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In vivo assessment of the gene encoding the membrane-bound protease Eep, in virulence

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Abstract

The increasing prevalence of antimicrobial resistance and the lack of innovation in developing new antibiotics has only emphasised the need to develop new antimicrobials. Therefore, research has focused on antimicrobials that target mechanisms other than those targeted by traditional antibiotics. A potential virulence enhancing extra cytoplasmicfunction (ECF) in *Enterococcus faecalis* was found to be targetable by bacteriocin EJ97, a promising antimicrobial candidate. In this study, we use the bacteriocin EJ97 to target the Eep, a membrane-bound proteinase. Using EJ97 as a selective agent on sensitive *E. faecalis* strains, we isolated thirty-six mutants resistant to the bacteriocin. The eep gene in the resistant mutants was DNA-sequenced, and nine of the thirty-six isolates were found to contain a severe frame shift mutation early in the gene, resulting in a stop codon, effectively truncating Eep. To investigate whether the eep gene was relevant in the virulence of E. faecalis, these mutants were included in a murine skin-wound model testing for *E. faecalis* virulence. After three days in the murine skin-wound model, the difference in colony-forming units between the mutants and the wildtype was investigated. No significant differences between the mutants and wildtype were found.

Sammendrag

Utbredelsen av antimikrobiell resistens blant mikroorganismer sammenfattet med mangelen på innovasjon og utvikling av nye antibiotika har formet et kraftig behov for utvikling av nye alternative antimikrobielle midler. Dette har ansporet forskningen til å fokusere på antimikrobielle midler rettet mot andre mekanismer enn de tradisjonelle antibiotika rettes mot.
I *Enterococcus faecalis*, ble en potensiell virulensfaktor Eep, en del av bakteriens «ekstra cytoplasmiske funksjon (ECF)» funnet å være reseptor for bakteriocinet EJ97, noe som gjør den til en lovende antimikrobiell kandidat i kampen mot multiresistente bakterier. I denne studien bruker vi bakteriocinet EJ97 som et selektivt agens på sensitive stammer av *Enterococcus faecalis* for å isolere trettiseks mutanter resistente mot bakteriocinet. Genet *eep* ble DNA-sekvensert i de resistente mutantene, og bekreftet at ni av de trettiseks isolatene inneholdt en alvorlig «frame-shift» mutasjon plassert tidlig i genet. Denne «frame-shift» mutasjonen resulterte i et stop-kodon, som medfører en avkortning av Eep. For å undersøke på hvordan måte og i hvilken grad *eep* er relevant som virulensfaktor i Enterococcus faecalis, ble mutantene inkludert i en hud-sårmodell i mus, som testet for deres virulens. Etter tre dager i hud-sår modellen i mus, ble forskjellen i «colony-forming units» mellom mutantene og villtypen undersøkt. Ingen signifikante forskjeller mellom mutanten eller villtypen ble funnet.

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Abbreviations

AMP	Antimicrobial Peptides
BEA	Bile Esculin
BHI	Brain Heart Infusion
CFU	Colony Forming Units
ECF	Extracytoplasmic Function
GRE	Glycopeptide Resistant *Enterococcus*
HAI	Hospital-Acquired Infections
LAB	Lactic Acid Bacteria
MIC	Minimum Inhibition Concentration
MSA	Multiple Sequence Alignment
ON	Overnight Culture
PCR	Polymerase Chain reaction
ROS	Reactive Oxygen Species
RIP	Regulated Intramembrane Proteolysis
VRE	Vancomycin-resistant *Enterococcus*

Introduction

One of the great predicaments of the 21st century is that the golden era of antibiotics came to an end (Aminov, 2010), an era governed by discoveries and enlightenment, in the field of molecular biology. In the golden age, new molecular targeting strategies for antibiotics were discovered rapidly while the library of synthetic antibiotics grew. However, the well of discovery that once seemed so profound is now close to dry, and new antibiotics are scarce and far in between (Conly & Johnston, 2005). While antibiotics were an important accomplishment for treating and preventing infections, innovation and development in the field have been lacking in recent years.

The drought in antibiotic innovation is threatening to take our bacterial relationship back to the pre-antibiotic era, and forcing scientists to start working in new directions. Scientists are developing new usage politics (Neu, 1992) and trying to understand the underlying mechanisms that make bacteria virulent in the first place (Silver & Bostian, 1993) — hoping that with this understanding new antimicrobials may be discovered, antimicrobials which can more precisely target these molecular pathways.

Antibiotic-resistance was never an unexpected side-effect of using the substrate, with the first cases of resistance being discovered a few years after penicillin's first implementation as a clinical treatment (Bush, 2004). However, antibiotic-resistance would not be considered a problem by the scientific community, until patients started to contract infections which were resistant to all available forms of antibiotics. When the scientific world finally started to realise the severity of the situation, they started advocating new politics regarding the use of antibiotics and emphasised the need to develop new forms (Neu, 1992).

Antibiotic-resistance is not limited to pathogenic bacteria, but also stems from the disruption of the human microflora by interfering with the host's innate colonisation resistance (Mullineaux-Sanders, Suez, Elinav, & Frankel, 2018). This interference is making the normal commensal flora a potential threat and is even putting the host at risk of colonisation by new types of bacteria. Colonisation is made possible by the changes in the stable and established community. This stable system serves as the main innate defence-system of the gastrointestinal tract (Mullineaux-Sanders et al., 2018). Therefore, colonisation and proliferation of new bacteria are usually associated with antibiotic treatment. Thus, the disturbance of the community structure associated with antibiotic use, makes opportunities for invading or for already colonised bacteria to acquire a broader niche. (Ubeda et al., 2010).

These new niches have recently been taken advantage of by certain strains of enterococci. Enterococci are normal colonisers of the human biome and gastrointestinal tract(Clewell, Gilmore, Ike, & Shankar, 2014) and will, under normal circumstances, live in a symbiotic relationship with its host. However, because of the use of antibiotics, enterococci are now starting to take advantage of their opportunistic side, allowing them to pursue niches made available by the higher usage of antibiotics. Therefore, the increase in antibiotic usage is making *Enterococcu*s one of the leading causes of nosocomial infections (Kristich, Rice, & Arias, 2014).

1.1 Enterococcus

The genus of *Enterococcus* can be found in many niches, varying from environmental to the gastrointestinal tract of mammals. This vast array of habitats allow *Enterococcu*s to express several tropisms, metabolic pathways, and phenotypic characteristics to cope with the varying environment (O'Driscoll & Crank, 2015). These different niches and characteristics of *Enterococcus* have divided the genus into more than 50 species. Although these species have been known for a decade, and even used in the food industry, it is considered a relatively new genus (Francois Lebreton, Willems, & Gilmore, 2014). This new characterisation is because enterococci were classified as a part of streptococci and were not considered as its own genus until the 1980s (Schleifer & Kilpper-Bälz, 1984).

The newly established genus *Enterococcus* consists of gram-positive, non-sporing bacteria, arranged in pairs or chains. *Enterococcus* is facultatively anaerobic and obligatory fermentative, although they are usually homofermentative. Glucose can be fermented to L-lactic acid by fermentative

processes, making them lactic acid bacteria (LAB) (Klein, 2003; Murray, 1990; Stiles & Holzapfel, 1997). Additionally, *Enterococcus* hydrolyse esculin in the presence of bile salts (Facklam, 1973). They are also catalase-negative, resulting in an insufficient amount of energy required to take up antibiotics, giving *Enterococcus* an intrinsic resistance to aminoglycosides, a commonly used antibiotic (Klare, Konstabel, Badstübner, Werner, & Witte, 2003). The physiology of *Enterococcus* allows them to grow at a pH ranging from 4 to 9.6 and at temperatures from 10 to 45C°, with survivability up to 30 minutes at temperatures set to 60 °C (Vu & Carvalho, 2011).

The intrinsic properties of the *Enterococcus* genus allow for their ubiquitous nature. While Enterococci is frequently isolated from several environments, such as plants, soil, water, fermented foods, and dairy (François Lebreton et al., 2013) the gastrointestinal tract of animals acts as the main reservoir (Klein, 2003; Toledo-Arana et al., 2001). *Enterococcus* serves as the predominant gram-positive coccus in faeces, with concentrations up to 10^7 CFU/g (Franz, Holzapfel, & Stiles, 1999), but still only respond to 0.1% of the healthy intestinal flora (Jett, Huycke, & Gilmore, 1994) (Francois Lebreton et al., 2014). Thus, Enterococci is most likely to be found in these environments due to contamination from faecal material (Kühn et al., 2003) (Gilmore, Clewell, Ike, & Shankar, 2014).

Although not considered a primary pathogen, enterococci's ability to acquire higher levels of resistance to antibiotic agents has allowed these bacteria to shift from being a commensal inhabitant of the gastrointestinal tract to a clinically important pathogen (Linden & Miller, 1999). In the 70s, enterococci became the first well established nosocomial pathogen and hence prevalent in hospitals (Jett et al., 1994). When *Enterococcus* is found in the hospital environment, it proves itself as a successful opportunist, becoming one of the leading causes of nosocomial infection (Hidron et al., 2008). However, perseverance in hospital environments occurs not only as a result of their physiology but as a result of their heightened innate ability to acquire mobile elements such as pathogenicity islands (PAI) and antibiotic-resistance(Manson, Hancock, & Gilmore, 2010).

1.2 Antibiotic-resistance in Enterococcus

The clinical importance of the *Enterococcus* genus is directly linked to the challenges of *Enterococcus*-resistance. Antibiotic resistance has become a compounded problem, due to *Enterococcus*' limited susceptibility to antibiotics, both as a result of their intrinsic and acquired antibiotic resistance. *Enterococcus* is intrinsically resistant to several types of antibiotics: chaplosporins, aminoglycosides, lincosamides, and streptogramins (Hollenbeck & Rice, 2012; Kristich et al., 2014; Rice, 2001). Also, *Enterococcus* has been successful in acquiring resistance to almost all clinically used antibiotics (Kristich et al., 2014).

Enterococcus' intrinsic and acquired resistance for antibiotics is well-documented, even showing resistances to glycopeptides. Glycopeptides, including vancomycin, are one of the most used antibiotics for an enterococcal infection. Because *Enterococcus* shows an inherent resistance to most other antibiotics (Rice, 2001), vancomycin is one of the few available treatment options.

Vancomycin was first introduced as a drug in the early 50s, yet, the usage of vancomycin was eclipsed by other antibiotics that were considered less toxic (Levine, 2006). However, the early 80s brought forth a dramatic increase in vancomycin usage because of a new oral formulation (Kirst, Thompson, & Nicas, 1998) and a decrease in effectiveness for the other antibiotics. The vancomycin usage caused an increase in resistance, leading to the discovery of vancomycin-resistant enterococci (VRE) in Europe in 1986 (Sahm et al., 1989). Since 1986, isolation rates for VRE have only increased and are now one of the most frequently isolated nosocomial pathogens. This rapid increase in resistance is raising alarms because it represents a dramatic loss of an essential bactericid used as the last line for treating VRE infections. This increase in resistance has caused treatment failure to increase by 20% and mortality to increase from 27% to 52% in the case of a glycopeptide resistant *Enterococcus* (GRE) (D. Brown et al., 2006).

However, vancomycin-resistant enterococci cause symptomless colonisation of the intestine in healthy humans (Patel, 2003). The VRE colonised intestines serve as a reservoir for further spread and even put the VRE-colonised person at potential risk for infection. Healthy carriers, coupled with enterococcus heightened antimicrobial resistance and its person to person transmission capabilities, make hospitals optimal breeding grounds for VRE (Boyce, 2007).

Once established in the hospital environment, VRE is hard to remove (Leclercq, 1997). Bacteria are transmitted through contaminated objects and healthcare personnel (Porwancher et al., 1997) and because of *Enterococcus'* physiology, it is usually resistant to common antiseptics, disinfection, UV, starvation, and antibiotics (Hartke, Giard, Laplace, & Auffray, 1998; François Lebreton et al.,

2017; Maraccini, Ferguson, & Boehm, 2012). Therefore, objects, personnel, and patients are possible reservoirs for dissemination (Andrup & Andersen, 1999). This allows for the presence of resistance and transfer of genetic-resistance elements within the hospital population.

To this date, nine distinct gene clusters give glycopeptide resistance in enterococci (Kristich et al., 2014). Six of these known clusters, VanA-VanG, cause VRE resistance (Fisher & Phillips, 2009). Van A and B are newly acquired gene clusters, previously not found in *Enterococcus* (Cetinkaya, Falk, & Mayhall, 2000). These two clusters are confirmed to be transferable by a plasmid, and the differences in the G-C content (Rice, 2001) supports that they have originated in other species. Thus, Enterococci only acquired VanA and VanB by horizontal gene transfer.

However, the fact that antibiotic-resistant bacteria are accumulating in close proximity is not only a problem because of acquired resistances but also because they might require other genetic elements like PAI. *Enterococcus* is known to have an active sex pheromone system (Varahan, Harms, Gilmore, Tomich, & Hancock, 2014) which puts them in a favourable position, capable of acquiring new resistances, virulence factors, and PAI on mobile genetic elements.

1.3 Enterococcus faecalis

E. faecalis is one of the most predominant species of enterococci and is in the forefront of enterococcal infections. *E. faecalis* is associated with 80-90% of all enterococcal infections (Kayaoglu & Ørstavik, 2004), in contrast to *Enterococcus feacium*, the second-most isolated strain of enterococci, which is responsible for 10-15% of all cases (Jett et al., 1994). The over-representation of *E. faecalis* is most likely due to the natural abundance of *E. faecalis* in the gastrointestinal tract where it outcompetes *E. faecium* 1 to 100 (Goh, Yong, Chong, & Kline, 2017). *E. faecalis* are capable of causing a wide array of diseases in humans, including urinary tract infections, bacteraemia, endocarditis peritonitis, and all forms of wounds (Jett et al., 1994). Specifically, *E. faecalis* is one of the most predominant species in hospital environments (Kühn et al., 2003) and is associated with 70% of all wound infections (Tien et al., 2017).

