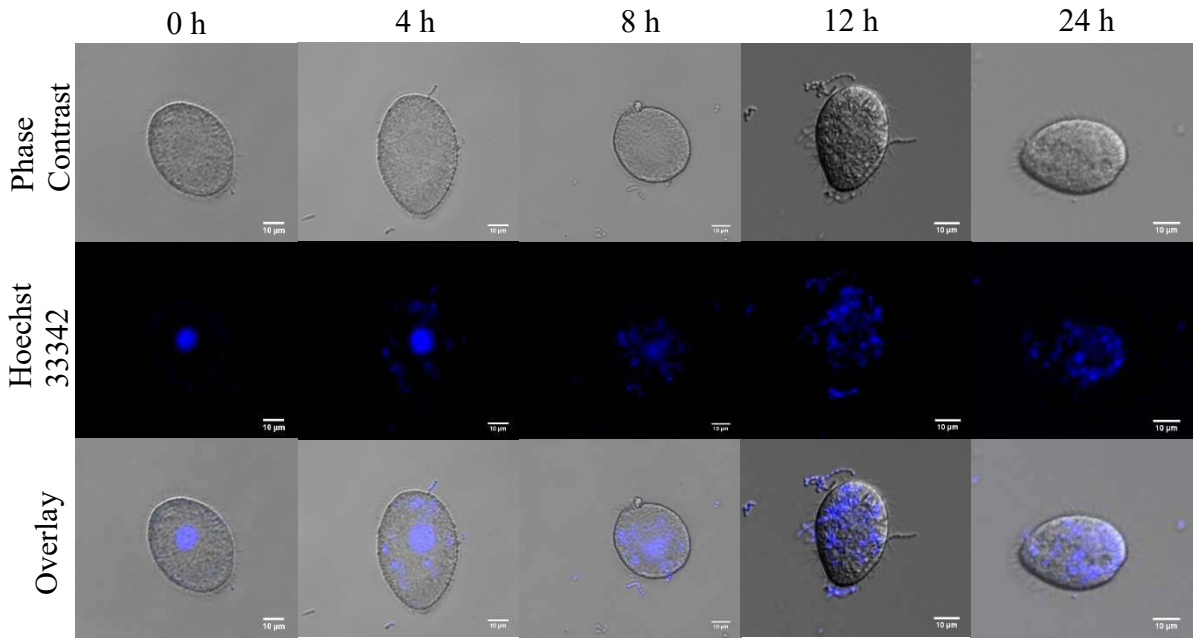

Figure 2-1: *T. pyriformis* ingests *E. faecalis*. *T. pyriformis* were fed with live *E. faecalis* and random samples of the co-culture transferred to a slide and viewed by microscopy every 4 h over a 24 h period. *E. faecalis* were observed to be internalised by the ciliate. Magnification: 40 ×. Scale bar: 10 µm

To determine if the internalised *E. faecalis* were packaged in to food vacuoles, thin section TEM were performed on co-cultured samples and revealed *T. pyriformis* cells containing bacterial cells in food vacuoles. Diplococci cells were observed entering the *T. pyriformis* buccal cavity (Figure 2-2 A). The average number of food vacuoles observed per section was between 4 and 7. Figure 2-2 B shows partial digestion of cargo within the food vacuole, as characterised by membrane whorls (black arrow, insert) (Nilsson 1977), while others contain intact *E. faecalis* (Figure 2-2 C, white arrow). Collectively, *E. faecalis* was observed to be ingested by the ciliate *T. pyriformis* and although some of the bacterial cells were being digested and destroyed, others stayed intact within the food vacuoles for up to 24 h.

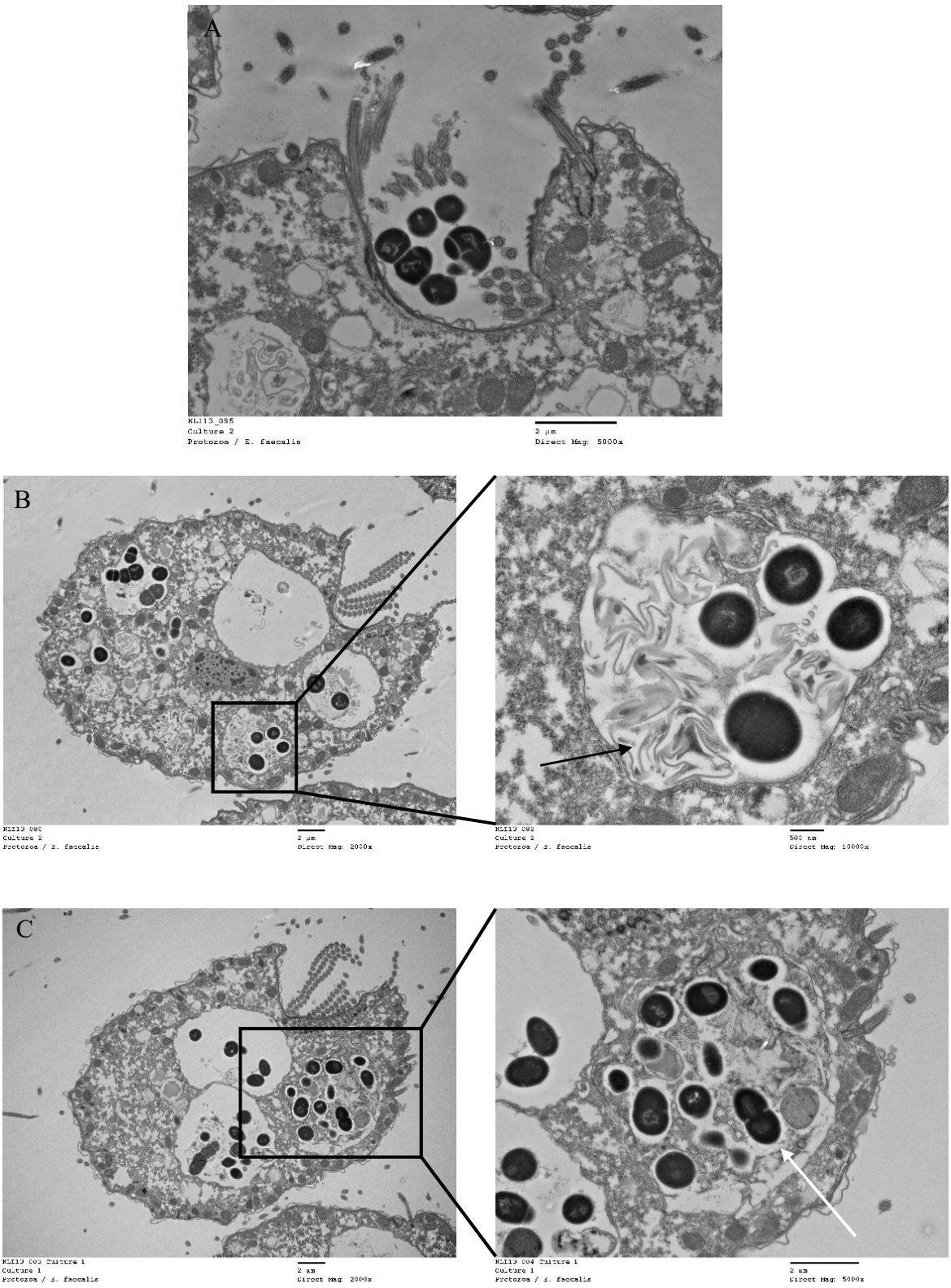

Figure 2-2: TEM of *T. pyriformis* containing *E. faecalis*. (A) *E. faecalis* entering the buccal cavity of *T. pyriformis*. (B and C) Food vacuoles contain a mixture of partially digested bacteria (black arrow) and intact diplococci (white arrow).

2.3.2) Lack of acidification in phagosomes containing *E. faecalis*

To explore the process of non-destructive ingestion of *E. faecalis* in *T. pyriformis*, acidic phagosomal/lysosomal compartments of *T. pyriformis* were stained with a red-fluorescent dye, LysoTracker™. Previous studies describe the use of LysoTracker™ to label acidified phagosomes/lysosomes in *Tetrahymena* spp for phagocytotic (Samaranayake, Cowan, and Klobutcher 2011, Bright et al. 2010) and autophagy (Akematsu et al. 2014, Akematsu, Pearlman, and Endoh 2010) pathways. Here, *T. pyriformis* was fed with heat-killed *E. faecalis* for 30 minutes and thereafter immobilised with $NiCl_2$ for microscopy. Co-localisation of red fluorescence (LysoTracker™) and blue fluorescence (Hoechst 33342) in food vacuoles indicated acidification of food vacuoles (Figure 2-3 panel B, white arrows). When *T. pyriformis* was fed with live *E. faecalis*, although acidic compartments within *T. pyriformis* were observed, weak red fluorescence was detected in the food vacuoles (Figure 2-3 panel C, yellow arrows). A starved *T. pyriformis* control was used to show no LysoTracker™ staining when there is no uptake of bacterial prey (Figure 2-3 A). Some ciliates, however, had odd morphology and displayed faint LysoTracker™ staining (data not shown). This could be the accumulation of lysosomes in the progression of autophagy and programmed cell death. Collectively, the data suggested that live *E. faecalis* were packaged into food vacuoles in *T. pyriformis* and may survive digestion by preventing acidification of phagosomes. This observation was similar to a previous study (Zou and Shankar, 2016) where they too observed lack of co-localisation of *E. faecalis*-containing compartments to the acidic lysosomal compartments in macrophages.

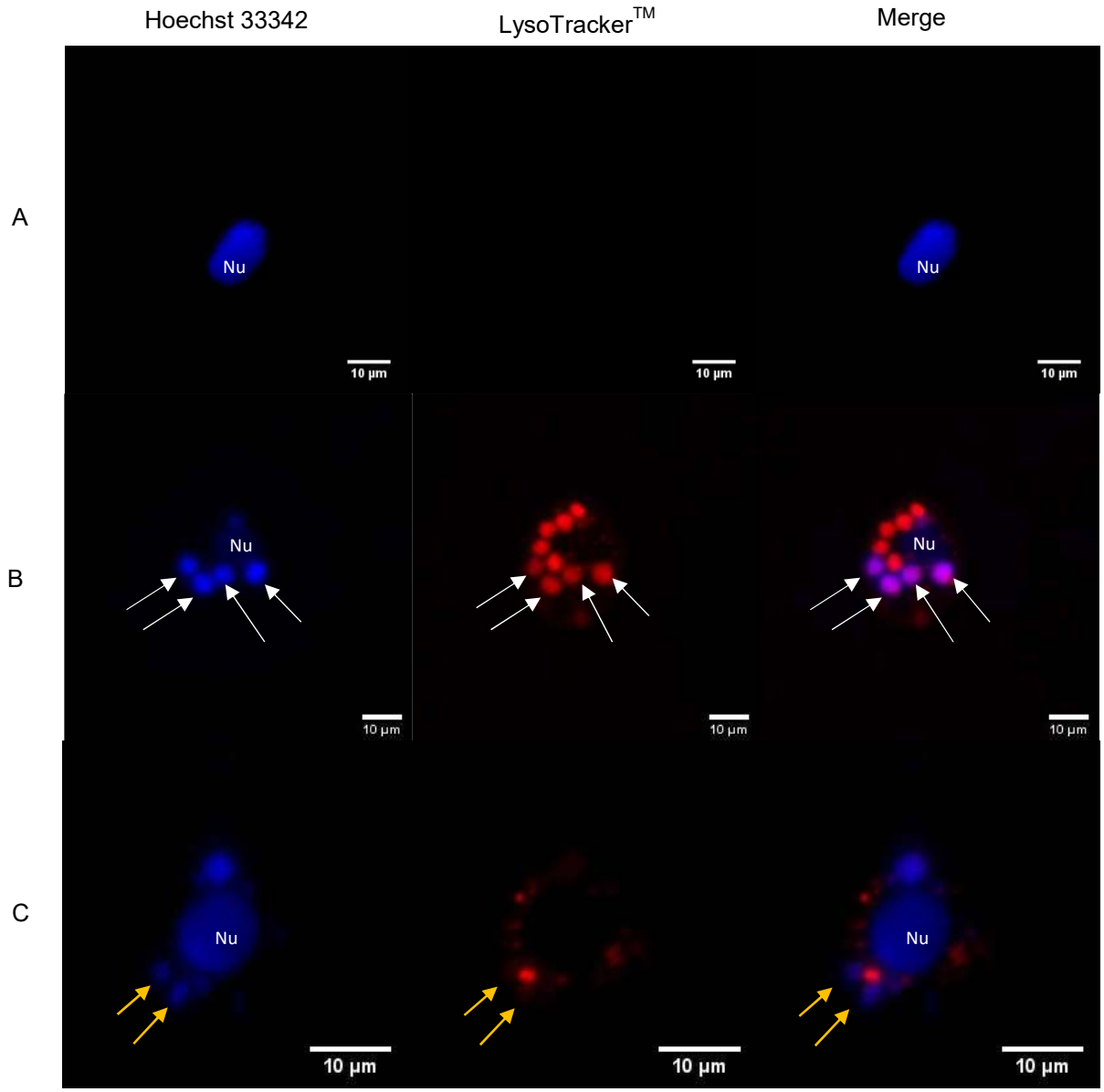

Figure 2-3: Live *E. faecalis* may resist digestion by preventing acidification of phagosomes. DNA was stained with Hoechst 33342 and acidic intracellular compartments were stained with LysoTracker™. (A) Starved *T. pyriformis* showing absence of food vacuoles and acidic lysosomal compartments. (B) *T. pyriformis* fed with heat-killed bacteria showed acidic compartments co-localising with food vacuoles (white arrows). (C) *T. pyriformis* fed with live *E. faecalis* showed multiple acidic compartments but food vacuoles containing *E. faecalis* were only weakly stained by LysoTracker™ (yellow arrows), suggesting resistance to digestion within *T. pyriformis*. Objective magnification: 40×. Scale bar: 25µm. Representative images were taken from 2 biological replicates. Nu: *T. pyriformis* nucleus