E. faecalis is like most enterococci, extremely hardy and shares most of its morphology and physiology across the "genus". *E. faecalis* is known to demonstrate a higher ability to survive in the presence of several environmental stress factors often associated with hospitals, such as temperature, acidic pH, and oxidative stress (Benachour et al., 2005). These stress tolerances are

associated with innate stress responses and are normally associated with chromosomal genes and give *E. faecalis* an increased intrinsic-resistance.

In contrast, virulence factors might be acquired by transposomes or plasmids (Huycke, Sahm, & Gilmore, 1998; Paulsen et al., 2003). In some species of *E. faecali*s over 25% of the genome consists of foreign sequences. A high amount of foreign sequences is associated with high virulence. These virulence factors seem to allow for better colonisation of the host. Virulence factors have been shown to increase enterococcal attachment, modulation, and even the evasion of the host's immune system (Tien et al., 2017).

Regardless of the negative sides, *Enterococcus* have potential benefits for human health. *E. faecalis* are currently and frequently used as starter cultures and probiotics. Additionally, because of its characterisation as a LAB, it is frequently used in milk products (Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). However, the prevalence of *E. faecalis* infections highlights the need for *in vivo* studies in order to replicate clinical scenarios (Goh et al., 2017).

1.3.1 Virulence factors of Enterococcus Faecalis

The dualistic nature of *E. faecalis*, as an inhabitant of the gastrointestinal tract and as one of the most predominant agents in nosocomial infections (Ch'ng, Chong, Lam, Wong, & Kline, 2018; Jett et al., 1994), makes *Enterococcus* a force to be reckoned with. Several studies have characterised a plethora of genetic traits that push *E. faecalis* to cause disease. However, the complex interactions between the host and *E. faecalis* are still poorly understood(Ch'ng et al., 2018). Genetic traits that cause enterococci to become pathogenic include; (1) secreted factors, such as enzymes and bacterial toxins (Garsin et al., 2014); (2) surface localised factors, like cell surface proteins or carbohydrates that mandate host attachment or evasion (Domann et al., 2007); and (3) transcriptional regulators. Therefore, the pathogenesis of enterococci is a complex interplay between these virulence factors and the host immune response.

Oxidative stress is an important factor in several diseases and has a clearly defined role in inflammation (Smith, 1994). *E. faecalis* produces Reactive Oxygen Species (ROS). However, the role of these secretions in virulence is unknown (Szemes et al., 2010). *E. faecalis* are registered to produce several oxidative molecules, like hydrogen peroxide and superoxide, which are directly produced through metabolic pathways (Huycke, Abrams, & Moore, 2002; Szemes et al., 2010). *E.*

faecalis also produces hydroxyl radical, which are made via aromatic hydroxylation (Huycke & Moore, 2002).

The *E. faecalis* production of ROS is interesting for the pathogenetic setting because neutrophils and macrophages also produce an array of ROS to combat invaders, which is part of the innate immune system. These immune cells' primary role in the innate immune system is to fight off invaders (Riboulet et al., 2007). The resistance *E. faecalis* expresses towards ROS is therefore essential for E. *faecalis* survival and propagation in a pathogenic niche. Systems and proteins that allow *E. faecalis* to cope with its oxidative stress, such as HypR, OxyR, and Eep can in many cases, be considered as virulence factors (Riboulet et al., 2007). Moreover, their ROS resistance allows for survival within macrophages and neutrophils for a prolonged time (Baldassarri et al., 2005; Gentry-Weeks, Karkhoff-Schweizer, Pikis, Estay, & Keith, 1999).

E. faecalis uses several immune evasion strategies to persist as a pathogen (Thurlow, Thomas, Fleming, & Hancock, 2009). In wounds, these systems include the production of biofilm, a common virulence trait among bacteria, which proves effective in preventing phagocytosis (Roilides, Simitsopoulou, Katragkou, & Walsh, 2015). *E. faecalis* is a known producer of biofilm. Biofilm is observed in several types of *E. faecalis* infections, ranging from the urinary tract, gastrointestinal tract, endocarditis, and skin wounds (Ch'ng et al., 2018). Biofilm serves as a means for bacterial dissemination and resistance within the infection and adds another level of resistance towards the immune system and clinical treatments (Ch'ng et al., 2018).

The low penetrability of biofilms increases *E. faecalis'* already high innate resistance to antibiotics and immune secretions (Foley & Gilbert, 1997). Although occurring in infections, biofilm can also occur on basically all surfaces, ranging from abiotic to biotic, making dissemination in hospital environments more feasible (Ch'ng et al., 2018; Joyanes, Pascual, Martínez-Martínez, Hevia, & Perea, 1999). The factors promoting *E. faecalis'* biofilm are unknown (Toledo-Arana et al., 2001) yet, their role in *E. faecalis* infections has been thoroughly studied (Ch'ng et al., 2018). Several genes in *E. faecalis* have been associated with biofilm production including, aggA, cylA, cylB, cylM, efaA, enlA, esp, gelE, and *eep* (de Marques & Suzart, 2004).

Moreover, comparing the genome of a clinical isolate to a commensal strain revealed that significant parts of the genome of the clinical strain consist of foreign sequences. This is why certain strains of the *E. faecalis* genome contains over 25% of mobile elements (Bourgogne et al., 2008; Paulsen et al., 2003). The foreign sequences mostly contain resistance genes, PAI (Bourgogne et al., 2008; Shankar, Baghdayan, & Gilmore, 2002), and several prophages (Matos et

al., 2013). This high plasticity towards foreign sequences may be explained by the plethora of mobile elements in the faecalis genome and its high usage of sex pheromones (Varahan, 2014). Therefore, the increase in the isolation rate of *Enterococcus* is partly because of its ability to incorporate foreign DNA (Huycke et al., 1998). This plasticity is even encouraged because the strains that show the highest amount of plasticity lack a Crispr-Cas-system (Palmer & Gilmore, 2010). Lacking a Crisper Cas-system reduces the cells' ability to recognise and cleave foreign DNA.

The increase in *Enterococcus* infections is attributed to the use of antibiotics; this usage has led to circumstances that facilitate pathogenic behaviour (Fisher & Phillips, 2009), allowing *Enterococcus* to pursue pathogenic niches. Niches have exposed *E. faecalis* to other virulent bacteria. The use of sex pheromones by enterococci has allowed enterococci to collect more virulence factors. This process occurs in a positive feedback loop, giving enterococci an increasing amount of new genetic elements. However, most virulence factors are poorly understood, and what we do know is extrapolated from animal models.

1.3.2 Infection models

Animal models have been an essential part in the study of *E. faecalis* pathogenesis. Several animal models have been employed to clarify the mechanisms of pathogenicity, host factors, and virulence factors (Goh et al., 2017; Yuen & Ausubel, 2014). The selection of these model organisms is primarily a result of the ethical, practical, and economic aspects associated with this sort of experimenting.

When deciding on a model, it is essential to understand the possibilities and limitations of that model organism. Moreover, in most cases, these limitations come from how well the model organism mimics the part of the human ecosystem being studied. There is no certainty that the bacteria play the same role in the different model organisms, or even within the same organism when exposed to different niches. Hence, there is no universal model that can address all aspects of host interactions, so the model has to be specifically chosen for what sort of response we want to facilitate (Goh et al., 2017). Therefore, a vast number of different models have been developed, ranging from vertebra to invertebrate.

Vertebrates, particularly rabbits, rats, and mice, are extensively utilised as model organisms to study bacterial-host interactions for *Enterococcus*. Their versatility in size, price, and storage allows for

more animals to be studied and allows for greater statistical analysis. In addition, due to the similarities of the immune system across mammalian species, these murine models mimic the human environment to a substantial degree, allowing for comparison (Garsin et al., 2014).

E. faecalis is one of the most frequently isolated bacteria in all types of wound infections, accounting for 15% of surgical site infections in hospitalised people (Chong et al., 2017). The need for animal models to study host interactions for these infections has been of importance. Animal models are needed to study host interactions in these wound niches. Mice have been frequently used for these types of studies because their immune system mimics human-immune systems and because they are more economically feasible (Chong et al., 2017; Malachowa, Kobayashi, Braughton, & DeLeo, 2013; Nizet et al., 2001; Wang, Ge, Tredget, & Wu, 2013).

When looking at bacteria's ability to survive different stresses and express several factors, they all seem to be linked to the same kind of system. Systems that efficiently control the gene expression to ensure rapid response to environmental and chemical changes (Benachour et al., 2005).

1.4 Extracytoplasmic functions of bacteria

As bacteria have little control over environmental changes and damages inflicted upon the cell membrane, they depend on reliable systems for sensing and effectively responding to changes (Ho & Ellermeier, 2012). Bacteria have therefore evolved signal-pathways that activate the use of alternative sigma factors, called extra cytoplasmic function (ECF) (Staroń et al., 2009). ECF allow the bacteria to change their gene expression in response to external stimuli, allowing the cell to make an appropriate modification at the molecular level. These systems are capable of detecting sudden changes in the environment like drought, starvation, temperature change, pH, salt concentration, antimicrobials, and in pathogenic settings, stresses put upon them by the immune system (Heimann, 2002). Because of these vastly different stresses, a numerous amount of responses are needed in each bacteria(Le Breton et al., 2003).

The ECF family of sigma factors controls the expression of genes that change function depending on the environment outside of the cell, increasing its fitness (Heimann, 2002). The best-studied ECF factor is the sigma E factor in *Escherichia coli* (De Las Peñas, Connolly, & Gross, 1997), where all enzymes, genes and phenotypic change have been characterized. However, it is assumed that most bacterial species share these features in *E. Coli* EFC.

In *E. coli*, the sigma E factor is controlled and tethered to a transmembrane protein, called an anti-sigma factor. In the absence of stress, Sigma E is rendered inactive by the anti-sigma factor. When stress occurs, the anti-sigma factor is degraded by membrane-bound and cytoplasmic proteinases, releasing the sigma factor and activating the ECF in a process called Regulatory Intermembrane Protolysis (RIP) (Ho & Ellermeier, 2012).

RIP is a system consisting of 2 membrane locked proteinases and a transmembrane protein (antisigma). When the site I proteinase receives a signal, it cleaves the extracellular part of the transmembrane protein. The cleavage of the transmembrane protein causes a protein change that allows for the cytoplasmic part of the anti-sigma to be cleaved by a site II proteinase, which in turn releases the active protein (Ades, 2008) (Figure 1.1).

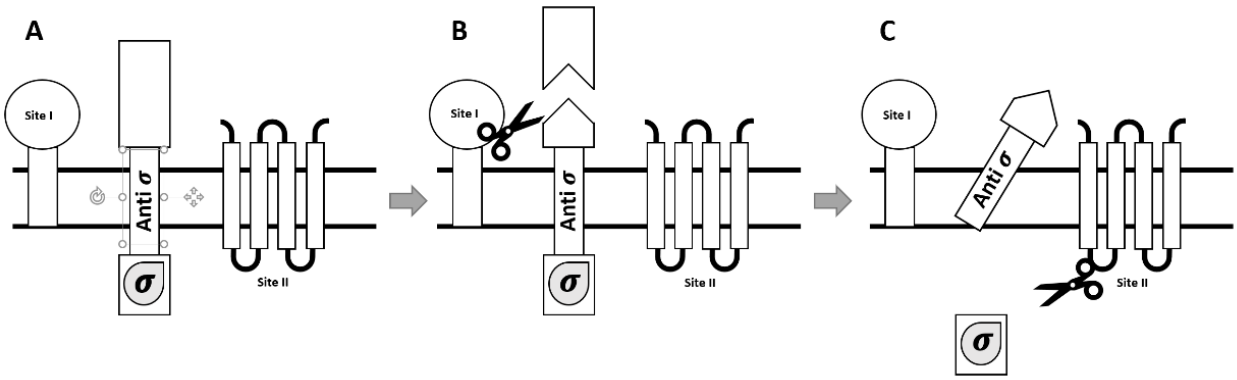

Figure 1.1: **Transmembrane signal transduction by regulated intramembrane proteolysis**. A. Inactive RIP system. The site 1 receives a signal, leading to the cleavage of the extracellular domain of the anti-sigma factor. C. The removal of the extracellular domain allows the site II proteinases to cleave off the cytoplasmic domain releasing it into the cytoplasm.

EFC pathways allow for rapid and concise information transfer to occur across biological membranes, which is why they are often associated with stress and other virulence factors (Giard et al., 2001). Transferring information across biological membranes is a difficult process, which is why regulatory proteolysis of a transmembrane protein has risen as a paradigm for transmembrane signal transduction (Barchinger & Ades, 2013). The strength of the RIP system comes from the fact that it can output a varying intensity signal, allowing for stochastic fluctuations in the environment (Ades, 2008).

1.5 Eep In Enterococcus faecalis

As previously mentioned *E. faecalis* is an extremely hardy bacterium and known to demonstrate a high degree of innate resistance to environmental stress factors including a heightened resistance towards lysosome (Benachour et al., 2005; Le Jeune et al., 2010). Many of these resistances have been linked to *E. faecalis'* ECFs(Varahan, Iyer, Moore, & Hancock, 2013). Eep is a member of the membrane-embedded, zink-metal-peptidases family (M. S. Brown, Ye, Rawson, & Goldstein, 2000). This protein family is often associated with ECF as part of the signal regulatory pathway, where it serves as a site II proteinase in the regulatory proteolysis of RsiV, one of the anti-sigma factors in *Enterococcus* (Varahan et al., 2013). However, the whole system is not characterized in detail. For instance, it is unknown which proteins serve as the site I proteinase, which genes are transcribed by the sigma factor, and if the cleavage of RsiV is the only function of Eep (Varahan et al., 2013). Although the complete role of the Eep associated system in *E. faecalis* is still unknown (Frank et al., 2013), recent significant contributions are finally starting to reveal the underlying mechanisms of the Eep associated system (Figure. 1.2). Removing any of the components of the Eep associated system does not seem to affect the cell in an unstressed environment. However, when inducing stress, the removal of Eep and sigma factor dramatically reduces the survivability of the cells by two orders of magnitude (Varahan et al., 2013). In the case that RsiV is removed, the cell seems unaffected in a stressful environment (Benachour et al., 2005).

While the structure of Eep remains unknown, we know that Eep has a zinc-binding motif HE(I/F/L)GH that seems to be shared across genus, including *E.coli* and *Bacillus subtilis,* ((Ades, 2008; M. S. Brown et al., 2000). The HE(I/F/L)GH motif is embedded in the hydrophobic environment near the N terminal, and it has been suggested that it serves as the active site (Ovchinnikov et al., 2017).

Because Eep is linked to so many responses, such as biofilm formation (Frank et al., 2013), sex pheromone secretion (Varahan et al., 2014), and as a stress response (Benachour et al., 2005; Frank et al., 2012; Ovchinnikov et al., 2017; Varahan, 2014; Varahan et al., 2014) it is clear that this is an important factor in the adaptation to environmental changes and is an important factor in infection settings (Frank et al., 2013).

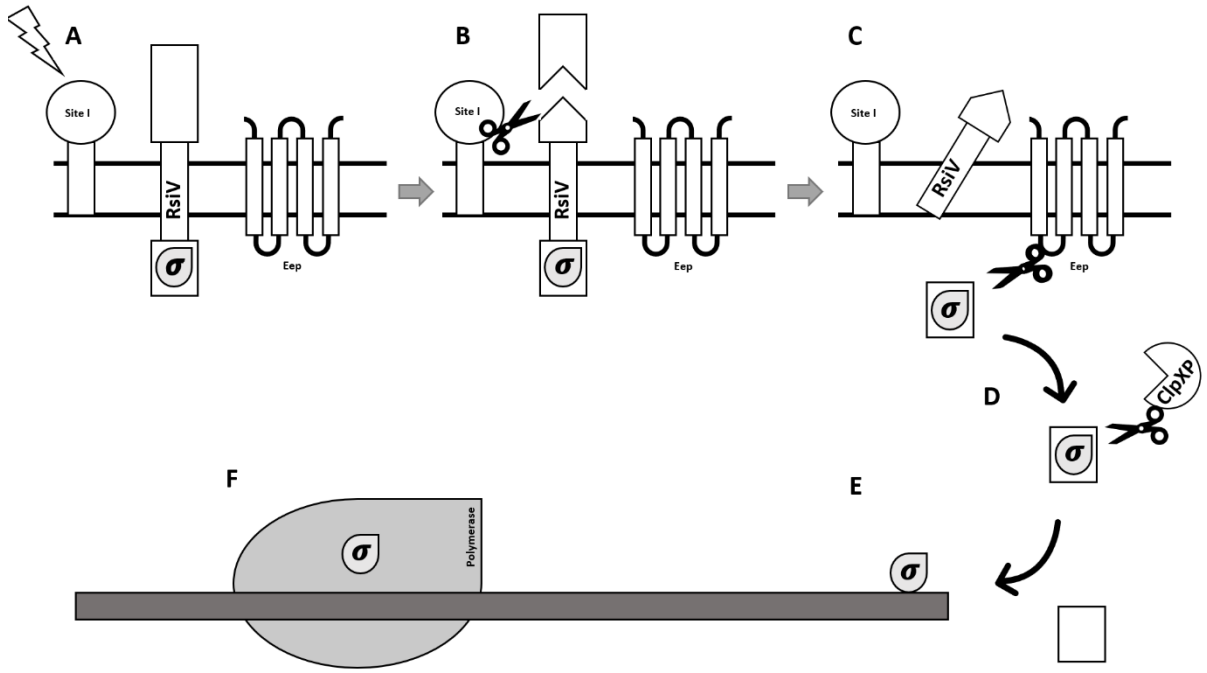

Figure 1.2: **The regulation of the *E. faecalis* sigma V.** The figure illustrates the proteolytic cascade involving Eep for the regulation of sigma V. **A**. *receiving signal.* A theoretical site I protein receives a signal. **B**. *activation of theoretical Site I*. Site I cleaves the Rsiv by releasing the ectoplasmic domain. ***C***. *activation of Eep*. After the exterior cleavage of Rsiv, the substrate is opened for Eep to cleave the cytoplasmic domain, releasing a Rsiv Sigma fragment. ***D***. *further degradation of Sigma V*. The cleavage of Rsiv generated a Rsiv-Sigma fragment. This is further degraded by ClpXP, releasing the free sigma V factor into the cytoplasm. ***E***. *Sigma attachment and translation*. The free sigma V activates the transcription of target genes.

1.6 Eep can be targeted by bacteriocin

Because of the importance of Eep in a variance of niches, it is of great interest that this proteinase is targetable by bacteriocins (Ovchinnikov et al., 2017). Bacteriocins fall into a large group of antimicrobial peptides (AMP) that are able to target mechanisms other than those targeted by traditional antibiotics (Zhang & Gallo, 2016). AMPs are a group of evolutionarily conserved molecules produced as a defence against a broad range of microorganisms including fungi, viruses, and bacteria (Zhang & Gallo, 2016). These AMPs may be an alternative to antibiotics (Cotter, Ross, & Hill, 2013).

Bacteriocin produced by gram-positive bacteria are a group of small antimicrobial peptides produced by bacteria to kill bacteria (Diep & Nes, 2002). In contrast to antibiotics, they are mostly ribosomally synthesised, with or without post-transcriptional modifications (Diep & Nes, 2002). Bacteriocins provide a selective advantage in the competition for niches because bacteriocins serve

as toxins towards other related bacterial species (Alvarez-Sieiro, Montalbán-López, Mu, & Kuipers, 2016) in their niche (Eijsink et al., 2002). Bacteriocins come with several advantages over traditional antibiotics, such as being antimicrobial active in the micro to the picomolar range and the difference in targeting strategy makes them unbiased against already antibiotic-resistant antibiotics (Cotter et al., 2013).

EJ97, which has been proven to target the Eep proteinase, is part of the LAB class IIc leaderless bacteriocins (Alvarez-Sieiro et al., 2016). Class II bacteriocins are membrane-active peptidases, meaning there mode of action is disrobing the integrity of the membrane, causing cell death by extracellular leakage (Kjos, Nes, & Diep, 2011). This group of leaderless bacteriocins from gram-positive bacteria seem to have a broader range than gram-negative bacteriocins, which is why they have increasingly received attention in the last decade because of the possibilities for drug development (Nes, Kjos, & Diep, 2011).

AIM of this study

The aim of this study was to investigate whether the Eep proteinase in *E. faecalis* played a large role in the colonisation of wounds, and to improve our understanding of the molecular mechanisms involved in enterococcal wound pathogenicity, which currently is lacking (Chong et al., 2017).

The importance of the Eep proteinase was tested by using bacterial isolates with a functionally disrupted Eep proteinase in a mouse wound trial, consisting of the following tasks.

1. The isolation of the mutants: The isolated process was done by naturally selecting for resistant mutants in the presence of bacteriocin.
2. Genotyping of mutants: Validating the presence of the disrupted *eep* gene in each isolate.
3. The approval of the Norwegian government: In Norway, all experimentation with animals needs the approval of the Norwegian government.
    - Qualified personnel: in accordance with Norwegian law, all personnel taking part in animal trials needs qualifications according to their role in the experiment.
    - Specific trial approval: in accordance with Norwegian law, all experiments need to be approved by the Norwegian Food Authorities, by the online tool FOTS.
4. Animal trial: Testing the functional Eep compromised *E. faecalis* in an approved mouse wound model.

Materials

2.1 Mediums and agars

Medium	Supplier
BHI (brain heart infusion) Lot: 2354995	Oxoid, USA
Growth medium: 18.5g BHI	
dH20 to 500 ml	
Sterilised in an autoclave for 15 min at 121 deg	
Soft Agar: BHI medium with agar (8g/L)	
Agar: BHI medium with agar (15g/L)	
BEA (Bile esculin agar) Lot: BCBV7559	Oxoid, USA
Agar: BHI medium with agar (64,5g/L)	
dH20 to 1L	
Sterilised in an autoclave for 15 min at 121 deg*	

*in contrast to the instructions for the manufacturer, however the manufacturer confirmed us that it wound not affect the selective capabilities of the of the medium.

2.2 Bacterial strains

Table 2.1: **Bacterial strains implemented in this study.**

LMG lab strain reference	Bacteria
LMG_3336	Enterococcus faecalis EF62
LMG_3388	Enterococcus faecalis EF Symbioflor
LMG_3593	Enterococcus faecalis HH22
LMG_3281	Enterococcus faecalis OG1RF
LMG_3566	Enterococcus faecalis Merz96
LMG_3088	Enterococcus faecalis V583
LMG_3560	Enterococcus faecalis D6
LMG_3569	Enterococcus faecalis T3
LMG_3351	Enterococcus faecalis MMH594(E0740)
LMG_3592	Enterococcus faecalis TX0104

2.3 Bacteriocins

Table 2.2: **Bacteriocins used in this study**

Bacteriocin	Amino acid sequence	Reference
EJ97	MLAKIKAMIKKFPNPYTLAAKLTTYEINWYKQQYGR YPWERPVA	(Gálvez et al., 1998)

2.4 Instruments

Function	Instrument	Developer
PCR	MyCycler	BioRad, USA
Gel visualisation	Molecular imager® Gel Doc™ XR+	BioRad, USA
Tissue Homogenizer	GentleMACS™ Dissociator	Miltenyi Biotec, USA
DNA Purity	NanoDrop 2000 spectrofotometer	ThermoFisher Scientific, USA

2.5 Software

software	Developer
Snap Gene	GSL Biotech, USA
image Lab™ 6.0	BioRad, USA
tm Calculator v.1.10.2	New England Biolab, USA
Primer Blast NCBI	The National Centre for Biotechnology Information, USA

2.6 Kits

Kit	Ref	Supplier
Nucleospinn® **Gel and PCR Clean-up**	740609.250	Macherey-Nagel, Germany
OneTaq® DNA polymerase **#M0480L**	10017332	New England Biolabs, USA

2.7 Chemicals and reagents

Chemical	supplier
Agar, powder	VWR, USA
agarose Ultrapure™	Invitrogen, USA
Loading buffer 6x	New England Biolabs, USA
50x TAE*	

* 50xTAE (tris-acetate-EDTA): 121 g Tris base is added to 28.5 ml acetic acid and 50 ml 0.5M EDTA pH 8.0. then milliq water is added to a final volume of 0.5 l

2.8 Primers

Table 2.3: **List of Primers.** shows a complete list of all primers used in this study, their sicqwnce and their application

Primer	Sequence (5'→3')	Application
PCR F	TAGGCGAAGTGGTCAAGTCC	Forward primer for PCR application of the *eep* gene
PCR R	GGTTTCTTCATGCGTTGGGC	Reverse primer for PCR application of the *eep* gene
Seq 1 F	TTTTACGAGACTTTCCCATGT	Forward primer for sequencing of the *eep* gene
Seq 2 F	ATTCTGTTTACGTTAGCGG	Forward primer for sequencing of the *eep* gene
Seq 3 R	TTTCATATAAGGATAAACGCCGACT	Reverse primer for sequencing of the *eep* gene
Seq 4 R	CTTCTGCATCATTTGGTACTTC	Reverse primer for sequencing of the *eep* gene

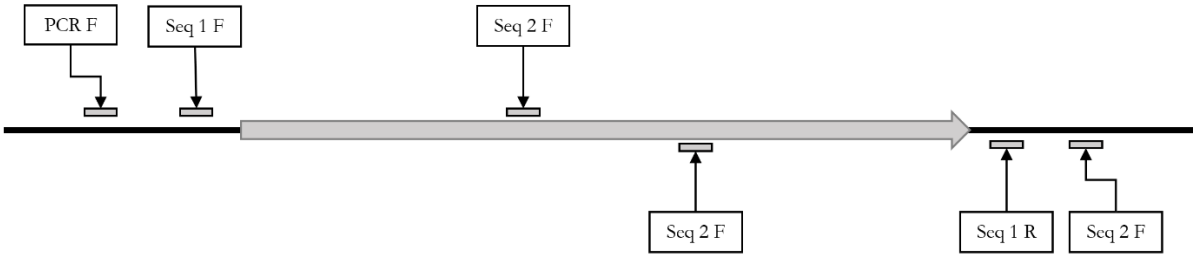

Figure 2. 1: **Primer positions to the *epp* gene:** shows all primers developed for this study in their relative position to the eep gene

2.9 Drugs

Table 2. 4: **Drugs used in this study.**

Name	Producer	Recipe
ZRF cocktail	Zoletil forte (Virbac, France), Rompun (Bayer, Germany) and Fentadon (Eurovet, The Netherlands) (ZRF cocktail) injected	ZRF cocktail contains zolezepam (~30 mg/kg), tiletamin (~30 mg/kg), xylacin (~4.5 mg/kg) and fentanyl (~26 ug/kg).
Tamgesic	(Indivior, Ireland)	
Isoflurane	(Baxter, Ill, USA),	

2.10 Animal lab mice pens and other utilities

Equipment and other utilities	Producer
Mice	Mice: BALB/cJRj Janvier Labs, Saint Berthevin Cedex France
Cages	Innovative, USA
Hair removal cream	Nair, USA
Food	Special diets service, UK
Ventilation/rack	Innorack IVC Mouse, Innovive, USA
Tegaderm dressing	M3, USA
Humidifier	Condair CP3 Mini humidifier

Methods

3.1 General

3.1.1 Sterile working techniques

Sterile working techniques were utilised throughout the laboratory part of this thesis. The appropriate countermeasures were taken to prevent possible contaminants in all procedures with inherent threats of contamination. All lab work was carried out in sterile workbenches. Before each sitting, all equipment, if not delivered sterile from the manufacturer, was autoclaved or ethanol and burning were used, depending on the equipment's material.