2.3.3) Non-destructive ingestion of *E. faecalis* results in formation of EFVs

Some pathogens are packaged in food vacuoles that are expelled out of the protozoan host (J.M. et al. 2014). Visual observation under bright-field microscopy of the co-culture after 24 h incubation shows the presence of EFVs in the extracellular milieu (Figure 2-4 A). EFVs were automatically detected using the MicrobeJ plugin of ImageJ, based on estimated Feret's diameter, area and circularity. EFVs were observed to have diameters ranging from 3 to 10 µm, and most were approximately 4 µm in diameter (Figure 2-4 B). EFVs were found both in the planktonic phase and on the bottom surface of the well. Both EFVs and *T. pyriformis* were enumerated by manual counting and it was found that on average, each *T. pyriformis* produced 7.4 EFVs in 24 h. Unfed *T. pyriformis* was used as a control and little or no EFVs were observed in these samples (data not shown).

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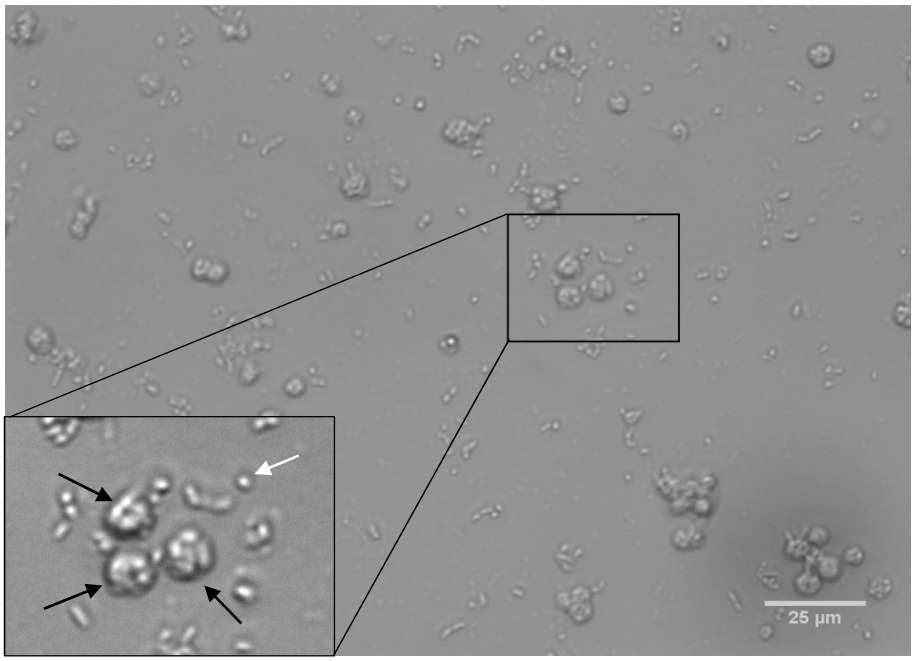

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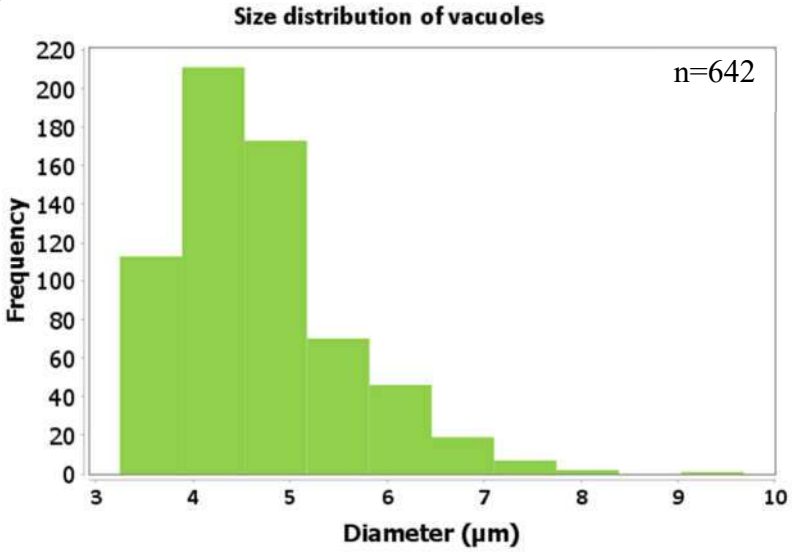

Figure 2-4: Co-culture of *T. pyriformis* and *E. faecalis* yielded EFVs. *T. pyriformis* was fed with *E. faecalis* at the ratio of 1:10000 over 24 h at 25 °C with gentle agitation. (A) EFVs (insert, black arrows) were formed as early as 2 h (data not shown). White arrow points to planktonic *E. faecalis*. Objective magnification: 40 ×. Scale bar: 25 µm. Representative image was taken from 3 biological replicates. (B) Measuring the longest distance from two ends, the size distribution of EFVs were found to be 3 – 7 µm, with most EFVs having a diameter of 4 µm.

2.3.4) Live *E. faecalis* was observed to be encased in EFVs

To confirm the viability of *E. faecalis* in the EFVs, EFVs were extracted from the co-culture by filtration which yielded planktonic bacteria as the filtrate and EFVs as the residue on the membrane filter. EFVs were treated with 1% Triton X-100 before CFU enumeration. Results show that CFUs were recoverable from EFVs (Figure 2-5 E), substantiating that live *E. faecalis* were packaged into EFVs and egested into the environment.

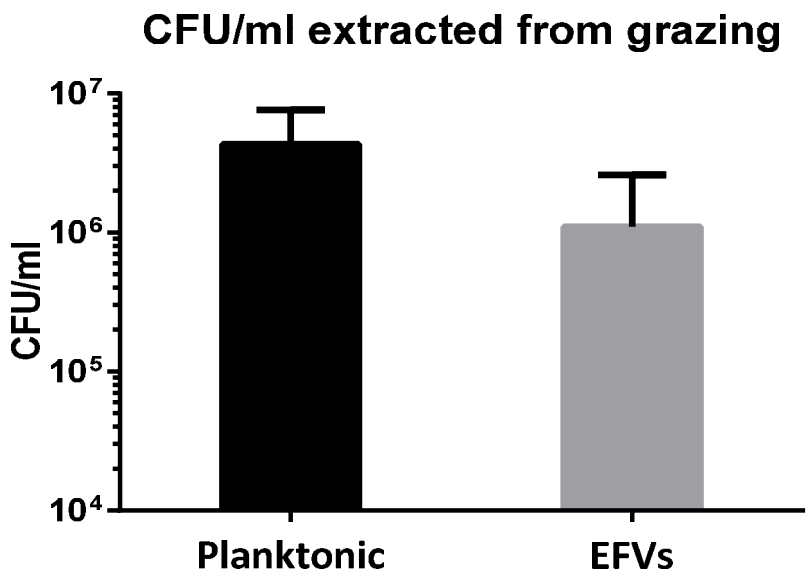

Figure 2-5: Live *E. faecalis* were present within EFVs. Colony forming units (CFU) recovered from EFVs that were purified by filtering the co-culture with 8 µm and subsequently 3 µm membrane filters, showing live cells were present in EFVs.

2.3.5) Bacteria are encased in membrane bound EFVs

CellMask™ Deep Red, an amphipathic lipid dye was used to pre-stain *T. pyriformis* before feeding on *E. faecalis*. CellMask™ stained the plasma membrane and the food vacuoles containing *E. faecalis*, showing that the dye can be endocytosed (Figure 2-6 A). To characterise EFVs, confocal laser scanning microscopy (CLSM) was used to image EFVs on multiple Z-stacks to form a composite three-dimensional image. Here, EFVs were shown to

be spherical membranous structures that encase bacteria (Figure 2-6 C). Mid-slices of the Z-stacks were also taken, and bacteria were observed to be arranged randomly within a "ring" of membranous structure (Figure 2-6 C, white arrow)

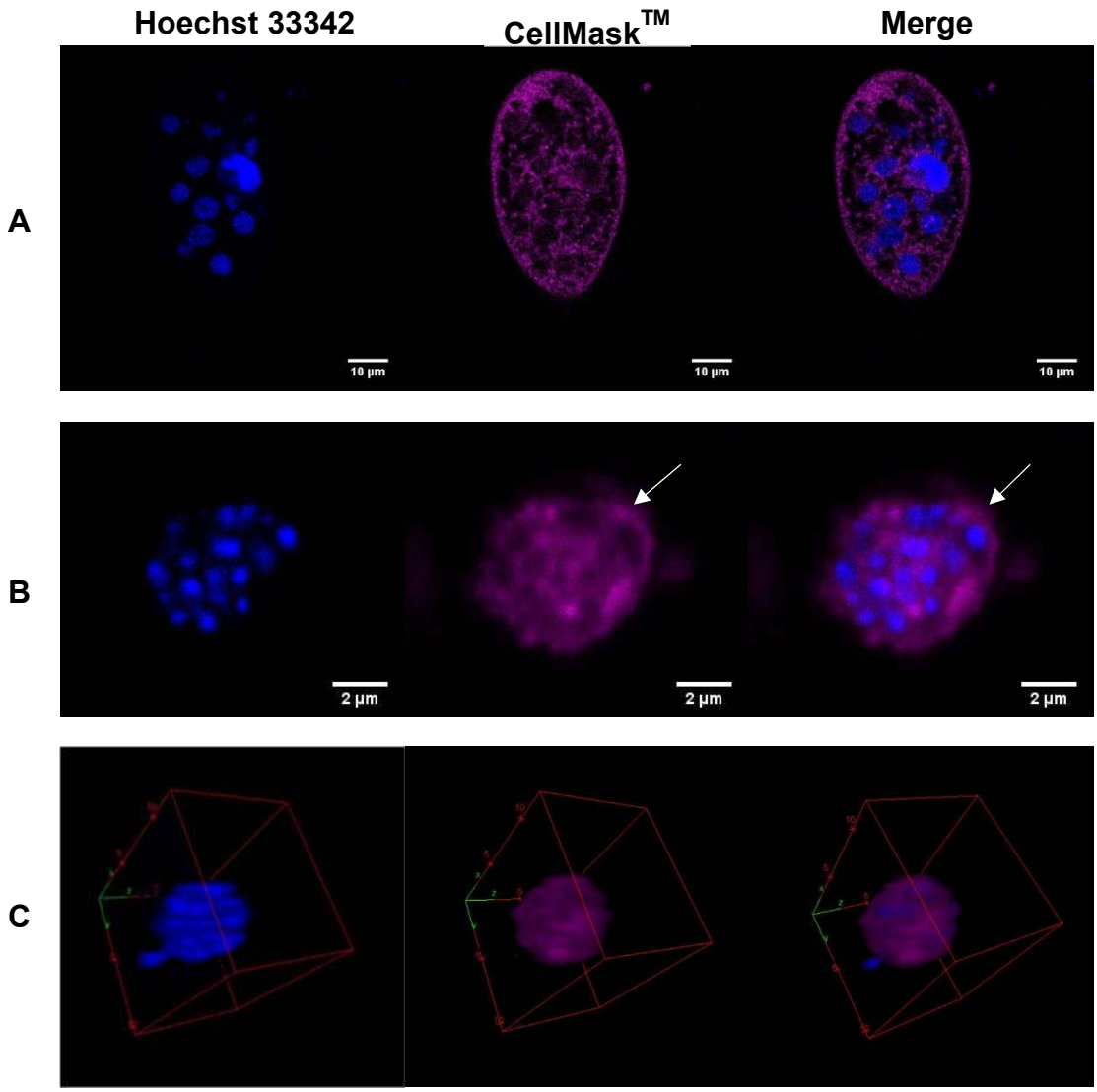

Figure 2-6: EFVs are plasma membrane bound. (A) *T. pyriformis* was pre-stained with CellMask™ before feeding on *E. faecalis*. Food vacuoles containing Hoechst 33342 stained bacteria and a "ring" of magenta CellMask™ was observed. (B) Planar view of EFVs stained with CellMask™ (magenta) and *E. faecalis* stained with Hoescht 33342 (blue). *E. faecalis* were observed to be encased in a random fashion within the EFV's membrane. White arrow shows a "ring" of magenta fluorescence around the encased

bacteria. (C) Z-series of EFVs were taken and represented in three-dimensional volume view. Objective magnification: 100×. Scale bar: 5µm. Representative images were taken from 3 biological replicates.