3.1.2 Media and incubation

All cultivation of bacteria requires a suitable growth media. When cultivating bacteria, we want bacteria to grow at a desirable rate, which is why we used Brain heart infusion (BHI) for the majority of our experiments. BHI is a general-purpose, nutrient media suitable for cultivating most bacteria. Another medium, Bile Esculin Agar (BEA) was also used in this study. This medium comes with a selective property, only allowing for the growth of *Enterococcus* related species(Lindell & Quinn, 1975). Thus, we used BEA in experiments where we wanted to discourage the growth of all other bacteria.

While 37°C is more suitable for enterococci growth, all media in this study were incubated at 30°C to avoid too much growth when left overnight.

3.2.3 Storage

All isolates presented in this thesis needed to be stored for a prolonged amount of time. Stock samples were made with a 15% glycerol stock solution to prevent ice crystals from disturbing the cell when stored at -80°C

3.2 Schematic of work progression

This study aimed to determine to what degree the removal of *eep* played a role in the virulence of wound infections, in order to better our understanding of the Eep associated stress response.

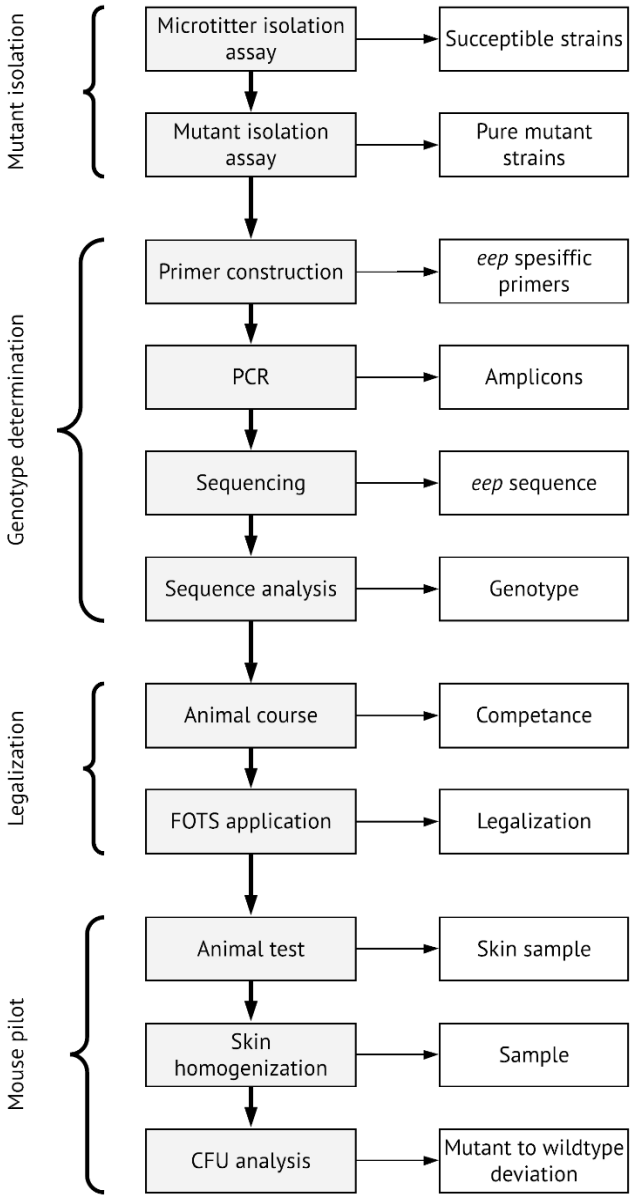

Figure 3.1: **Schematic outline of the workflow**. This figure shows the schematic workflow of this study, including the four main goals and their according outcomes.

3.3 Mutant isolation

In this study, we chose to select for bacteriocin resistant bacteria, by exposing *E. faecalis* to the class IIc bacteriocin EJ97 (Alvarez-Sieiro et al., 2016). Class II bacteriocins are membrane-active peptidases, meaning there mode of action is disrobing the integrity of the membrane, causing cell death by extracellular leakage (Kjos et al., 2011). Thus, only bacteria with an unavailable Eep are capable of growth (Ovchinnikov et al., 2017). Although exposing bacteria towards bacteriocin gives no control over the mutation that is selected for, this method gives a bacteriocin resistant phenotype. However, since the growth and selection are set to occur under favourable non-stressing conditions, it is theorised that because there is no need for the *eep* gene, it is likely that the source of resistance would come from a naturally occurring defect gene.

Although more precise methods of gene modulation exist, the bacteriocin approach was used because this thesis is part of a project which has a primary goal to investigate whether certain bacteriocins can be developed into drugs for therapeutic usage.

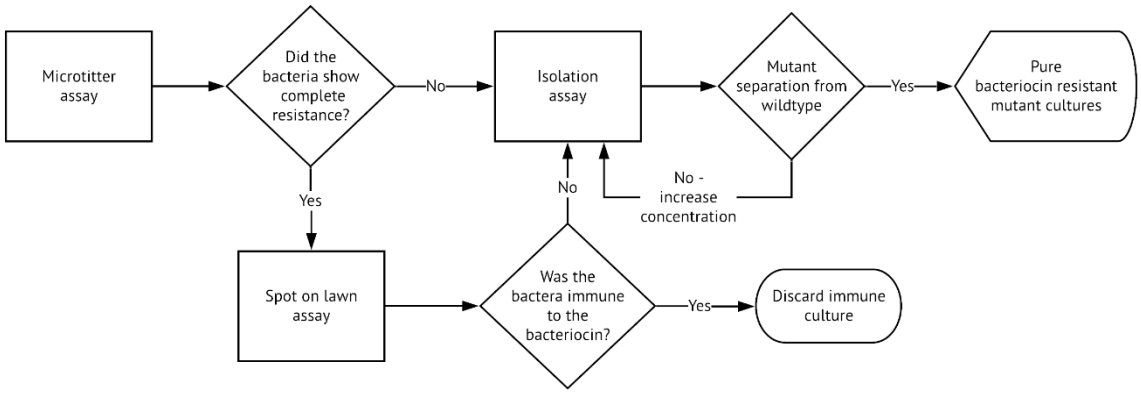

Figure 3. 2**: Mutant isolation by bacteriocin-selection flowchart**: Shows the flowchart used for bacteriocin creation in this study.

3.3.1 Microtiter-inhibition-assay

To investigate to what degree the isolates at our disposal were susceptible to the EJ97, we used a minimal inhibition concentration assay. These assays use a two-fold dilution of any given substrate to obtain the minimum inhibition concentration (MIC). This assay allows for the calculation of the minimum inhibition concentration needed to reduce growth by 50% (MIC_{50}) (Ovchinnikov et al., 2017) (Figure 3.1).

For the assay, we diluted 1mg/ml EJ97 in a BHI medium. The assay concentration started at 50 µg/ml in column 1, with a two-fold dilution ending in the eleventh column at 0.02 µg/ml and the twelfth column served as the positive control in the assay. Each row served as a different bacterium, and row H served as a negative control. All wells had a final bacterial concentration 1/50 of the overnight culture (ON). The plate was then incubated at 37°C for 4h and read in a SPECTROstar nano absorbance plate reader with optical density (OD) 600nm.

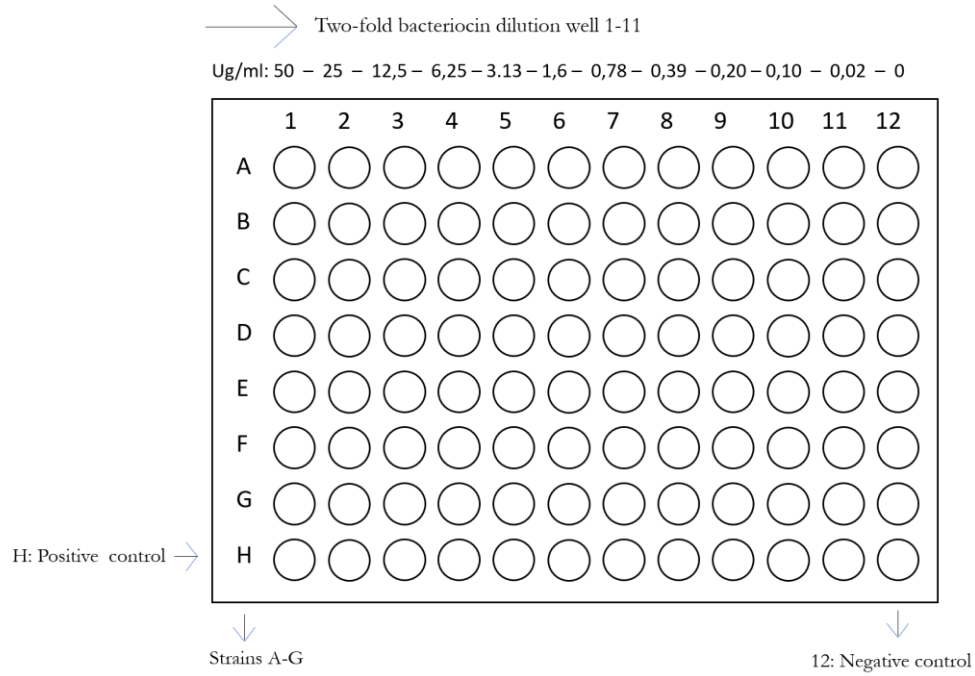

Figure 3. 3: **Diagram of the 96- well minimum inhibition assay:** Diagram of a 96-well microtiter plate, used to determine the bacteriocin-EJ97 resistance. EJ97 was diluted twofold for each column 1- 11, leaving column 12 for negative control(without EJ97) and row H for positive control and allowing for the estimation of the MIC_{50}-values.

3.3.2 Spot-on lawn-inhibition assay

The spot-on-lawn assay is a technique utilised to test a bacteria's behaviour towards a given substrate or another microorganism. This technique was used to test whether the certain strains of *E. faecalis* can survive EJ97 at concentrations outside the spectrum of the (3.3.1) method. The microtiter-inhibition-assays are mostly to confirm the EJ97 resistance in our strains, even at the highest possible concentrations.

In preparation of the assay, 5ml of BHI soft-agar was re-heated at 50°C. The soft-agar was then gently mixed with 70µl of ON culture. (bacterial culture containing approximately 10^8 CFU per ml). The bacterial soft-agar mix was then poured onto a BHI 25 ml agar-plate and left to settle. After 10 minutes, 10 µl bacteriocin was dropped onto the plate following the desired concentration and patterns, before being incubated overnight at 30°C. The concentration used in this study were 1mg/ml, 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml in the pattern illustrated in Figure 3.3.

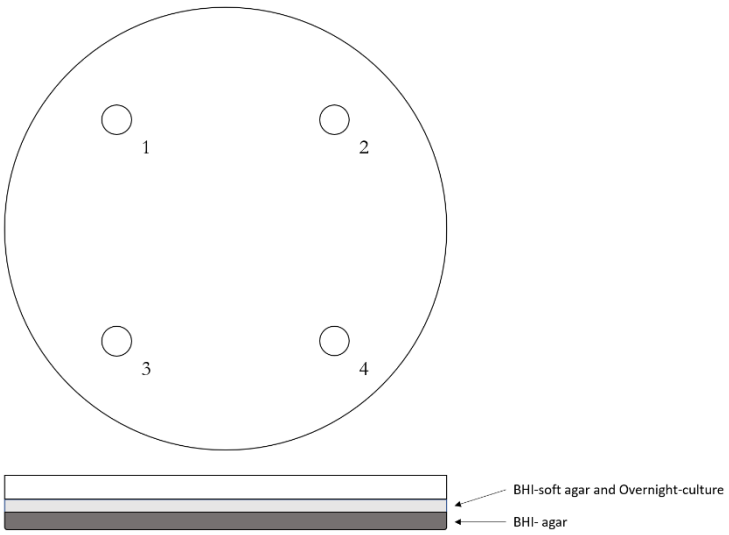

Figure 3. 4: **Spot-on-lawn inhibition assay.** Shows an illustration of a possible organisation pattern of the spot on lawn assay, as seen from above. The illustration of agar and soft agar from the side indicates the layering of the different media.

3.3.3 Mutant isolation

To isolate pure mutants, we used the bacteria that had shown susceptibility to the bacteriocin (Table 4.1.1.) in a somewhat modified version of the (3.3.2) spot-on-lawn inhibition assay. This method allows for the selection and allowed us to isolate natural bacteriocin-resistant mutants occurring in the wild type culture.

In preparation of the assay, 5ml of BHI soft agar was heated up to approx. 50°C. The soft-agar was then gently mixed with 70µl of ON bacterial culture and 70 µl of 1mg/ml bacteriocin EJ97. The soft-agar, bacteria, and EJ97 mix were then poured onto a normal 25ml BHI agar-plate, and let to settle before being incubated overnight at 30°C.

After 24h, bacteriocin resistant single colonies should have emerged on the plate. Sterile loops were used to pick four different colonies, which were streaked onto new BHI agar plates to create single pure colonies. These four plates were created to ensure pure colonies for the creation of pure glycerol stock.

Figure 3. 5: **Mutant isolation assay:** illustrates the layering of the mutant-creation-assay, as seen from the side.

3.4 Genotype determination

Because of the type of selection assay we used, the reason for the resistance was unknown and we needed to determine the genotype. Ideally, we wanted these mutants to have a deleted *eep*. However, because of the shortcomings of the bacteriocin-method, before genotyping, we only knew the bacteriocin-resistant phenotype, which required us to determine the reason for the resistance. For this study, we chose only to genotype the *eep* gene, arguing that although other mutations might be responsible for the resistance, we are only interested in those that have a confirmed defect protein. Therefore, any other mutations were of little interest because we set out to determine what happens when the Eep proteinase is disrupted.

3.4.1 Primer construction

In order to obtain complete and reliable coverage of the *eep* gene, we needed a total of six primers, two for the Polymerase chain reaction (PCR)and four additional for sequencing, as illustrated in Figure 2.1.

In order to get complete coverage of the *eep* gene. The PCR primer pairs needed to be placed outside of the *eep* gene, at least 250 base pair upstream and 200 base pair downstream, preferably within conserved regions, in order to remain functional across several strains of *E. faecalis*. To obtain these conserved flanking regions, we used a blueprint sequence from the Uniport database (*Q9RPP2)*. The *Q9RPP2* sequence was Blasted (Basic Local Alignment Tool) to obtain a Multiple Sequence Alignment (MSA) containing several *E. faecalis species*. The MSA allowed us to identify several conserved regions flanking the *eep* sequence.

Like the PCR primers, sequencing primers needed to be placed in conserved regions. Two of the sequencing primers needed to be placed outside of the *eep* gene, to obtain the beginning and the end of the *eep* sequence, yet needed to remain inside the amplicon of the PCR primers. The last two sequencing primers needed to be placed somewhere inside the *eep* gene. These four primers collectively enabled a minimum of two amplicon overlaps when sequenced.

With these conserved flanking regions of the *eep* gene, we used the NCBI's primer design tool (Primer-Blast) to search for the most optimal primers in the designated conserved regions. This tool locates the best spots based on balancing the G/C content, working around self-complementing, hairpin structures, target specificity, and complementing between primer pairs, all working around a set optimal melting temperature (T_m) (Ye et al., 2012). Thus, the primer-blast was used to give the most optimal locations and sequence for the primers. Finally, the primer sequences were sent to the New England Biolabs, where the primers were assembled.

3.4.2 DNA extraction

In order to perform PCR, DNA had to be extracted from the bacterial cells. For this study, we chose to lyse the cells by microwaving. Microwaving is a rough, rapid, and cheap technique, rapidly reducing the amount of time needed in comparison to chemical or physical lysis. The DNA was extracted by picking a single bacterial colony from an agar plate, placing it into a PCR tube, and microwaving it at 800 W for 1 minute.

3.4.3 Polymerase chain reaction

We used PCR in order to obtain concentrations of *eep* amplicons suitable for sequencing for all *E. faecalis* isolates. The *eep* gene was amplified using regular PCR, utilising OneTaq® DNA polymerase amplification kit (New England Biolabs). We made a PCR reaction mix (master mix), according to Table 3.1, containing the OneTaq Buffer, nucleotides, primers, and polymerase (New England Biolabs) with our PCR primers, created in section 3.4.4 (Table: 2.3).

Table 3. 1: **Reaction setup for PCR**; table show the proportion of components with their corresponding concentrations and volumes.

Component	For a 50 ul tube	Final concentration
5x PCR buffer[1]	10 µl	1x
10nm dNTP	1 µl	200 µl
10 nM Forward Primer	1 µl	0,2 µM
10 nM Reverse Primer	1 µl	0,2 µM
One Taq Polymerase	0,25 µl	1.25 units/50 u PCR[2]
dH2O	36.75 µl	
Bacteria	One colony	
Total volume	50 µl	

1. OneTaq Gc action buffer and Hight Gc enhancer can be used for difficult applications
2. For applications between 3-6 kb, use 2,5-5 units

Table 3. 2: **PCR program for DNA amplification**. The PCR program used to amplify the *eep* gene with OneTaq® DNA polymerase and tm adjusted for the PCR primers (Table 2.3)

STEP	TEMP	TIME
Initial denaturation	94 C	30 seconds
35 cycles	94 C	30 seconds
	51 C	45 seconds
	68 C	2.1 minute
Final Extention	68 C	5 minutes
Hold	4 C	Infinity

3.4.4 Sequencing

PCR product clean-up and Nanodrop

The PCR product was purified using Nucleospinn® Gel and PCR Clean-up up kit according to the manufacturer's instructions. Next, a NanoDrop ND-1000 (NanoDrop technologies) measurement was utilised to quantify the DNA concentration and to measure the purity after the PCR clean-up.

Agarose gel electrophoreses

To visualise the amplicons, we used agarose gel electrophoresis; this method allows for the confirmation of the correct amplification of the PCR product, by comparing the indicated length of the amplicon to the theoretical length.

All samples in this study were run on 1% agarose. This 1% concentration was chosen for its versatility and easy assembly, and because 1% of agarose gels proved sufficient in separating the bands. In order to create a 100ml gel, we used 1g agarose mixed with 100ml TAE-buffer and 4 µl of Peq-Green. The electrophoresis was set up with a Bio-Rad electrophoresis kit. After placing the gel into the electrophoresis chamber, it was filled with 1xTAE (Tris-acetate-EDTA) buffer. The clean PCR products were mixed with 50% loading buffer for visualisation and were loaded onto the gel in 5 µl concentrations with a 1kb ladder on each side. The agarose gel electrophoresis was run at 100v for 20 min; this voltage and time period were sufficient in separating the bands. For visualisation, the gel was placed in a photographic chamber (Bio-Rad) and visualised under UV light.

Sequencing

After the NanoDrop measurement and visualisation of the gel, samples were diluted with dH20, to meet the preferred concentration of 20 -80 ng/µl, which was needed for sequencing. For each cleaned PCR product, in order to get a complete read of the entire *eep* gene, a total of four samples were sent to sequencing. Each of the four sequence samples were prepared by mixing 5 µl of the newly diluted DNA samples (20-80 ng/µl) with 5 ul from one of the four sequencing primers. Thus, each of the four sequence samples added up to a total volume of 10 µl. Moreover, each sample was sent out of house to be sequenced by Eurofins Genomics Sanger sequencing services by GATC Light Run, allowing for reads up to 1100bp.

Sequence processing

After the samples were processed by Eurofins Genomics, the sequenced reads were downloaded from the Eurofins Genomics website. The generated reads were processed using Snapgene®, allowing assembly of continuous sequences and for MSA between strains.

3.5 Legislation of animal experiments

Animal experiments require the approval of the Norwegian food safety authority (Matilsynet). To acquire authorisation from Mattilsynet, a certified laboratory (§ 5), qualified personnel (§ 24), and approval of the specific experiment (§ 6 and § 7) are required before one can perform any type of animal experiments (forskrift om bruk av dyr I forsøk FOR-2015-06-18-761).

Applicant and applications

Personal qualifications can be obtained by two means. First, one can obtain a temporary approval by a leading scientist who has the appropriate qualification to grant such approval. Alternatively, one can complete the "course in laboratory animal science for researches, 10 ECTS" or any corresponding foreign courses. The Norwegian course is based on the recommendations from FELASA category C and covers the Norwegian and European regulations for performing animal experiments. Of these two options, the FELASA C course was chosen.

Finally, all experiments need direct approval by the Mattilsynet. In Norway, all authorisations are granted through Forsøksdyrforvaltningens tilsyns og søknadssystem (FOTS), an online application processing system. Prior to the experiments a FOTS application was therefore submitted and approved.

Ethics

Animal ethics is an essential issue in all animal associated studies, building on the fact that animals have the capacity to suffer, a trait that species other than man commonly share. Therefore, animals are protected from suffering unnecessarily under European law. This protection makes it mandatory that all animal experimentation in Norwegian territories is approved and, to some degree, will produce results which can provide benefits for their field of study. In this study, the two important ethical considerations were met. First, Mattilsynet approved the experiments. Second, the procedures performed on animals were all performed in compliance with Mattilsynet's approved guidelines.

3.6 Mouse pilot experiment

Vertebrates, particularly rabbits, rats, and mice, are extensively utilised as model organisms to study bacterial-host interactions. These interactions are meant to mimic human-bacterial interactions due to the similarity of the innate and adapted immune systems (Garsin et al., 2014). Because of this, animal models have been increasingly used to understand the pathogenicity of enterococci. Moreover, animal models have provided several advancements in this field (Clewell et al., 2014).

Therefore, we wanted to test our truncated *eep* mutants and their corresponding wildtype to determine if there is a difference in their ability to colonise a mouse wound. Therefore, a mouse-wound-pilot was carried out in this study, modified from Chong and coworkers (Chong et al., 2017) to accommodate our bacteria and available equipment. Skin serves as a mechanical barrier and is the primary interface between the body and the environment, as well as playing an essential role in the innate and adaptive immune response. Additionally, it serves as an early warning system for infections (Kupper & Fuhlbrigge, 2004).

3.6.1 Experimental design of the pilot

Experimental setup of the pilot trial

Thirty-six female BALB/cJRj(Janvier Labs, Saint Berthevin Cedex France) mice were divided into three experiments (n=12): experiment 1 (LMG_3560), experiment 2 (LMG_3569) and experiment 3 (LMG_3592). Each experiment consisted of 3 groups (n=4). Each experiment was set to test one *Enterococcus* mutant and its corresponding wildtype. Therefore, each experiment contained the following: cage 1, LMG_wildtype (n=4); cage 2, LMG_mutant (n=4); and cage 3, a saline control group (n=4) (Figure 3.5).

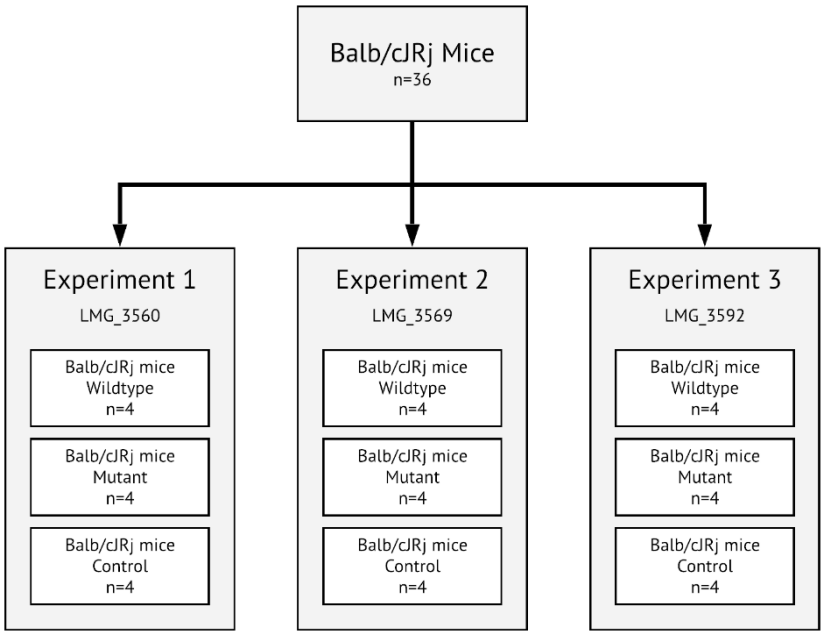

Figure 3. 6: **Division of the mice experiment**. Mouse groups, with a three experimental grouping, containing n=12 mice each. Each grouping was further subdivided into three groups of n=4: group 1 consisting of wildtype, group 2 Mutant strain, and group 3 consisting the Saline control.

Animals and housing

The mice used were of the BALB/cJRj variant. The BALB/c is an inbreed strain dating back to 1935. These mice are a well-established model organism for skin infections (Chong et al., 2017; Malachowa et al., 2013; Nizet et al., 2001; Wang et al., 2013).

The 36 female mice included in this experiment were bought from Janvier Labs. Upon arriving at our facility, the mice were acclimatised for a minimum of two weeks, before the experiment started. The mice were 10-13 weeks old at the start of the experiment. The animals were housed in individually ventilated cages, where the temperature was set to keep at 24°C, the humidity was set to 45-55%, and with a 12h light-dark cycle. Moreover, food and water were given *ad libitum* and the cages were equipped with everything needed for standardised mice husbandry, including a running wheel and house. Before each experiment, the mice in each experimental group were relocated at random to remove any potential cage effects, like grooming or dietary habits.

3.6.2 Wound creation and inoculation

Mouse welfare is always an utmost priority, and because of these concerns, we separated the depilation (hair removal) step from the wound creation step. These steps were separated to ensure the time and drugs needed to perform both exercises would not put the mice under unnecessary stress.

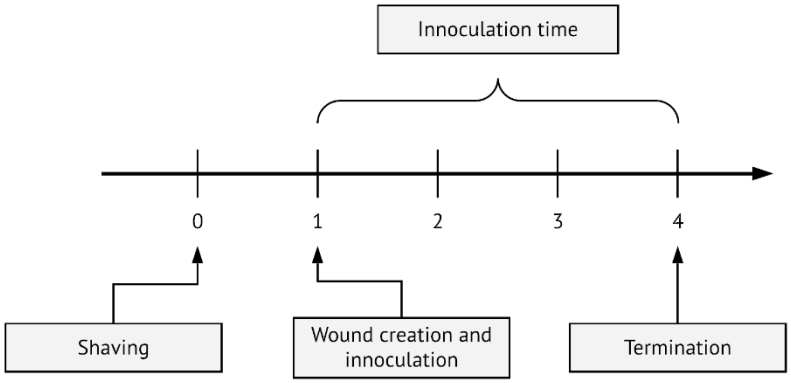

Figure 3. 7: **Mouse pilot experimental timeline.**

3.6.2.1 Depilation T0

To ensure a clean wound and an adequate seal for a wound dressing, the dorsal regions of the mice where depilated one day before wound creation. During depilation, all mice were anaesthetized by the use of 2.5% isoflurane (Baxter, Ill, USA), delivered through a face nozzle. The majority of the hair was removed using an electric hair trimmer, and the remaining hair was removed with a hair removal cream (Nair,USA),. After cream removal the mice were taken off the isoflurane and allowed to make a recovery in their corresponding cages, with a wake-up time of approximately 30 seconds.