2.3.6) EFVs lyse to release bacteria in the milieu

It has been hypothesised that for effective dissemination of *E. faecalis* in the environment, bacteria within the EFVs must be able to escape. To simulate different environmental conditions, EFVs in co-culture were washed to remove the ciliates and planktonic cells, before replacing the media with either fresh BHI, spent BHI or M9. Fresh BHI simulates a nutrient-rich environment, while spent BHI and M9 simulate a nutrient-poor environment and absence of nutrients respectively. Live cell imaging (LCI) was performed to identify when EFVs lyse and release the bacteria. EFVs were observed to lyse at beginning of 114^{th} min, 135^{th} min and 126^{th} min in fresh BHI (Figure 2-7 A), spent BHI (Figure 2-7 B) and M9 (Figure 2-7 C) respectively.

To determine whether cells within the EFVs are protected against environmental stresses, the EFVs were incubated in bactericidal concentrations of a mixture of gentamycin and penicillin G and survival was compared to planktonic cells (Figure 2-8 A). To verify that bacteria within EFVs were protected against the antibiotics in the medium, EFVs were collected by filtration and plated on agar plates for CFU enumeration. With Figure 2-5 E as a reference, planktonic bacteria cell CFUs fell approximately 3 $\log_{10}$ fold from expected ~10^6 to ~10^3 CFU mL^{-1}. Cells encased in EFVs, on the other hand, stayed viable with no decrease of CFU mL^{-1} (Figure 2-8 B). Taken together, EFVs confer antibiotic protection to the enclosed bacteria, and release of bacteria from EFVs depends on the environmental conditions but not by nutrient availability.

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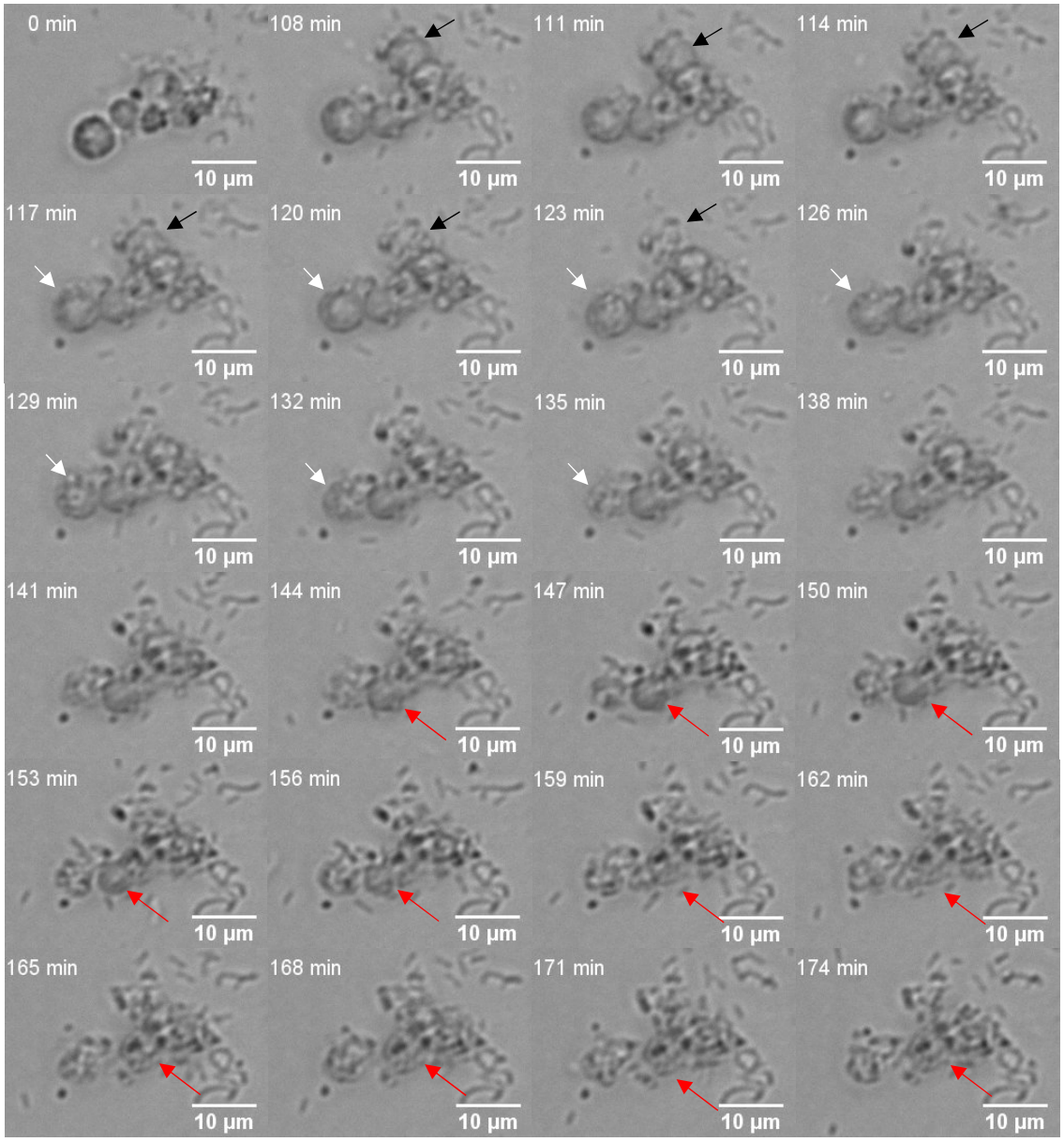
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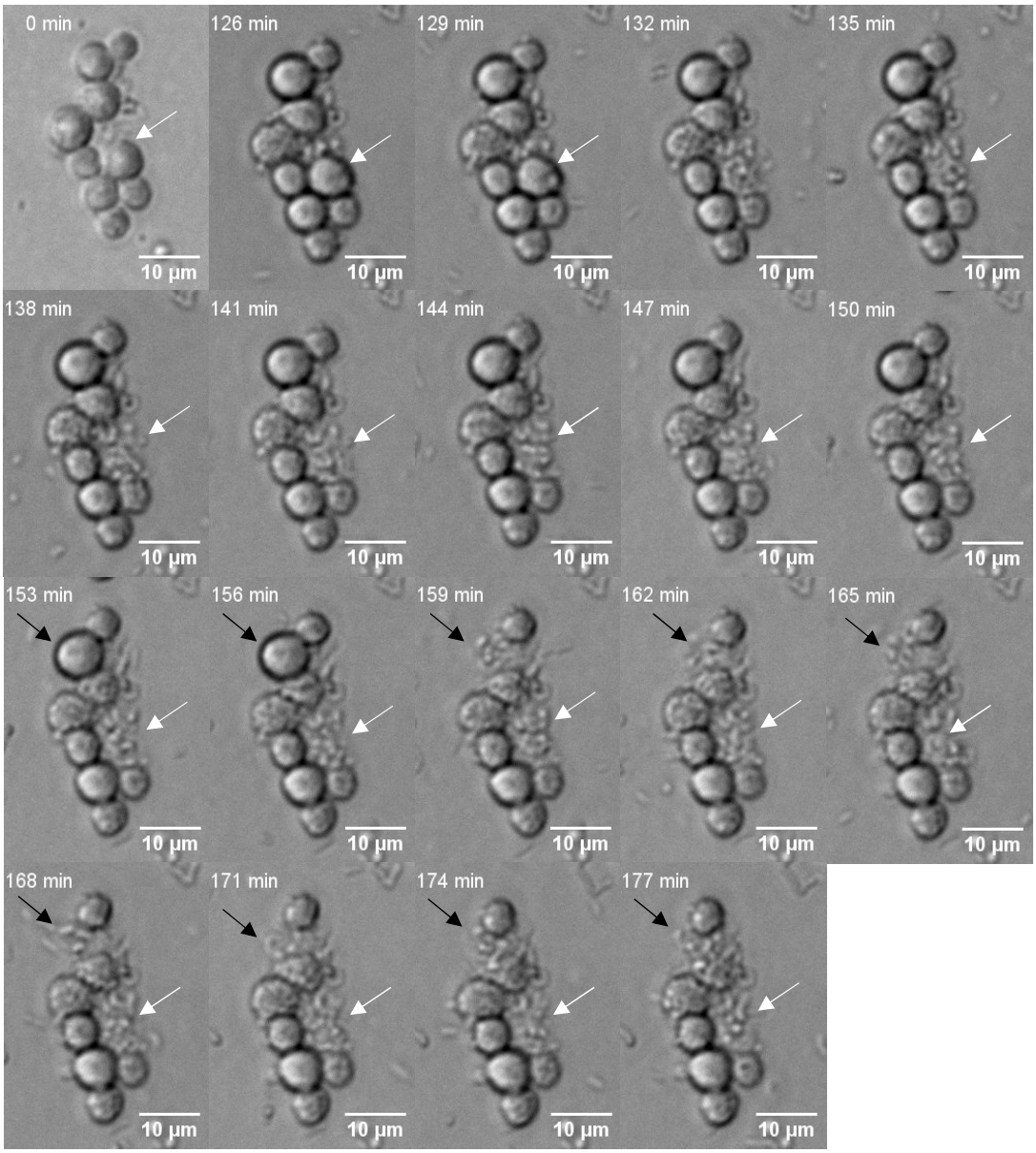
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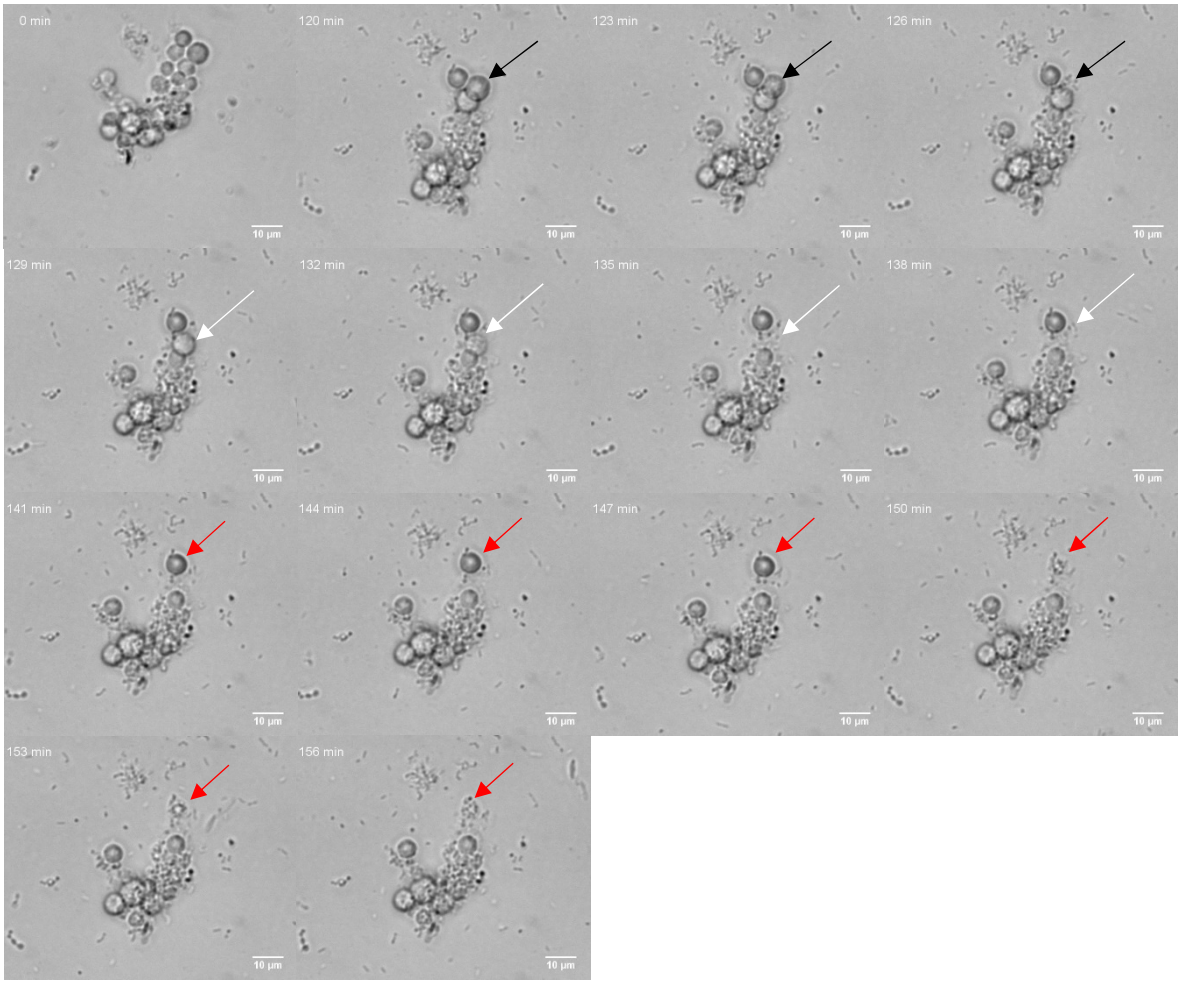