3.6.2.2 Wound creation T1

On the day of surgery, the mice were anaesthetised with ZRF 100 µl/10g by intraperitoneal injection, as this method did not need additional equipment and allowed us to perform the experiment in a sterile environment. Alternatively, isoflurane could have been used (Wang et al., 2013), allowing for more adjustable anaesthesia and quicker recovery. However, intraperitoneal injections with ZRF allow for deeper sleep, making the surgery less painful, and allow the operation to be performed in a sterile hood, reducing contamination risks. To counteract the deeper sleep and longer recovery time, which reduce the mice's ability to control body temperature, the mice were kept on a heating pad and closely monitored during the entire operation and recovery time.

Before surgery, the skin was disinfected with ethanol, and the procedure was performed on a sterile bench. The wound was placed in the centre of the shaved area and was created by pinching the dorsal skin cranially and caudally and making a double skin fold. From this double fold, a crescent was punched out with a 6 mm biopsy punch, creating a full-thickness circular wound across the midline of the back.

The wound was then inoculated with 10 µl of its corresponding determined solution (saline, mutant or wildtype strain), containing 10^6 CFU. This concentration of *E. faecalis* was chosen because it has proven sufficient to infect wounds and cause a long-lasting infection (Chong et al., 2017). Lastly, the wound was sealed using Tegaderm dressing (M3,USA) as a countermeasure to prevent any contamination of the wound.

3.6.2.3 Surveillance

Because of the nature of the experiment, the mice were under strict and constant surveillance. Surveillance was done to ensure that the experiment never inflicted unnecessary levels of distress. Moreover, the mice were scored in compliance with the scoring sheet in the FOTS application to determine if the experiment ended up causing distress. As an additional measure for pain management, the mice were given additional painkillers, Temgesic (Indivior, Ireland) 1 µl/g of mice, subcutaneously every 12h for the first 24h of the experiment.

Figure 3.8: **Mice with a full-thickness wound and Tegaderm.**

3.6.3 Tissue processing

All animals were terminated three days after the bacterial inoculation. Similar infection studies of wildtype *E. faecalis* showed that three days seemed to stabilise the number of bacteria in the wound (Ch'ng et al., 2018), minimising the bacterial variance between mice.

All mice were euthanised by cervical dislocation before sampling.

3.6.3.1 Tissue collecting

The dorsal region and Tegaderm were swabbed with ethanol shortly after euthanasia to avoid contamination from the skin. A square 1x1 cm piece of skin/Tegaderm surrounding the wound was rapidly cut out. Moreover, the skin/Tegaderm needed to be cut up into small pieces before being placed in an M-tube (the proprietary tubes accompanying gentleMACS-dissociater, USA) and mixed with 1 ml of saline solution. This additional cutting-measure was taken to ensure that we did not clog the M-tube.

3.6.3.2 Homogenising of the tissue

In order to get an adequate read, a thorough homogenization of the skin was needed. To homogenise the tissue, we used the gentleMACS-dissociate, with RNA.01.01 program. Although this is a machine generally used for homogenising multicellular tissues, this program has been established to be able to separate bacteria from the host tissue. The RNA.01.01 program was run twice, before being filtrated through a 40 μm sieve, in order to obtain an adequate homogenisation of the tissue. The filtration was done in order to remove the last of the larger pieces and the unprocessed Tegaderm.

3.6.3.3 Agar spreading

In order to quantify the number of bacteria in the wound, we chose to use selective BEA to assess the number of CFU. This method allowed us to obtain the exact number of bacteria in the wound.

For the counting, homogenised skin tissue was diluted in a twofold dilution series, with each dilution spread upon BEA plates. The BEA plates were then incubated at 30°C for 18h. 18h is enough time for colonies to create the stereotypical black haze, but not in amounts that would negatively interfere with counting. By counting the single forming units on each dilution, we could calculate the number of bacteria in the original sample and therefore, the amount of CFU in the wound as a whole.

Results

4.1 Mutant creation

4.1.1 Microtiter-plate-assay.

In order to isolate pure mutant cultures, we needed to know which of the *E. faecalis* strains were susceptible to EJ97. Using the microtiter-plate-assay, we obtained a distinct OD-well growth pattern for each of the ten strains. The measurements from these wells allowed us to obtain the EJ97 MIC_{50} values for each of the wildtype strains, as seen in Table 4.1. Although not evident in the MIC_{50} values, we did observe a clear difference in each strain susceptible to EJ97, because no bacteria reacted the same way in the presence of EJ97.

However, we were not able to obtain a MIC_{50} for LMG_3088, because none of the EJ97 concentrations presented in the assay allowed for a measurable reduction in growth. Thus, we needed to determine whether the lack of inhibition in LMG_3088 was a result of low EJ97 concentrations in the assay, or if it was because of immunity towards EJ97. To investigate the source of this resistance, we used a spot-on-lawn assay. This assay confirmed that even at the highest possible constrictions of EJ97, growth remained unaffected, suggesting that if Eep is present in the cell, it is unavailable for extracellular EJ97, or it has obtained immunity by some other means.

Table 4.1: **EJ97 Mic_{50} values for *E. faecalis* wildtype strain**: obtained by a MIC_{50} inhibition assay.

***E. faecalis* strain**	**EJ97 MIC_{50} (μg/ml)**
LMG_3336	1.56
LMG_3388	0.13
LMG_3593	3.125
LMG_3281	0.26
LMG_3566	1.56
LMG_3088	x*
LMG_3560	0.26
LMG_3569	0.26
LMG_3351	0.78
LMG_3592	0.78

*not obtained

4.1.2 Mutant-isolation-assay

Phenotypic mutants were isolated using the selection assay described in chapter 3.3.3 and applied to the nine wildtype strains that were susceptible to EJ97. Only six strains adequately reduced wildtype growth, as illustrated in Figure 4.1.A, while the other four strains still showed growth of wildtype bacteria, as illustrated in Figure 4.1.B. Thus, for the remaining four bacteria, we were not able to obtain pure-mutant cultures.

The growth of wildtype bacteria most likely was caused by an insufficient concentration of EJ97. In order to obtain single mutants, the experiment was repeated with higher bacteriocin concentration (Figure 3.2). The isolation assay was repeated until all strains had a concentration of bacteriocin high enough to separate the mutants from the wildtype. Furthermore, the increase in concentration allowed us to obtain mutants from all the EJ97 susceptible strains (Table 4.2). We picked four different mutants from each of the assay plates, giving us a total of 36, distinct isolates over nine different *E. faecalis* strains (Table 4.2).

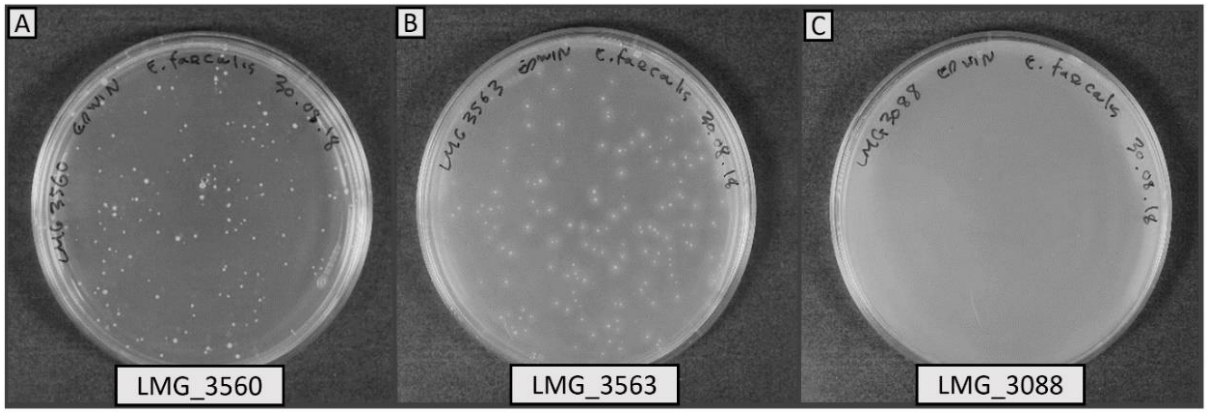

Figure 4. 1: **Mutant-isolation-assay plates**: Three isolation assay plates are showing three different strains of *E. faecalis* in the presence of a low amount of bacteriocin (70µl of stock solution). **A**: LMG_3560 showing clear separated single mutant colonies. **B**: LMG_3563 showing colonies, but only reduced of wildtype growth, resulting in insufficient separation. **C**: LMG_3088 showing full growth with no visible separation.

Table 4. 2: **Isolated *E. faecalis* mutants with EJ97 resistance phenotype:** All isolated *E. faecalis* mutants with the accompanying concentration of EJ97 needed for the selection.

Laboratory strain	**µl of 1mg/ml of EJ97**
LMG_3336-m1	350
LMG_3336-m2	350
LMG_3336-m3	350
LMG_3336-m4	350
LMG_3388-m1	70
LMG_3388-m2	70
LMG_3388-m3	70
LMG_3388-m4	70
LMG_3593-m1	350
LMG_3593-m2	350
LMG_3593-m3	350
LMG_3593-m4	350
LMG_3281-m1	500
LMG_3281-m2	500
LMG_3281-m3	500
LMG_3281-m4	500
LMG_3566-m1	70
LMG_3566-m2	70
LMG_3566-m3	70
LMG_3566-m4	70
LMG_3560-m1	70
LMG_3560-m2	70
LMG_3560-m3	70
LMG_3560-m4	70

LMG_3569-m1	70
LMG_3569-m2	70
LMG_3569-m3	70
LMG_3569-m4	70
LMG_3351-m1	70
LMG_3351-m2	70
LMG_3351-m3	70
LMG_3351-m4	70
LMG_3592-m1	70
LMG_3592-m2	70
LMG_3592-m3	70
LMG_3592-m4	70

4.1.3 Confirmation of resistant phenotype

To confirm the bacteriocin resistant phenotype, all mutants were tested for bacteriocin resistance with the MIC assay. All assays showed a high resistance towards EJ97, indicating that the isolated mutants were indeed expressing bacteriocin-resistance. As an additional confirmational step, selected-mutants were tested with the spot-on-lawn inhibition assay. This showed that the mutants (later chosen for the animal experiment) were indeed resistant, even to high concentrations of EJ97, as illustrated in Figure 4.2.

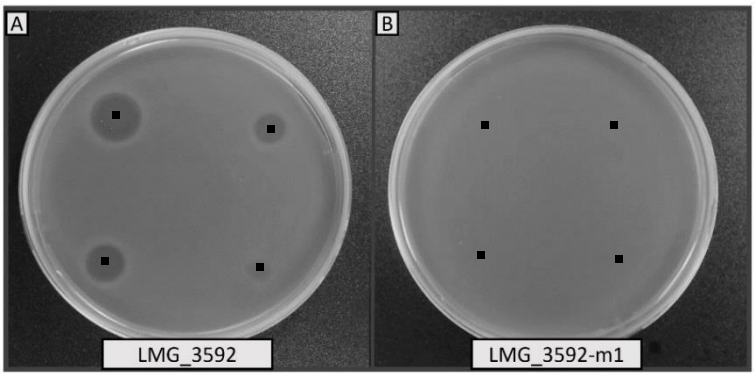

Figure 4. 2: **Spot-on-lawn-assay LMG_3592 and LMG_3592-m1:** These two images illustrate the difference in EJ97 susceptibility between LMG_3592 and LMG_3592-m1. The dark circles around the black square illustrate susceptibility towards EJ97 in concentrations 1mg/ml, 0.1mg/ml, 0.01mg/ml and 0.001 mg/ml.

4.2 Genotyping

4.2.1 PCR

The OneTaq-PCR amplification served as the base for the upcoming sequencing, as well as confirming the presence of the desired sequence. By visualising the PCR product with a gel run, we confirmed the presence of a similar size sequence within each strain of *E.faecalis*. The length seemed to be conserved across all wildtype strains (Figure 4.3), including those not susceptible to the bacteriocin.

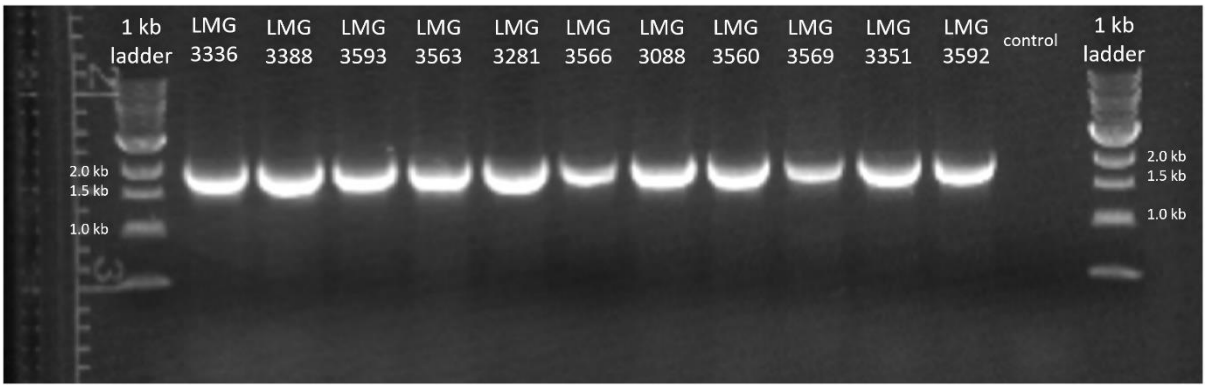

Figure 4.3: ***eep* gene visualisation for wildtype strains:** Figure shows gel image of the PCR amplification, using the *eep* specific PCR primers (Table 2.3) on all 11 LMG wildtype strains (Table 2.1). Expected amplicon size was 1800. OneTaq-PCR band for the amplification of the *eep,* with a 1Kb ladder on each side, indicating a total length of 1800.

The gel visualisation was also implemented for all of the 36 isolated mutant strains (Table 4.2). Gel visualisation also gave an early indication for whether the mutant gene had any significant insertions or deletions. However, no size variation was detected between the *E.faecalis* wildtypes and their corresponding mutants, as illustrated in Figure 4.4 and Figure 4.3. The amplicon length for all strains matched the theoretical length of the amplicon (1800bp), within the precision level of the visualisation. However, the presence of an amplicon of the expected length, only confirms the presence of a similar-sized sequence

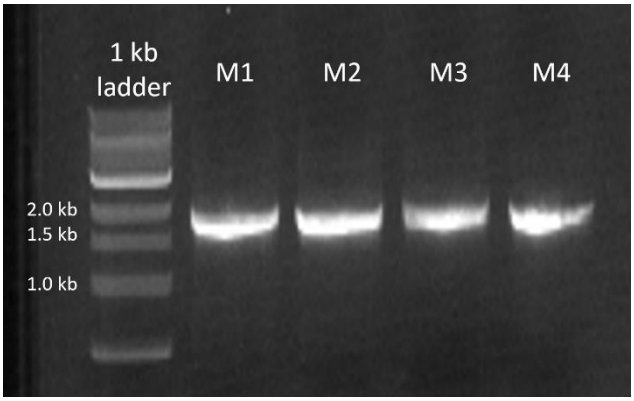

Figure 4.4: ***eep* gene Visualisation for LMG_3592 mutants 1-4:** The OneTaq-PCR band for the amplification of the *eep*, with a 1Kb ladder on each side, indicating a total length of 1800. Using the *eep* specific PCR primers (xx) on LMG_3592 mutants 1-4. Expected amplicon size was 1850. Indicating no significant insertion or deletion between the primer locations.

4.2.2 Sequencing

The wild type strains

The sequencing results allowed us to assemble a complete continuous sequence for the *eep* gene within all the wildtype strains, confirming the theoretical length of 1269 bp and 423 aa, across all wildtype strains. Using a multiple-sequence-alignment (MSA), all wild-type strains had a high degree of conservatism with little to no deviation among the strains, corresponding to as little as one nonsense mutation. This conservation gives a strong indication that a functional *eep* gene is present in all Wildtype strains, including those who were not susceptible to EJ97. Thus, the variation in resistance to EJ97 does not come from the *eep* gene sequence but most likely occurs because of changes in the gene expression or other cellular processes.

Identifying mutants

Unfortunately, during the sequencing of the mutants, only two-thirds of the amplicon were sequenced. However, we chose only to re-sequence those strains that had favourable gene-terminating mutations within the available sequence, arguing that mutations early in the gene are the most favourable because they have a higher chance of rendering the gene non-functional.

By performing an MSA, we were able to compare the first two-thirds of the mutant *eep* sequence to their corresponding wildtype, MSA allowed us to identify several of the mutations in the mutant *eep* gene. Most mutations seemed to be missense-mutation or mutations with an unclear effect, such as mutations in the Shine-Dalgarno sequence. However, nine strains (Table 4.3) showed clear mutations that would affect the translated protein. In all cases, these mutations resulted in a stop codon halfway in the protein, effectively truncating the protein by removing half of the translated aa. However, the cause of the stop codon was due to three different types of mutations.

Table 4.3: **Listing of mutant isolates with severe *eep* gene mutation**: Each strain is listed with their type of mutation, location within the gene, and their resulting change in sequence.

Strain	Type of mutation	Resulting in
LMG_3388-m1	c.708_715del	p.I236fsX21
LMG_3388-m2	c.708_715del	p.I226fsX21
LMG_3560-m1	c.708_715dup	p.S239fsX19
LMG_3560-m2	c.708_715dup	p.S239fsX19
LMG_3560-m3	c.708_715dup	p.S239fsX19
LMG_3560-m4	c.708_715del	p.I236fsX21
LMG_3569-m1	c.708_715del	p.I236fsX21
LMG_3551-m3	c.708_715dup	p.S239fsX19
LMG_3592-m1	c.625C>A	p.G209X

For LMG_3592-m1 the stop codon occurred as a result of a missense mutation from a Gly – Stop at placement 209 aa, truncating the protein. However, the remaining eight strains (Table 4.3.) were the result of a mutation in the variance of an eight bp (CAAAAAAT) repeat sequence. The CAAAAAAT mutations were observed as a repeat variance of the same eight bp repeat sequence, starting at 708 bp. Within this repeat, the mutations occurred either as a deletion or as an insertion.

Both genotypes were observed to occur in the same wildtype strain (Table 4.3). Both the insertion and deletion caused a frameshift, successfully frameshifting the translation of the next 19-21 aa before resulting in a stop codon. The stop codon caused a truncation of 214aa of 422aa

Although we only managed to obtain a total of nine confirmed protein-alteration mutations, these nine do not necessarily represent the full list of severe alteration mutations, because we were only working with two-thirds of the gene. Thus, other types of mutation might be prevalent. However, we can conclude that in compliance with the visualisation of the amplicons, no major insertion or deletion occurred within this unknown part of the *eep* sequence.

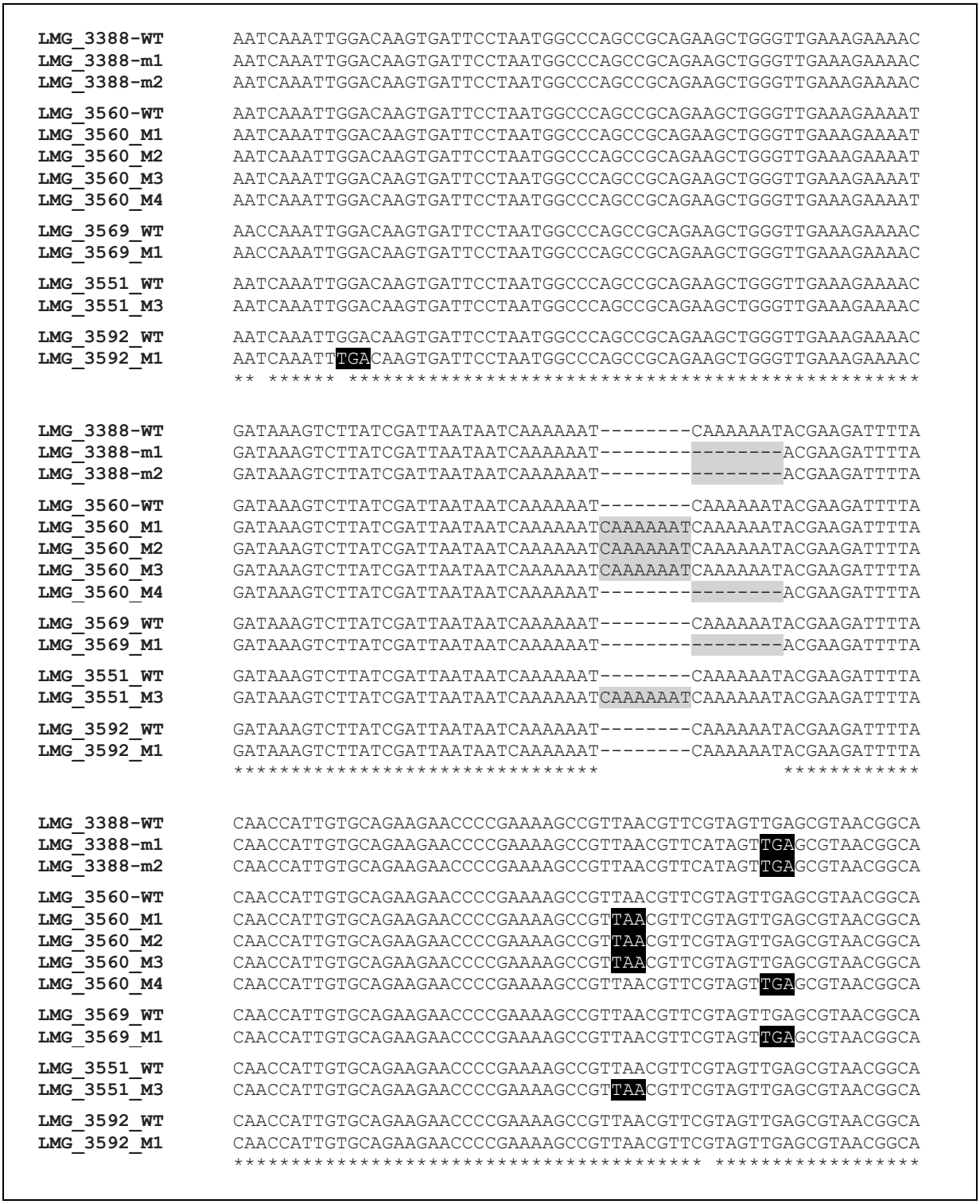

Figure 4.5: **Placements of confirmed severe alteration mutations in mutants strains:** All severe alteration mutants compared to their wildtype strains. In the figure, black marks the placement of the stop codon.

4.3 Animal experiment

Due to the novelty of this research, this experiment was only a pilot. The pilot was meant to indicate whether the *eep* gene plays a role in the difference in the number of *E. faecalis* in a wound model. Hence, we hypothesised that *eep*-mutant strains would lead to fewer bacteria in the wound than the wildtype strain with an intact Eep.

Due to unexpected obstacles experienced during the homogenisation of the substrate, a revision of the protocol was necessary after the first trial. This revision was necessary because we were experiencing that homogenisation of the substrate was insufficient. We experienced a significantly lower number of bacteria than previously observed with similar models (Ch'ng et al., 2018). At the time, we believed the remaining bacteria were still in the unhomogenized samples. In an attempt to increase the bacterial count, we added an additional homogenisation step to the protocol. We hoped this would further homogenise the tissue and release the remaining bacteria. In addition, we also added a filtration step, in order to remove the larger pieces of Tegaderm, because they clog up the pipets and smear the agar plates with its larger pieces. These additions to the protocol, as explained in the method section, were only applied to the last two experiments.

4.3.1 Visual infection of the wound

To assess the infection caused by different strains, we performed a visual inspection of the wound based on specific criteria and characteristics (visual-wound-characteristics) as described here:

1. Type I: no signs of redness, swelling, inflammation or necrosis, other than the observation of small amounts of pus under the Tegaderm, as illustrated in Figure 4.7.B.C.
2. Type II: with signs of redness and no swelling, inflammation or necrosis, other than the observation of large amounts of fluid under the Tegaderm, as illustrated in Figure 4.7.D.
3. Type III: no signs of any bacterial growth or immune response by the host.

When observing the spread of these infections, type II was only observed in two mice, across two experiments, where one was a mutant. However, in most cases, the mice showed type I characteristics (Table 4.4). In experiment 1, the control mouse 1 showed type II characteristics, while control mice 2, 3, and 4 showed the type III characteristics. The wildtype mouse 3 showed type II characteristics, while the remaining mice had type I characteristics. In experiment 2, the control mice 1, 2, and 3 showed type III characteristics, while control mouse 4 showed type I characteristics. Mutant mouse 4 showed type II characteristics, while the remaining mice showed

type I characteristics. However, in experiment 3 only one mouse showed deviation from type I, where control mouse number 4 exhibited type III characteristics.

Table 4.4**: Infection types across mice:** Illustrating the visual characteristics of each wound on each mouse, for all experiments with results for wildtype, mutant, and control.

		Mouse 1	Mouse 2	Mouse 3	Mouse 4
Experiment 1	Control	+	-	-	-
	Mutant	+	+	+	+
	Wildtype	+	+	++	+
Experiment 2	control	-	-	-	+
	Mutant	+	+	+	++
	Wildtype	+	+	+	+
Experiment 3	Control	+	+	+	-
	Mutant	+	+	+	+
	Wildtype	+	+	+	+

\+ Type I characteristics
++ Type II characteristics
\- Type III characteristics

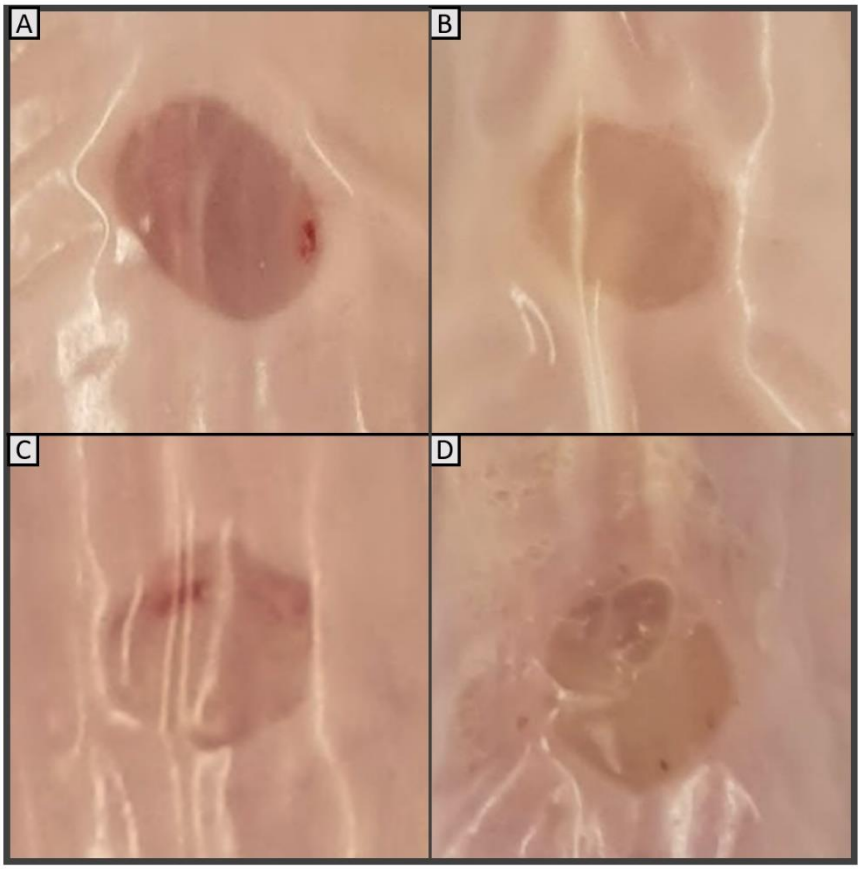

Figure 4.6: **Illustration of the different wound infections:** Pictures of wound infections, in BALB/cJRj c mice three days after inoculation of 10^6 CFU. **A**. Experiment 3; 3592 control mouse 4, type III infection. **B**. Experiment 3; 3592 wildtype mouse 1, type I infection. **C**. Experiment 3; 3592-m1 mouse 4, Type I infection. **D**. Experiment 2; 3569, mouse 4, type II infection.