Figure 2-7: EFVs lyse to release *E. faecalis* into the environment. EFVs were first cultivated by co-culture between *T. pyriformis* and *E. faecalis*. Co-culture media was replaced with (A) fresh BHI, (B) spent BHI, or (C) 1×M9 and was viewed under microscope over 9 h. Arrows of different colours point to individual EFVs which was observed to lyse. *E. faecalis* was observed to escape from EFVs in all above mentioned conditions. Objective magnification: 40 ×. Scale bar: 10 µm.

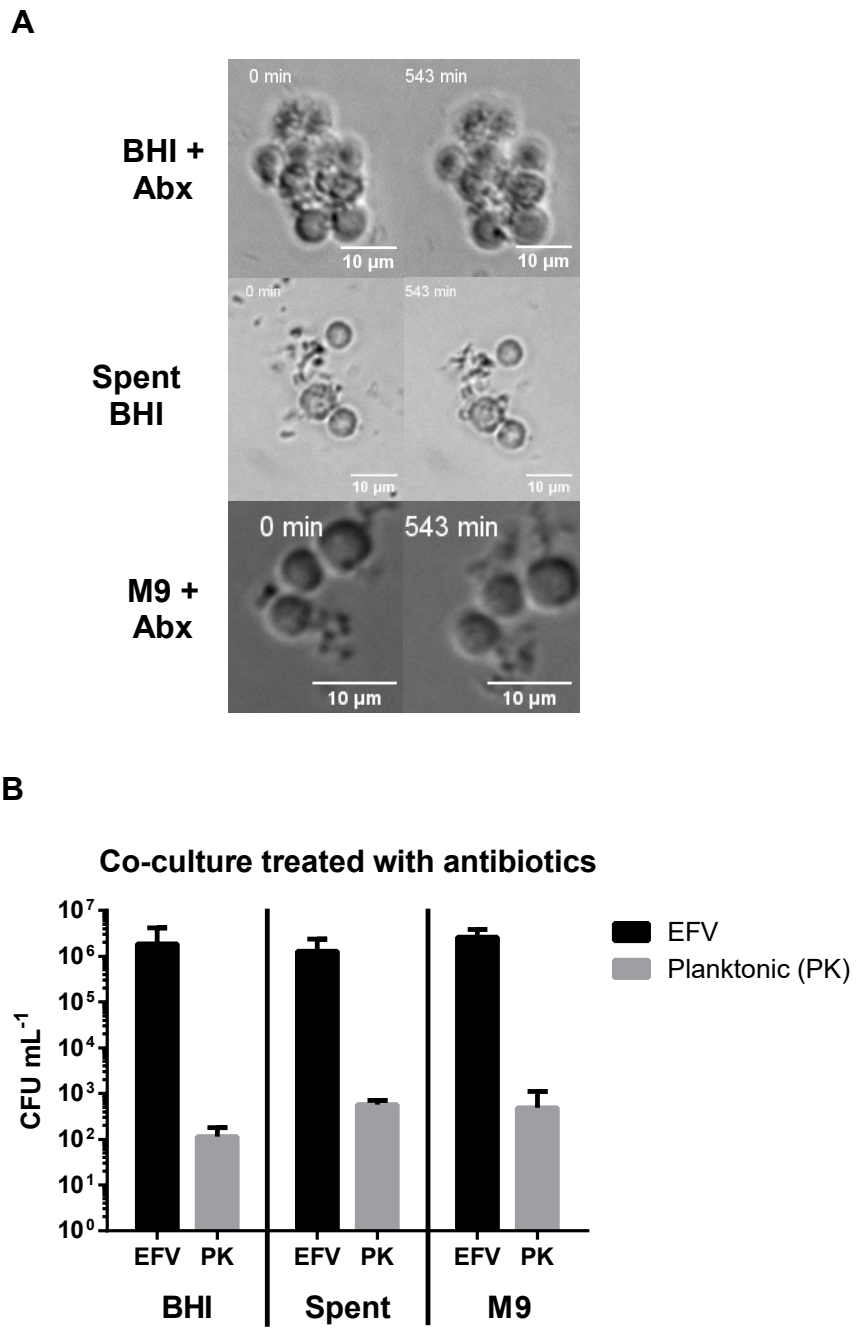

Figure 2-8: *E. faecalis* do not release from EFVs when antibiotics were present in the replaced media. EFVs were cultivated by co-culture for 24 h and the media replaced with fresh BHI with antibiotics, spent BHI with antibiotics and 1 × M9 with antibiotics. Antibiotics refer to a mixture of 500 µg/ml of gentamicin and penicillin G. (A) After replacement of media, EFVs were viewed under the microscope for 9 h. *E. faecalis* did not appear to escape from the EFVs over these 9 h. Objective magnification: 40 ×. Scale bar: 10 µm. (B) EFVs were purified by filtration and plated for CFU. High CFU numbers in EFVs

as compared to planktonic bacteria suggested EFVs provided protection against antibiotics in the medium.

2.4) Discussion

E. faecalis is a Gram-positive bacterium that is commonly found in the gut. However, it is also an opportunistic pathogen that causes human and animal infections globally. Although it is recognised as one of the main indicators of waterborne pathogens in recreational water (Byappanahalli et al. 2012), little is known about the interactions between enterococci and bacterivorous predators in the environment. Previous studies have shown that *Salmonella enterica* and *Legionella pneumophilia* are expelled in vesicles post ingestion by *T. pyriformis* (Berk et al. 2008, Brandl et al. 2005). However, there is little knowledge on the fate of *E. faecalis* after non-destructive ingestion by environmental predators. In this study, the effects of the interactions between *E. faecalis* and *T. pyriformis* were closely examined.

It was shown that although *E. faecalis* is not an obligate intracellular bacterium, it can persist within *T. pyriformis* for long periods of time without being digested. The inability to digest *E. faecalis* could be explained by the lack of acidification in the food vacuoles (Figure 2-3). In mammalian phagocytic cells, phagosomes are progressively acidified by a series of endosomal and lysosomal fusion events. Acidification of phagosomes creates a low pH environment which has a bacteriostatic or bactericidal effect. Furthermore, it is necessary for activation of host enzymes that digest bacteria in the phagosomes (Hackam, Rotstein, and Grinstein 1999, Ip et al. 2010). In this study, although it was shown that there was lack of phagosome acidification, it is not known at which stage does the acidification was prevented. To answer this, future experiments could be designed to label the phagosome pathway with antibodies that are specific for protein markers on the phagosome surface at different stages

of endosomal pathway. For example, Rab5 and Rab7 are found on early and late endosomal membranes, respectively.

Also, it was demonstrated that when *T. pyriformis* was co-cultured with *E. faecalis*, EFVs were expelled into the media (Figure 2-4) while starved *T. pyriformis* did not release EFVs, suggesting that this process requires the presence of bacteria. In addition, live *E. faecalis* is needed for EFV production as little or no EFVs were formed when *T. pyriformis* was fed with heat-killed *E. faecalis*. This suggests that the formation of EFVs does not involve the expression of bacterial factors on the cell surface, but instead secreted factors may be involved. These secreted factors may be key in intracellular survival. For example, *L. pneumophilia* utilises the Dot/Icm Type IV secretion system to modify the endosomal pathway and prevent bacterial cell digestion (Berk et al. 2008, Hilbi, Segal, and Shuman 2001, Qiu and Luo 2014).

Using various chemical dyes, EFVs were shown to be made up of membranes and each EFV contains live *E. faecalis* (Figures 2-3 to Figure 2-6). The presence of live bacteria in the vacuoles was verified by purification of EFVs from the co-culture, lysing them with Triton X 100 and plating the released cells on BHI agar plates (Figure 2-5 E). Here, CellMask^{TM} was used as a dye to stain for plasma membranes. The plasma membrane of *T. pyriformis* was stained as well as the membranes of food vacuoles (Figure 2-6 A). EFVs were stained as well and the staining pattern of a "ring" wrapping around bacteria suggested that they are membrane bound (Figure 2-6 B). This was also observed in *Legionella*-containing pellets where partial digestion of bacteria accumulates membranous material in the pellet (Berk et al. 2008). In the same study, ultrastructural analysis of pellets showed membrane fragments lining the pellets originated from bacteria (Berk et al. 2008). Here, the data suggests that EFV is surrounded by a single membrane. Therefore, to identify the membrane donor, a dye that

stains only eukaryotic cell membranes will be used to stain the EFVs in the future. As cholesterol is absent in prokaryotic cells, it may be a target for staining to better visualise the vacuolar membrane.

Different species of bacteria can be expelled in EFVs after ingestion by protists. For example, ciliates have been suggested to expel *L. pneumophila* in spherical pellets as a food stockpile for the ciliates' growth and survival (Hojo et al. 2012). Previous studies have shown that cells within the *Legionella*-laden pellets could survive long term starvation in low nutrient medium (Koubar et al. 2011) while it has been shown that *S. enterica* in vesicles were resistant to calcium hypochlorite treatment (Brandl et al. 2005), suggesting that EFVs can provide protection against unfavourable environmental conditions. The vacuoles may also allow transmission of the bacteria from one place to another, be it in the environment or into a host, potentially aiding in the colonisation of new sites. To be ecologically relevant in the dissemination of bacteria via EFVs, bacteria must be able to release from the EFVs. Here, it was demonstrated that regardless of the amount of nutrients in the environment, EFVs will lyse and release bacteria into the milieu. The protective function of EFVs was also examined by exposing the co-culture to high concentrations of antibiotics and subsequently isolate EFVs for CFU plating. When exposed to antibiotics, bacteria within EFVs were shown to remain viable as compared to planktonic cells from the same co-culture. Taken together, packaging of bacteria into EFVs can be a method of dissemination, and due to its protective nature against antibiotics, EFVs can complicate treatment options during an infection.

Overall, it was shown that grazing of *E. faecalis* by *T. pyriformis* resulted in non-digestion passage through the ciliate and the bacteria were packaged into EFVs. These EFVs were able to release the encased bacteria in the environment, suggesting a possible route of transmission in the environment. EFVs can also protect bacteria by conferring them a physical barrier protection from antibiotics in surrounding medium.

Chapter 3 Effects of long-term predation on *E. faecalis* by *Acanthamoeba castellani*

3.1) Introduction

In the environment, due to strong predative pressure exerted, bacteria have developed various evasion and antipredator mechanisms. One such mechanism is the formation of microcolonies or bacterial aggregates that are resistant to protozoan uptake (Hahn and Hofle 2001, Hahn, Moore, and Hofle 2000). For instance, *Vibrio cholerae* forms biofilms that are resistant to grazing by surface-feeding protozoa (Matz et al. 2005). Biofilms contain one or more communities of bacteria which associate with each other in a matrix of extracellular polysaccharide (EPS). Biofilms protect bacterial cells from stresses such as antibiotics and protozoan grazing (Matz et al. 2005, Erken et al. 2011). Biofilm formation is a critical step in establishing enterococcal infections, because it can protect the bacteria from antimicrobials and phagocytosis (Dunny, Hancock, and Shankar 2014). In this study, it was hypothesised that acclimation to grazing would cause phenotypic changes in biofilm formation. The data showed that biofilm formation was decreased in acclimated (A+) isolates while non-acclimated (A-) isolates maintained similar biofilm production as parental *E. faecalis* OG1RF.

Intracellular survival in eukaryotic hosts is another spectrum of defence against protozoan killing. Previous studies have hypothesised that intracellular survival mechanisms in mammalian cells was evolved from interactions with primitive unicellular eukaryote and strong selective pressure maintained the adaptations through the generations (Strassmann and Shu 2017). In this study, the aim was to study the effects of acclimation to constant predatory pressure in *E. faecalis*. To test this hypothesis, intracellular survival assays were performed to determine whether acclimation resulted in a phenotype that extends intracellular survival in mouse macrophages.

Here, a co-evolution experiment was successfully initiated where *E. faecalis* was constantly exposed to an amoeba predator, *A. castellanii* for a period of 30 days. This long-term co-culture generated *E. faecalis* populations that were acclimated to predation by *A. castellanii*. In parallel, non-acclimated populations were generated by continuous subculture of the bacteria in the same growth media in the absence of amoeba. Isolates were collected by random picking of single colonies arising from these populations on designated days. Whole genome sequencing of these isolates was performed to investigate genetic changes occurred during acclimation.

Overall in this chapter, the genetic and phenotypic changes that occurred in *E. faecalis* during 30 days of constant co-culture with *A. castellanii* was examined. The data suggests that acclimation to predation can result in changes biofilm formation. Genetic analysis, though preliminary, showed that accumulation of mutations unique to acclimation to amoeba were present. A larger sample size is required for definitive analysis of the genetic changes that occurred between A+ and A- co-cultured isolates, and over the period of exposure to predation. Nevertheless, this information is important in beginning to understand the evolution of virulence in pathogens and the relation between protozoan and mammalian hosts.

3.2) Materials and Methods

3.2.1) Organisms and culture conditions

Enterococcus faecalis OG1RF (ATCC 47077) and its derivative mutant strain, *ΔsrtA* mutant (sortase A deletion mutant) were grown and cultured as the same method described in Chapter 2.

Acanthamoeba castellanii was obtained from American Type Culture Collection (ATCC 30234) and maintained in PYG media as a monolayer in a tissue culture (T25) flask. Cells

were passaged weekly by gently tapping the flask to dislodge the cells and transferring 500 µl to 10 ml of fresh PYG media. To prepare *A. castellanii* for experiments, the amoebae were first dislodged by tapping the T25 flask and pelleted at 700 × *g* for 5 min. The supernatant was removed, and amoeba resuspended in PPY (2% peptone and 0.1% yeast extract; Becton Dickinson, USA). The amoebae were enumerated by microscopy using a hemocytometer and adjusted to 10^4 cells/ml in PPY media. Following adjustment, 1 ml of the amoebae were seeded into a new T25 flask and incubated statically at room temperature for 30 - 60 minutes to allow the amoebae to adhere to the flask surface.

3.2.2) Cell culture

Raw 264.7 mouse macrophages (ATCC® TIB-71™) were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA, Austria). Cells were kept in T75 or T175 culture flasks as adherent monolayer at 37 °C, 5% CO_2 and were routinely passaged once confluency of 80% was reached.

3.2.3) Co-culture assay

Overnight *E. faecalis* broth cultures were pelleted and resuspended in M9 to an $OD_{600\ nm}$ of 0.125. *A. castellanii* concentration was adjusted to and 10^4 cells/ml and added to T25 flasks for 30 minutes at room temperature as described above. To achieve a multiplicity of infection (MOI) of 1:10, 10 µl of adjusted *E. faecalis* was added.

3.2.4) Generation of acclimated *E. faecalis* isolates

Co-cultures of *A. castellanii* and *E. faecalis* were set up as described previously and intracellular *E. faecalis* were collected and re-inoculated into fresh cultures of *A. castellanii* in PPY every 3 days. Briefly, every 3 days, the co-culture was passed through a 3 µm pore size nitrocellulose filter membrane (Merck Millipore, Germany). Amoebae that were trapped

on the filter were resuspended in 3 ml PBS, before lysing with 0.5% (v/v) triton-X for 1 minute. The suspension was pelleted and washed twice with PBS. Finally, the pellet was resuspended in 1 ml PBS and denoted as an acclimated (A+) population. One hundred µl of the sample was pelleted and resuspended in 30% glycerol-BHI for long term storage at -80°C. The remaining bacteria were transferred to a fresh culture of *A. castellanii* (seeded at final number of cells to 10^5 cells). This process was repeated for 30 days.

In parallel, a non-acclimated population (A-) was established as a control. Briefly, *E. faecalis* (adjusted to final number of cells to 10^4 cells) was inoculated into a T25 flask containing 10 ml PPY without amoebae. Every 3 days, 100 µl of *E. faecalis* culture was harvested, pelleted and resuspended PPY, and reinoculated into a T25 flask containing 10 ml of fresh PPY media. Another 100 µl of the culture was pelleted, resuspended in 1ml of 30% glycerol-BHI and stored at -80°C for long term storage. This was denoted as non-acclimated (A-) population. The process was repeated for 30 days.

To obtain individual isolates from A+ or A- population, frozen stocks were spread plated onto BHI agar plates and incubated statically overnight at 37°C. A numbered plate guide and a random number generator (https://www.random.org/integers/) was used to randomly select 10 different colonies from each population. Each colony was stored in 30% glycerol-BHI for future experiments. Due to time constraints, only Day 3, 15 and 30 isolates were used in experiments documented in this report to represent early, mid, and late acclimation.

3.2.5) Intracellular survival studies

Intracellular survivability was first accessed in *A. castellanii* by co-culturing *E. faecalis* and *A. castellanii* as described in Section 3.2.3, for 4 h. A combinatory treatment of 500 µg mL^{-1} gentamycin and penicillin G at room temperature for 1 h was used to remove extracellular and adherent bacteria. Cells were washed with 1 × PBS thrice before 0.1% Triton X-100 was

added to lyse the amoeba and release *E. faecalis* at different timepoints over 24 h. *E. faecalis* was plated on BHI plates for enumeration of CFU.

For intracellular survivability studies in mouse macrophages, macrophages were grown in DMEM-FBS to 80% confluency in 6-well plates. *E. faecalis* was added in the multiplicity of infection (MOI) of 1:10, where in theory, every macrophage will encounter 10 bacteria. Co-culture was incubated for 3 h and 6 h. One hour before harvesting the macrophages for intracellular bacteria, 500 µg mL^{-1} gentamycin and penicillin G was added and incubated at 37 °C for 1 h. Cells were washed thrice with 1 × PBS to remove the antibiotics and 1% Triton X-100 was used to lyse the macrophages to release intracellular bacteria. Recovered bacteria were plated on BHI plates for CFU enumeration.

3.2.6) Growth kinetics

To determine if acclimation of E. faecalis to A. castellanii had any effect on its growth, overnight cultures of A+, A- and overnight planktonic E. faecalis cells were adjusted to OD600 nm to 0.05 (c. 4 x 107 cells mL-1) in BHI and 300 µl was transferred into 96-well plates in triplicate. Absorbance at OD600 was measured every 30 minutes over a period of 18 h at 37oC using a microplate reader (Tecan Infinite® M2000; Tecan, Switzerland).

3.2.7) Biofilm assay

Overnight cultures of A+, A-, the parental strain OG1RF and the *ΔsrtA* mutant (final concentration of 1.6×10^8 cells/ml) were incubated statically in TSB supplemented with 0.25% glucose for 24 h at 37ºC. The attached cells were washed twice with 1× PBS to remove planktonic bacteria and stained with 200 µL of 0.1% crystal violet for 30 min at 4ºC. Unbound crystal violet was removed by washing twice with 1× PBS and bound CV stain solubilised in 200 µL of ethanol:acetone (80:20) for 30 min with shaking at 50 rpm. Absorbance at 595 nm was determined using a microplate reader (Tecan Infinite® M2000).

3.2.8) Extraction of genomic DNA

Genomic DNA of A+ and A- and parental *E. faecalis* were extracted using DNeasy Blood & Tissue Kit (Qiagen, Germany) with slight modification of the manufacturer's protocol. Overnight cultures were adjusted to an $OD_{600\ nm}$ of 0.7 in PBS and pelleted. The pellet was resuspended in 200 µl of enzymatic lysis buffer (20 mM tris-HCl pH8.0 (Life Technologies, USA); 2 mM EDTA (Ambion, USA); 1.2% triton-X and 20 mg/mL lysozyme (Sigma-Aldrich, USA) in heat block at 37°C for 45 minutes. The lysate was then subjected to treatments described in the manufacturer's protocol.

Extracted genomic DNA was then checked for purity by spectrophotometry (Nanodrop™ 2000 UV-Vis; ThermoScientific, USA). DNA samples outside of acceptable ranges (absorbance ratio at 260/280 nm between 1.8 to 2.0 and 260/230 ratio ranges between 2.0-2.2.) were further purified using Genomic DNA Clean & Concentrator™-10 (Zymo Research, USA). Concentrations of DNA were determined by fluorometry (Qubit ® 2.0; ThermoFisher Scientific, USA) following manufacturer's protocol for high sensitivity DNA concentration quantification. These samples were sent for whole genome sequencing in house (Singapore Centre for Environmental Life Sciences Engineering).

3.2.9) Whole genome sequencing and analysis of variants

Sequencing libraries were prepared using the TruSeq DNA sample preparation kit (Illumina) and 150 bp paired-end reads sequenced on a MiSeq V2. Four random A+ and A- isolate sequences from Day 3, 15 and 30 were trimmed using BBMap (University of California, USA) and sequence quality was checked with FastQC (Babraham Institute, UK). A+ and A- isolate sequences were aligned to the *E. faecalis* OG1RF reference genome (taken from NCBI) using CLC Genomics Workbench 8 (Qiagen, Germany). Mutations were identified using CLC's basic fixed ploidy variant detection algorithm and filtered against known mutations in the parental strain. Unique mutations were identified manually by comparing

isolates common mutations that were present in all three time points and comparing A+ isolates with A- isolates.

3.3) Results

3.3.1) Identifying genomic changes in *E. faecalis* due to acclimation to *A. castellanii*

It was hypothesised that long-term predation will exert a selective pressure on *E. faecalis*, resulting in accumulating mutations that will aid its survival. To test this hypothesis, I selected for and passaged bacteria that survived within *A. castellanii* to ensure recovery of bacteria that were exclusively in contact with the predator. This was achieved by co-culturing *E. faecalis* with *A. castellanii*, and intracellular *E. faecalis* was recovered by lysing *A. castellanii* every three days. These intracellular bacteria were re-inoculated into fresh batch of amoeba to resume co-culture until 30 days. A portion of these intracellular *E. faecalis* from each time point were frozen in glycerol stocks and termed as acclimated populations (A+ population). Acclimated populations Days 3, 15 and 30 were then streaked onto BHI agar plates and ten single colonies were randomly selected and termed as acclimated isolates (A+ isolates). Non-acclimated populations (A- populations) and non-acclimated isolates (A- isolates) were generated in a similar way by inoculating *E. faecalis* in co-culture media without *A. castellanii* and collected by centrifugation every three days for 30 days. Days 3, 15 and 30 timepoints were chosen to represent early, mid and late adaptations. Four out of ten isolates were chosen randomly from Days 3, 15 and 30 for whole genome sequencing (WGS). Selected isolates were grown in overnight culture and genomic DNA was extracted for WGS.

Using CLC Workbench 8 software, trimmed reads were aligned to parental *E. faecalis* OG1RF genome. Resultant aligned reads were used to detect single nucleotide polymorphisms (SNPs) and InDels. In total, among all four of isolates sequenced, 11 non-synonymous mutations, 4 synonymous mutations and 3 mutations that are not in the coding

region were detected. Five out of eleven non-synonymous mutations were found in all isolates that were sequenced. One mutation on a gene coding for a hypothetical protein and one mutation that was not in coding sequence were detected in all sequenced isolates as well. Unique mutations were detected in some of the sequenced isolates. Day 3 A+ Isolate 1 accumulated two non-synonymous mutations; Day 3 A- Isolate 1 accumulated one non-synonymous mutation and one mutation not on coding sequence; Day 15 A+ Isolate 4 accumulated one mutation on a hypothetical protein; Day 15 A- Isolate 4 accumulated one mutation on a hypothetical protein; Day 30 A+ Isolate 1 accumulated one non-synonymous mutation and one mutation not on coding sequence; and Day 30 A+ Isolate 2 accumulated one mutation on a hypothetical protein (Table 3-1). Non-synonymous mutations refer to the changes in the DNA sequence that results in change of encoded amino acid. In this study, non-synonymous mutations that occurred in *E. faecalis* during acclimation were focused on because changes in the amino acid sequence may alter protein structure and folding, which likely to affect protein function.

In the examination of the genes containing non-synonymous mutations, five mutations that occurred throughout all samples sequenced were detected, regardless of timepoint or presence of acclimation to amoeba. This suggests that neither the conditions of the co-culture nor predation contributed to the mutations of the genes. Of the five genes, two genes were identified related to metabolism, one gene involved in DNA replication, recombination and mismatch repair (MMR), one gene coding for a hypothetical protein, and one mutation that is not found on the coding sequence (Table 3-1).

Unique mutations were identified in some isolates, namely, Day 3 A+ Isolate 1, Day 3 A- Isolate 1, Day 15 A+ Isolate 4, Day 30 A+ Isolate 1 and 2 (Table 3-1). Day 3 A+ Isolate 1 accumulated mutations in the *feoB* and *rnz* genes, which code for ferrous iron transport protein B and ribonuclease Z. FeoB is involved in iron acquisition and homeostasis in blood,

and can be important in establishing *E. faecalis* gut colonisation (Vebø et al. 2009). Ribonuclease Z is an endoribonuclease which process tRNAs for maturation in prokaryotes. However, its exact function is poorly characterised (Redko, Li de la Sierra-Gallay, and Condon 2007). Day 3 A- Isolate 1 has accumulated mutations on two genes, *marR* and an unannotated gene. *MarR* encodes for Multiple Antibiotics Resistance Regulator (MarR), a ubiquitous family of transcription factors that regulates the transcription of various virulence genes in *E. faecalis* (Michaux et al. 2011). Day 30 A+ Isolate 1 accumulated mutations on *pepV* gene and an unannotated gene. In *Streptococcus gordonii, pepV* encodes for dipeptidase V that hydrolyses hydrophonic dipeptides exclusively (Goldstein et al. 2005), but it has not been characterized in *E. faecalis*. The remaining isolates have mutations in either hypothetical proteins or regions that are not in a coding sequence.

In summary, all isolates carried common mutations in *chiC*, *atoB*, and *hexB*/*mutL*, suggesting that these were spontaneous mutations that were accumulated were neither due to culture conditions nor the presence of an amoebal predator. Because MutL is involved in DNA mismatch repair, mutation of MutL leads to formation of hypermutable strains in *Escherichia coli*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecium*. Hypermutation may play a major role in acquisition of antibiotic resistance and can result in worsened outcome in disease progression (Guarné 2012, Prunier and Leclercq 2005, Willems et al. 2003). Mutation in *mutL* gene may form hypermutable *E. faecalis* strains and may have increased the rate of accumulation of mutations. Unique mutations occurred in some of the A+ isolates that were not detected in A- isolates, suggesting these mutations could be due to adaptations to predation. Inversely, mutations that were specifically accumulated in A- isolates suggested these mutations were due to the culture conditions, but not the presence of the predator. Assuming it was loss-of-function mutations that occurred, mutations in some of these genes appeared to reduce virulence in *E. faecalis*. For example, *feoB* is important in

iron acquisition and loss of this function will result in poor growth and virulence when iron is limited. As MarR is an important transcription factor regulating virulence genes, loss of this function may result in reduced expression of virulence factors.

Table 3-1: List of non-synonymous mutations occurred during long-term acclimation. Shaded squares indicate presence of mutation. *CDS: coding sequence. [+] DNA Mismatch Repair.

			Day 3 Isolates								Day 15 Isolates								Day 30 Isolates							
			Acclimated				Non-acclimated				Acclimated				Non-acclimated				Acclimated				Non-acclimated			
Locus tag	**Gene**	**Category**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Not on CDS*	NA	NA																	■							
OG1RF_RS01395	*chiC*	Metabolism	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OG1RF_RS02165	*pepV*	Metabolism																	■							
OG1RF_RS03870	-	Hypothetical protein												■												
OG1RF_RS01950	*feoB*	Metabolism	■																							
OG1RF_RS05760	-	Hypothetical protein																■								
OG1RF_RS13460	*atoB*	Metabolism	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OG1RF_RS07120	*marR*	Transcription					■																			
OG1RF_RS07235	*rnz*	Poorly characterised	■																							
OG1RF_RS07750	-	Hypothetical protein																		■						
Not on CDS*	NA	NA					■																			
OG1RF_RS13585	-	Hypothetical protein	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OG1RF_RS12440	*hexB*/*mutL*	MMR system[+]	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Not on CDS*	NA	NA	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

3.3.2) Intracellular survival is neither enhanced nor reduced after acclimation to grazing

To first establish whether *E. faecalis* acclimated for growth in *A. castellanii* could survive better intracellularly, an internalisation assay was performed. The ability of the parental *E. faecalis* strain to survive intracellularly was first assessed by incubating it with a monolayer of *A. castellanii* for 4 h, before treating with an antibiotic mixture of gentamycin and penicillin G to kill both planktonic and extracellular adhered bacteria. Intracellular bacteria were then released by 0.1% Triton X-100 treatment from samples starting at4 h, and every 4 hours until 24 h (Figure 3-1 A). At t = 0 h, there were approximately 10^3 CFU mL^{-1} intracellular *E. faecalis*. These numbers increased constantly over the period of 24 h. By 24 h, the number of viable bacteria increased 4 log folds to ~10^7 CFU mL^{-1} (Figure 3-1 B). Since these increases occurred in the presence of antibiotics, these results suggest that not only *E. faecalis* is able to survive and persist in *A. castellanii*, it can also replicate within the amoeba.

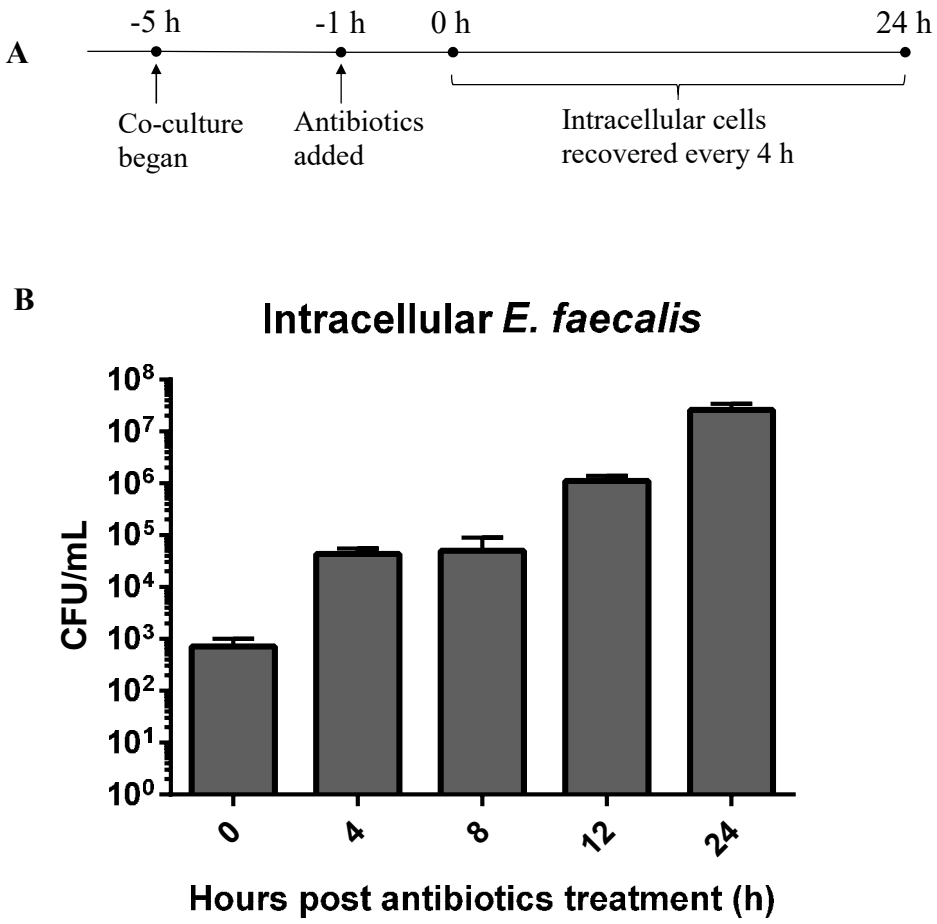

Figure 3-1: *E. faecalis* can survive and replicate within *A. castellanii*. (A) Schematic diagram of experiment procedure. Antibiotics treatment refers to a combinatory treatment of 500 µg mL^{-1} (B) *E. faecalis* was co-cultured with *A. castellanii* for 4 h before treating with antibiotics to remove all extracellular bacteria. Antibiotics refer to a mixture of gentamycin and penicillin G. Data showed there were increase in viable bacteria cells from the point of antibiotics treatment (t = 0 h) to t = 24 h time-point, suggesting intracellular replication.

It was next hypothesised that acclimation to amoeba may enhance intracellular survival of *E. faecalis* in macrophages. As such, RAW 264.7 mouse macrophages were infected with A+ and A- populations to test this hypothesis in a mammalian host. These data showed that *E. faecalis* can survive and persist intracellularly in mouse macrophages and maintained at high CFU mL^{-1} of 10^6 at 3 h post infection and 10^7 at 6 h post infection, consistent with previous report (Gentry-Weeks et al. 1999). However, there was no significant difference in viable

CFU recovered between A+ and A- populations in the intracellular survivability in mouse macrophages across all time points. These findings suggest that acclimation to amoeba grazing may not enhance nor reduce the intracellular survivability of *E. faecalis*.

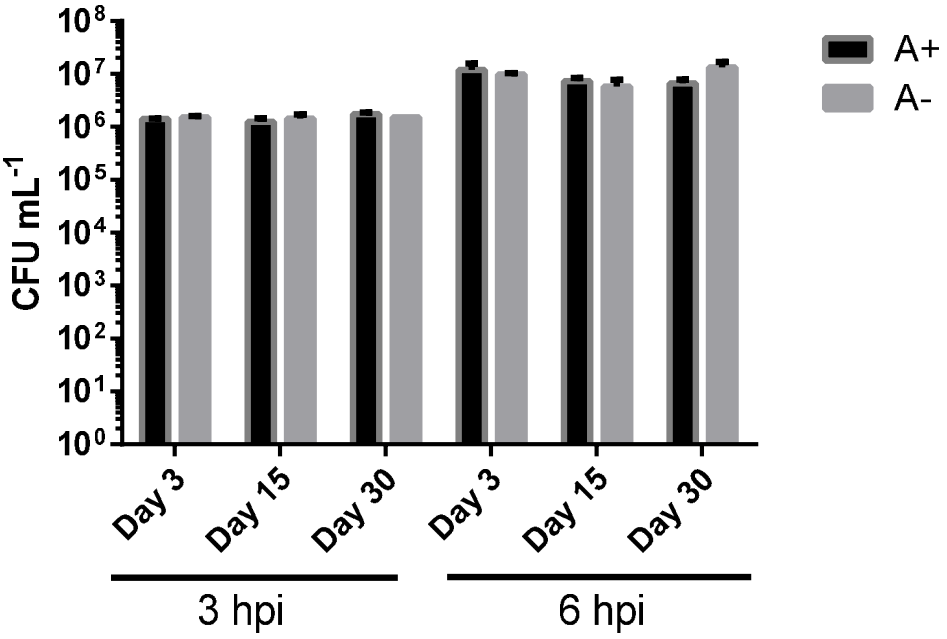

Figure 3-2: Acclimation to grazing do not affect the ability for intracellular survival in mouse macrophages. Raw 264.7 macrophages were infected with *E. faecalis* that were acclimated or non-acclimated over 3 and 6 h at MOI of 1:10. At each time point, cells were incubated in DMEM with Gentamicin and Penicillin G for 1 h at 37°C to kill extracellular bacteria. CFU was recovered and enumerated after lysing the cells with Triton-X. Intracellular CFU of both acclimated and non-acclimated population showed no differences over 3 and 6 h. Mean and standard deviation were calculated over 2 biological replicates.

3.3.3) Long term co-culture with *A. castellanii* led to reduced biofilm formation as compared to non-acclimated *E. faecalis*

In this study, it was hypothesised that long-term adaptations to amoebae would cause phenotypic changes in *E. faecalis* that allow for better survivability under predatory pressure. One anti-predation phenotype is biofilm formation, thus crystal violet staining method was

used to assess the differences in biofilm formation between A+ and A- isolates. Planktonic growth kinetic assay of each isolate was first performed to ensure that none of the isolates were growth defective (data not shown). A sortase A deletion mutant (*ΔsrtA*) is a strain that has poor anchorage of virulence factors on its cell wall (Kristich, Manias, and Dunny 2005), leading to poor biofilm formation. Therefore, this strain was selected as a low biofilm former control in this assay (Selvaraj et al. 2014). The parental *E. faecalis* OG1RF strain was used as a positive control for biofilm formation.

Data shows that acclimation to amoebae resulted in a reduction of biofilm formation by *E. faecalis* when compared to non-acclimated isolates (Figure 3-3). The data also suggests that reduction in biofilm formation in A+ isolates were temporally dependent, as the effect increased significantly only from Day 15 to Day 30. A- isolates formed similar amounts of biofilm biomass as parental OG1RF, suggesting that long term culture in media did not alter their phenotype. Therefore, any changes in A+ isolates were likely due to predation pressure by the amoebae. Though there was a reduction in biofilm formation in Day 15 A+ and Day 30 A+ isolates, none of them were recorded to have lower biomass than the *ΔsrtA* mutant.

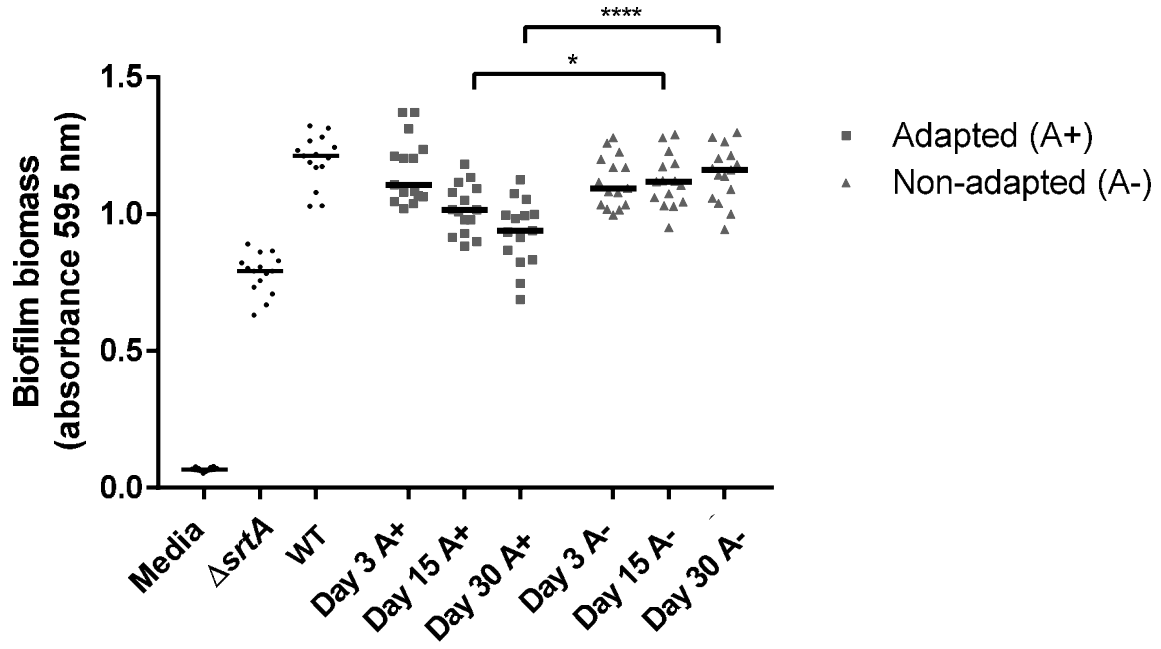

Figure 3-3: Biofilm formation of A+ isolates decreased from Day 3 to Day 30 as compared to A- isolates. Ten isolates (both A+ and A-) from Day 3, Day 15 and Day 30 co-cultures were inoculated onto 24-well plates containing TSBG and were left to grow biofilms in static 37°C conditions. OG1RF served as positive control, while *ΔsrtA* and media control as reference for low biofilm formation and no biofilm formation respectively. Median was calculated from a pool of 10 isolates and with 3 biological replicates. Level of significance was measured by two-way ANOVA, followed by Bonferroni's *post hoc* test (*p<0.05, ****p<0.001).

Next, the stability of biofilm formation phenotype was investigated in the populations. After *in vitro* passage, crystal violet staining were performed using the A+ and A- population. Data showed that on the first day of subculture (Figure 3-4, black bars), biofilm formed on Day 3 A+ populations were lower than A- populations; biofilm formed on Day 15 A+ populations was similar to Day 15 A- populations; and Day 30 A+ populations formed more biofilm than Day 30 A- populations. Biofilm formed by the populations on the first day of subculture were also noted to be higher than positive control, parental OG1RF. However, when A+ and A- populations were cultured continuously for three days, the difference in biofilm formation observed in the first day was no longer observed on the second and the third day (Figure 3-4,

dark grey bars and grey bars), where after passaging, biofilm formation in Days 3, 15, 30 reverted to the levels similar to parental OG1RF This suggested that the amoeba-acclimated biofilm phenotypes were not stable.

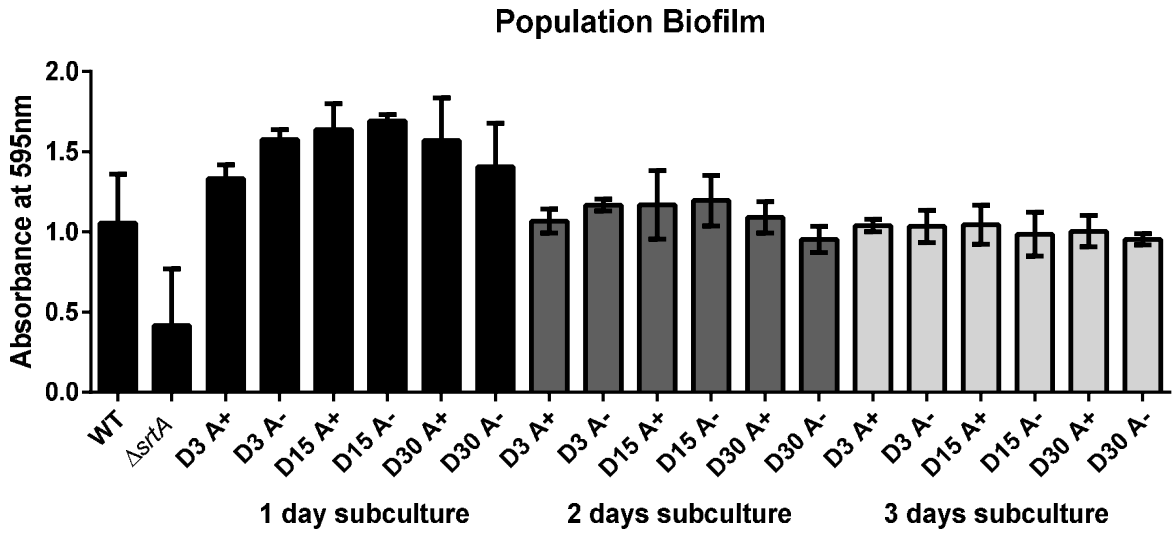

Figure 3-4: Amoeba-acclimated biofilm formation phenotypes were not stable. A+ and A- population were inoculated on 24-well plates containing TSBG to form biofilms. Each day before inoculation, an aliquot of overnight culture was taken and diluted in fresh BHI for subculturing. Mean and standard deviation were calculated over 2 biological replicates.

3.4) Discussion

In this chapter, the effect of long-term acclimation of *E. faecalis* to *A. castellanii* was investigated. It was hypothesised that long-term grazing would exert an intense selective pressure on *E. faecalis* and drive them to acquire anti-predator mechanisms.

Both A+ and A- populations were generated successfully over 30 days of co-culture. By random selection of single colonies, 10 isolates were picked from each population. To determine how predation shapes bacterial acclimation to amoeba, whole genome sequencing was performed on A+ and A- isolates to identify mutations that were accumulated during the experiment. Isolates were sequenced to understand in detail which mutations occurred during

acclimation process. In theory, population samples have a mixture of different isolates that may accumulate different mutations at different frequencies. As such mutations that occurred at a lower frequency may be masked by those with a higher frequency. By sequencing and analysing isolates, each mutation that has been accumulated from acclimation could be investigated, without underestimating the effect of some mutations. However, a few limitations were noted in this study. Firstly, although ten isolates of each condition were chosen, they might not be representative of the whole population. A larger sample size of isolates would be required to represent the phenotypic differences in populations samples. Further examination of the scatterplot in Figure 3-3, where each point represents one isolate, wide range of scattering of data points were noted within each condition and timepoint. This could be indicative of a possible masking effect of phenotypic changes by diverse isolates within a population. Secondly, during long-term co-culture, populations of different timepoint were generated. In this experimental design, populations recovered from previous timepoint were re-inoculated to fresh amoeba. Isolates, on the other hand, were isolated from these populations and were not used for re-inoculation. This resulted in genetically different isolates generated from different timepoints, instead of originating from a single isolate. For example, Day 3 A+ Isolate 1 is genetically different from Day 15 A+ Isolate 1 and Day 30 A+ Isolate 1. Therefore, in this study, it was not able to observe accumulation of mutations in any isolate from Day 3 to Day 30, and these isolates could not be compared amongst one another temporally. To address these limitations, populations from different timepoints could be sequenced and be compared with the isolates data. More isolates could be generated to increase the sample size and become more representative of the population. By comparing both population and isolate genomic data, a more comprehensive outlook of genetic adaptations due to strong selective pressure from predation can be achieved.

The intracellular survivability of *E. faecalis* in both environment and mammalian predator was also examined. In *A. castellanii*, it was demonstrated that *E. faecalis* can survive and replicate within amoeba. *E. faecalis* was also observed to be protected from antibiotics by staying within amoeba, agreeing with previous studies (Barker and Brown 1994, King et al. 1988). This suggests *A. castellanii* can be a reservoir for *E. faecalis* and may play a role in dissemination in the environment. Although macrophages and amoeba are evolutionary distinct, their phagocytotic pathways are similar. It was hypothesised that acclimation to predation enhanced intracellular survivability in macrophages. RAW 264.7 mouse macrophages were infected with A+ or A- *E. faecalis* and the data showed that there was no significant difference in recovered CFU mL^{-1} between A+ and A- (Figure 3-2). This could be because the infection period was too short (3 h and 6 h) to see a phenotypic difference. Although it was reported that *E. faecalis* could survive up to 72 h in macrophages (Gentry-Weeks et al. 1999), long infection times was found to result in sloughing and detachment of macrophages (Data not shown). This would lead to underestimation of intracellular counts, as some macrophages would have been washed away.

Previous studies have shown that in *Vibrio cholerae* and *Pseudomonas aeruginosa* respond to protozoan grazing by forming biofilms that are difficult to phagocytose (Erken et al. 2011, Matz and Kjelleberg 2005, Sun, Kjelleberg, and McDougald 2013). Hence, it was hypothesised that *E. faecalis* may employ a similar strategy to evade predation. Interestingly, it was found that the longer the bacteria have been acclimated to amoebae, the less biofilm is formed. However, it should be noted that the experiment was designed to specifically select for intracellular *E. faecalis*. This may lead to isolating *E. faecalis* that may prefer an intracellular lifestyle. Therefore, these isolates were found to form lesser biofilms as they do not need to evade phagocytosis as described in previous studies. Although this limited the examination of antipredator mechanisms to post ingestional acclimation, the selection of

intracellular *E. faecalis* was an unavoidable step in order not to recover bacteria that did not encounter the predator at all, which possibly lead to false negatives.

Lastly, the data showed that the biofilm formation phenotype was not stable as biofilm biomass of all populations reverted to parental OG1RF when populations were subcultured for three days. However, even though the phenotype was unstable, the mutations occurred may not be lost from subculturing. To check whether the mutations are stable, subcultured populations can be sequenced and compared with populations without subculture. If the same mutations could be found in the subcultured populations, then it could suggest that the mutations had no effect on the phenotype. Inversely, if the mutations were lost, then it could suggest that the mutations influence the biofilm formation phenotype.

In summary, it was reported that bacterial adaptations to amoebae leads to changes in virulence traits. In this study, it was investigated how acclimation to amoebae changes biofilm formation, which is one of many virulence traits *E. faecalis* possesses. Biofilm formation decreased over time during co-incubation, suggesting that acclimated *E. faecalis* may prefer to be taken up by the amoebae and live intracellularly within the amoeba, and thus biofilm formation is no longer favoured. Other than biofilm formation, there may be other phenotypic changes as a result of acclimation of *E. faecalis* to *A. castellanii*. Additional phenotypic studies are required to elucidate what other phenotypes are altered during acclimation to amoeba predation.

Chapter 4: General discussion and Conclusions

4.1) General Discussion

Protozoan grazing is one of the key factors in maintaining and shaping bacterial communities in the environment (Adiba et al. 2010, Erken, Lutz, and McDougald 2013, Servais, Billen, and Rego 1985). Predation can control bacterial populations and is deemed as a major mortality factor for bacterial (Servais, Billen, and Rego 1985). As such, predation exerts a strong selection for fitness and traits to avoid or survive ingestion (Abrams 2000). Bacteria developed different strategies and mechanisms to defend against protozoan grazing. In general, extracellular pathogens seek to evade predation by altering their morphology, like forming filaments that are difficult to ingest (Hahn, Moore, and Höfle 1999), forming bacterial aggregates, microcolonies or biofilms to prevent phagocytosis (Hahn, Moore, and Hofle 2000), increasing motility to prevent capture (Matz and Jürgens 2005) or secretion of quorum sensing mediated toxic antipredator compounds (Matz, Bergfeld, et al. 2004) Intracellular pathogens develop mechanisms to enhance their survival within protozoa. Some bacteria, such as *Legionella pneumophilia*, resist phagosomal digestion by using an elaborate Dot/Icm Type IV secretion system that secretes effectors that all the bacterium to persist within the host (Hilbi, Segal, and Shuman 2001, Qiu and Luo 2014).

Another recently recognised mechanism of predation resistance is the ability of some pathogens to pass through the digestive system of protozoa unharmed in the form of expelled food vacuoles (EFVs) (J.M. et al. 2014). EFV formation by were studied intensively in Gram-negative pathogens, such as *Salmonella enterica*, *Escherichia coli* (diarrheic strains), and *L. pneumophilia*. *S. enterica* and *E. coli* are both well characterised enteric pathogens causing food-borne diseases, while *L. pneumophilia* is the causative agent for Legionnaire's disease. This study is the first examination of interactions of *Enterococcus faecalis* and two model

protozoa, *Tetrahymena pyriformis* and *Acanthamoeba castellanii*. Unlike other studies that focused on the mortality of *E. faecalis* by predation in the environment (Menon, Billen, and Servais 2003, Boehm, Keymer, and Shellenbarger 2005), it was shown that in Chapter 2 and 3, *E. faecalis* can persist in *T. pyriformis* and *A. castellanii* for 24 h.

In this study, two different protozoa were used as model organisms. They were chosen based on their distinct and different feeding modes. *T. pyriformis* is generally a filter feeder that feed on planktonic bacteria. As there is no evasion strategy to evade from a filter feeder, the interactions between the protist and prey are limited to the *E. faecalis* being taken up by *T. pyriformis*. *A. castellanii*, on the other hand, are surface feeders and feed on biofilms or bacteria on surfaces, resulting in interaction between predator and prey that affects uptake and persistence in *A. castellanii* as *E. faecalis* can adopt evasion strategy.

In Chapter 2, the persistence of *E. faecalis* in *T. pyriformis* and expulsion in EFVs was examined. A pH sensitive probe, LysoTracker™, was used to label *T. pyriformis* cells and found that the lack of acidification of phagosomes could be the reason for intracellular survival of *E. faecalis* in *T. pyriformis* food vacuoles (Figure 2-3 C). Weak LysoTracker™ staining was observed to co-localise with the food vacuoles containing live *E. faecalis*, suggesting a lack of acidification within, while phagosomes containing heat-killed *E. faecalis* were stained. However, LysoTracker™ stains all acidic compartments, including lysosomes, maturing phagosomes, and late endosomes. Hence, to track at which stage of the endocytic pathway acidification is inhibited, immunofluorescent staining using different endosomal markers would be required. For example, Rab 4 and Rab 5 could be tagged for early endosome, Rab 7 and Rab 9 for late endosomes, and cathepsin for lysosomes (Eskelinen 2006, Gruenberg and van der Goot 2006, Jacobs et al. 2006). Colocalisation of the markers with the food vacuoles with LysoTracker could elucidate when acidification of food vacuoles

was prevented. As Rab proteins in the endocytic pathway are functionally conserved (Bright et al. 2010), antibodies could be obtained from other sources and check for cross reactivity in future experiments.

The structure and form of EFVs are extensively studied, but the mechanisms of packaging bacteria into EFVs are unknown. By understanding the mechanisms driving EFV formation and expulsion, chemical or small molecule regulators could be designed to encourage or inhibit the process. This will allow us to better address the impact of bacteria packaging in the environment and their potential role in disease transmission. The first step could be to determine the rate of passage of EFVs through the phagosomal trafficking pathway by performing a series of pulse-chase experiments. A fluorescently labelled bacteria could be co-incubated with *T. pyriformis* to allow for uptake, and then removed and non-labelled bacteria added. The rate of fluorescent loss could be inferred to be the rate of bacterial passage through *T. pyriformis*. Secondly, proteins or markers involved in the process could be identified and used to label the machinery required for packing bacteria. This will give us information of the molecular process of bacterial packaging and egestion of EFVs.

It has been hypothesised that EFVs can serve as a protection against desiccation, biocides and chemical stresses, thereby making them apt candidates for dissemination into the environment (Bouyer et al. 2007, Koubar et al. 2011, Brandl et al. 2005, Gourabathini et al. 2008). However, this hypothesis was not fully explored as dissemination of bacteria was assumed based on the better survivability and persistence of packaged bacteria compared to planktonic ones. To identify EFVs as a method of dissemination of pathogenic bacteria, EFVs must show not only enhanced survival for encased bacteria, but the release of bacteria from EFVs. However, no studies so far had characterised the release of packaged bacteria into the environment. Here, it was shown that EFVs provides protection for encased bacteria

against antibiotics treatment (Figure 2-8 C). More importantly, new evidence was provided, via live cell imaging, that EFVs can lyse and release bacteria into the milieu, regardless of nutrient presence in the environment (Figure 2-7 A to C). However, when antibiotics were present, lysis of EFVs was not observed (Figure 2-8 A). As of now, it is inconclusive what the biological trigger for EFV lysis is.

In addition, it is important to investigate the impact of EFVs in the context of pathogenesis and human health. It was demonstrated as early as 1980, *L. pneumophilia* could be transmitted from the environment to humans by inhaling aerosolised vesicles packaged with the bacteria (Rowbotham 1980). A later study reinforced the concept of infectious EFVs by showing *L. pneumophilia* extracted from EFVs were more infectious than non-packaged bacteria in human pneumocytes *in-vitro* (Koubar et al. 2011). Here, it was demonstrated that EFVs protects bacteria against antibiotics and natural release of bacteria from EFVs was observed. However, the infectability of the released *E. faecalis* and its infection dynamics in animal models have not been explored yet. *Enterococcus* spp is one of the most commonly isolated pathogens in invasive infections such as bacteraemia, wound infections (Fisher and Phillips 2009, Agudelo Higuita and Huycke 2014). To examine the impact of packaged *E. faecalis* on infections, infection studies *in-vivo* could be performed in future experiments. Murine model on wound infections (Chong et al. 2017) is well established and could be used to monitor disease progression and immune response. Fitness of packaged bacteria can be compared with parental cells to determine if they have an advantage over parental cells in establishing colonisation and persistence during wound infections. For example, in Chong et al. (2017), the authors demonstrated that *mprf1/2* mutant, a strain that is defective in resisting antimicrobial peptides, was found to be fitness defective when co-infected with OG1X, a commensal strain. Therefore, similarly, co-infection using extracted bacteria from EFVs with parental OG1RF could be performed to test this hypothesis on wound infections.

In Chapter 3, the genetic and phenotypic changes of *E. faecalis* in a small-scale co-evolution experiment were examined. A single batch of *E. faecalis* that was continuously cultured with the amoeba predator, *A. castellanii* for a period of 30 days. It was hypothesised that constant predation by *A. castellanii* will exert a strong selective pressure on *E. faecalis*. Four isolates out of ten from acclimated and control populations (A+ and A-) on days 3, 15 and 30 were isolated and analysed the mutations in these isolates. Common mutations amongst all four isolates that were not a result of acclimation nor culture conditions including two metabolic genes *chiC* and *atoB*, and a DNA mismatch repair (MMR) gene, *hexB*/*mutL*. As for mutations unique to A+ strains, a ferrous iron transport gene *feoB*, ribonuclease *rnz*, and dipeptidase gene *pepV* were detected. In A- isolates, mutations in Multiple Antibiotics Resistance regulator (MarR) was detected. Other mutations detected were either not within coding sequence or coded for hypothetical proteins.

Here, genetic changes were shown occurred during long term co-culture of *E. faecalis* and *A. castellanii*. However, nothing is known currently about the immediate response of the *E. faecalis* to *A. castellanii*. Therefore, it would be interesting to perform RNA sequencing on A+ and A- isolates. Transcriptomic analysis can reveal which genes were upregulated or downregulated in response to predation. Concurrently, *A. castellanii*'s transcriptome profile during predation could be analysed to identify target genes that were affected by *E. faecalis* to prevent digestion.

Studies have shown that *L. pneumophilia* uses the same genes to survive and replicate in both amoeba and macrophages, even though the hosts were evolutionary distinct (Hilbi, Segal, and Shuman 2001, Segal and Shuman 1999). Little is known in *E. faecalis*, however, about the genes involved in intracellular survival in eukaryotic hosts. Currently, two genes (*hypR* and *asa1*) have been identified as contributing factors in intracellular survival in macrophages

(Verneuil et al. 2004, Sussmuth et al. 2000). To date, there are no reports on the genes required for intracellular survival in *A. castellanii*. As such, a transposon library screening of *E. faecalis* mutants that result in augmented intracellular survivability in both macrophages and amoeba could be carried out. Genes that are important in both macrophages and amoeba may further suggest that *E. faecalis* could have evolved intracellular survival from interactions with primitive unicellular eukaryotes.

4.2) Conclusions

In summary, it was established that *E. faecalis* was ingested by *T. pyriformis* but was able to resist digestion. Passage through *T. pyriformis* yielded EFVs. Staining of the EFVs with various chemical dyes showed that they were surrounded membranous structures and contained live *E. faecalis*. Interestingly, the data showed that these EFVs provided protection to antibiotics. *E. faecalis* in EFVs could also be released, suggesting a possible dissemination method. However, further studies are required to elucidate the mechanism of bacteria packaging into EFVs and evaluate the impacts of EFVs on human health.

Long-term acclimation studies demonstrated that *E. faecalis* could survive intracellularly for long periods of time (up to 30 days). Continuous selection for intracellular *E. faecalis* yielded isolates that form less biofilm biomass compared to non-acclimated isolates and the parental strain. However, there was no difference in intracellular survival in macrophages between acclimated and non-acclimated strains. As such, it is inconclusive as of now whether acclimation to constant predation contributes to the changes in virulence of a pathogen. Nevertheless, this study has shed light on how acclimation to environmental predators may shape the virulence of a pathogen.

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