When visually comparing the type I infections for the three wild type strains (LMG_3060, LMG_3069 and LMG_3592), no distinctive variation between the three E.*faecalis* strains was observed. This lack of variation indicated that the visual-pathogenicity-characteristics for early wound colonisation were the same across the three strains. These visual characteristics were also observed when comparing the three mutant strains (LMG_3060-m1, LMG_3069-m1 and LMG_3592-m1). When comparing the mutants to the wildtypes, no differences, could be noticed between them. The lack of deviation indicates that if there is a difference between the mutant and the wildtype, it is not visible in the wound-infection-characteristics. However, type I infection was observed in 5 of the 12 controls, across all control groups (Table 4.4). This indicates that some bacteria still found a way into the wound, although measures were taken to make sure the wound stayed sterile.

4.3.2 The number of bacteria in the wound

The mouse model was designed to visualise the difference in the amount of *Enterococcu*s CF*U* in each mouse wound after three days.

In experiment 1, the control behaved as expected. There was some growth of bacteria, but the amount is negligible. The wildtype also behaved as expected, but with a somewhat larger spread between the CFU/wound results. Our mutants seemed to fall within the same range as the wildtype results but with a smaller spread between wounds. These results indicate no variation between the wildtype and the mutant isolate in the number of CFU/wound.

In experiment 2, the control showed no significant bacteria in the wound. The wildtype strain in experiment 2 had a smaller spread but fell within the same range as experiment 1. However, the mutant strain had two significant outliers, one far under and one far above the median from experiment 1.

In experiment 3, however, the control showed significant amounts of *Enterococcus* bacteria in two of the mice. The wounds in these mice seemed to have a type I infection as described above. The highest count of 3500 CFU/wound in control mouse 1 is comparable to some of the mice inoculated with bacteria. The wildtype and control results all fell within the same range as the wildtype in experiment 2, but with a lower spread. The average count for mutants was slightly higher than that of the wildtype.

After comparing the data in Table 4.5. with Figure 4.8, there is no apparent difference in the amount of *Enterococcus* CFU, regardless of which strain the mice were inoculated with. There is no difference in CFU, between the mice inoculated with *E. faecalis* wildtypes and mice inoculated with that strainss corresponding mutant. Thus, showing no meaningful difference in the survival rate or the proliferation of the different strains. Although working with a small dataset, we still believe there is no difference in the survivability of our mutants. If the *eep* gene has an essential role in the proliferation of this niche, the removal of the gene would cause the difference in CFU to be much more significant than what we have observed in this study. However, we also found some *Enterococcus* in the controls, with varying numbers as indicated in Table 4.5.

Table 4. 5: ***Enterococcus* CFU in each mouse wound table:** This table shows the amount of *Enterococcus* CFU for each mouse, in each of the experiments for the wildtype, mutant, and control.

		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Average
Experiment 1	control	10	0	0	40	10
	Mutant	2700	4000	2600	9100	4600
	Wildtype	1900	8900	26600	1500	9700
Experiment 2	control	0	0	0	60	15
	Mutant	4300*	8100	500	128400	35300
	Wildtype	2300	1800	7200	9700	5200
Experiment 3	control	3500*	0	800	0	1100
	Mutant	4000	7700	3900	2800	4600
	Wildtype	3700*	2100*	3600*	2500*	5200

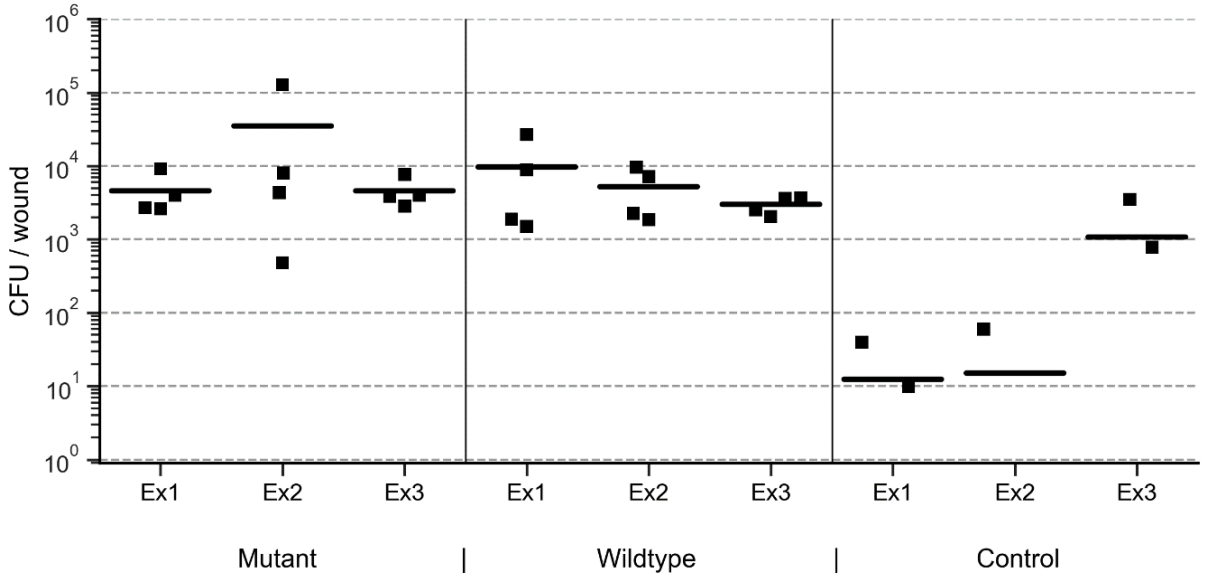

Figure 4.7: ***Enterococcus* CFU in each mouse wound plot:** Data plot categorised by mutant, wildtype, and control - enterococci's CFU per wound.

Discussion

5.1 Mutant Isolates

In this study, *eep* mutant isolates of *E. faecalis* were obtained through the selection-assay using the EJ97 bacteriocin. This method was adopted to enable the isolation of single colonies of pure phenotypic resistant bacteria that naturally occur when the bacteria grow freely in a stress-less environment. This selection method is intended to give a non-functional version of the Eep proteinase, presumably because of changes in the *eep* gene (Ovchinnikov et al., 2014).

All of the ten wildtype bacteria used in this study were first screened for their unique growth pattern in the microtiter-inhibition-assay. This was a necessary step in order to determine which bacteria were susceptible for the mutant-isolation-assay, as well as the calculation of the MIC_{50} value. Although not present in the MIC_{50} values, the individual strains all showed a unique growth pattern, which means that these strains had a variation in their susceptibility for the bacteriocin. Comparing this variance to the sequencing results showed that the gene was highly conserved across all wildtype strains, indicating that the sequence is not the factor responsible for the variation in susceptibility. Thus, indicating that variation in susceptibility between the *E. faecalis* strains is because of other variations in the cells.

However, one wild type strain, LMG_3088, was not susceptible in the microtiter assay and showed full immunity with the spot-on-lawn assay. When comparing LMG_3088 to the genes in the other wildtype bacteria, it is clear the sequence is indeed present in the genome and that the immunity does not come from changes in the gene sequence. Instead, the resistance might stem from differences in gene expression, other genetic mutations, or immunity proteins. We wanted to know whether this immunity might stem from an immunity protein. Bioinformatic analysis of available sequences showed that the LMG_3088 strain had previously been identified to harbour the

sequence necessary for transcribing EJ97. Since bacteriocin genes manifest in gene clusters, one can assume it also includes all the necessary proteins to produce, transfer, and resist self-targeting. Thus, it is reasonable to believe that LMG_3088 is resistant because of an immunity protein. Although this bacteria could not be used further in our study, this immunity should be researched further if EJ97 is to be developed into a clinical drug.

With the strains that have proved susceptible to the bacteriocin in the minimum-inhibition-assay, we managed to isolate 36 distinct mutant strains, across nine strains of *E. faecalis,* by using the mutant-isolation-assay. All 36 mutant strains were picked because they were expressing the phenotypic trait of growing in the presence of bacteriocin. The bacteriocin-resistant phenotype was then confirmed by running all the bacterial isolates in the microtiter-plate-assay, which means that a bacteriocin-resistant phenotype had been successfully selected for.

In order to obtain the genotype for these 36, we chose only to sequence the *eep* gene. If a mutation correlating to the resistance phenotype was to occur somewhere else within the *E. faecalis* genome, we would not be able to obtain this mutation type or their location. However, we argued that other mutations were irrelevant because we were only looking for *eep* mutations that result in a stress intolerant phenotype. Specifically, we were looking for a mutation that, to a certain degree, would alter the translated proteinase to such an extent that it presumably would no longer be functional and stop the theorized associated stress response.

However, because of the lack of knowledge of the Eep proteinase, we do not know the functions of the different parts nor the structure of the Eep proteinase. It is known that the HE(I/F/L)GH motif is located near the N terminal and the LDG motif is located close to the COOH terminal, and these motifs are of importance based on similarities in homolog genes. These two motifs likely work together and are necessary for the regulation and function of the active site in site II proteinase' (M. S. Brown et al., 2000). Therefore, a mutation that causes a large change in the protein, as well as inhibiting proteinase function, was preferred. Ideally, we wanted these mutants to have a deleted *eep*.

Because the selection-assay allows for the growth of phenotypic mutants that naturally occur, this method might not be the most optimal way of obtaining a particular genotype (Dominguez, Lim, & Qi, 2016; Joung & Sander, 2013; Urnov, Rebar, Holmes, Zhang, & Gregory, 2010), which preferably would be the removal of the entire gene. In addition, this isolation assay also allows for other mutations that might give immunity but still contain a functional Eep proteinase, which

previously has been observed in other species (unpublished data). However, before genotyping, we only knew the bacteriocin-resistant phenotype: this phenotype indicates that the part of the Eep protein that serves as the binding place for EJ97 may no longer be available for EJ97. This uncertainty in the phenotype does not allow for the concrete identification of whether the mutation had occurred in the *eep* gene.

Unfortunately, we were only able to obtain two-thirds of the *eep* sequence during sequencing. This sequencing problem was easily handled because, as stated previously, the mutations we wanted to obtain would most likely occur early in the *eep* gene. Therefore, we only re-sequenced those isolates that had favourable confirmed mutations. Even with a reduced amount of sequences, we were still able to identify nine serious mutations, across five *E. faecalis* strains. These alterations would hopefully inhibit the function of the proteinase. All of our mutations share a common trait, namely that they result in a stop codon somewhere between the second and third transmembrane area of the Eep proteinase, effectively removing half of the protein. Removing the last half of the protein also removes the LDG motif that has been deemed important for the function of the active site of the Eep proteinase (M. S. Brown et al., 2000).

Of our nine mutant strains showing a severe alteration in the Eep proteinase, we observed that our mutations seemed to manifest in one of two ways. The first was a missense that led to a direct stop codon. Second, we had the CAAAAAAT repeat variance. This repeat variance has been observed before in *Enterococcus* (Jensen, 2014) and proved to result in bacteriocin-resistant strains, giving further insight into the third transmembrane area as a potential important attachment site for the EJ97 (Holth, 2017).

The fact that both the repeat variances occur in LMG_3560, indicates that this strain has both the capabilities for deletion and insertion, which perhaps may be some sort of replication slippage (Chen, Chuzhanova, Stenson, Férec, & Cooper, 2005; Viguera, Canceill, & Ehrlich, 2001). Replication slippages are quite interesting, in some cases making it possible for bacteria to mutate back to their original sequence when the selective pressure from bacteriocins are diminished. The replication slippage can, in certain instances, change the repeat number without the loss of information, making it possible for the bacteria to re-gain the information and use the replication slippage as a switch, which can be toggled on and off. Thus, our mutants were checked to see if they had re-mutated after the wound inoculation, which they had not done.

Our lack of sequencing also illustrates the fact that the remaining 27 bacteria had no confirmed mutations in the first two-thirds of the gene. However, as in the case of LMG_3569, we can extrapolate from the missing data. The only mutation observed in the available sequence was the CAAAAAAT deletion mutation. If this is a slippage, then we can assume that the insertion might also be prevalent because strains that have one might also have the other, gaining further insight from our small sample size. Because three out of four mutant strains of LMG_3569 were in the unknown sequence, the immunity comes from mutations in the last third of the gene or as a result of alternative mutations in other genes that give bacteriocin-resistance. Unfortunately, we do not have the last sequence which could have provided insight about where the bacteriocin attaches, how mutants become immune, and if our strains can gain immunity by other alterations outside of the gene.

In order to test the importance of the *eep* gene in a murine-wound environment, it was essential to identify the mutants that caused severe alterations in the *eep* gene, preferably by complete deletion, resulting in certain non-functionality of the proteinase. However, with our small library of confirmed severe alteration mutants, we needed to decide which strains we were going to test in our animal model. We hoped to use LMG_3088 and LMG_3281 because these strains had already been established as virulent and previously used in mice wound models. These strains have an established infectious dose and growth curve (Chong et al., 2017), making them the perfect candidates for our trial. Unfortunately, although we managed to obtain phenotypic strains from LMG_3281, we were not able to confirm any severe alteration genotypes, and LMG_3088 was bacteriocin immune, forcing us to consider the other strains in our library.

With the limited amount of data about the virulence of the remaining bacteria, we searched for sources which had previously isolated our strains, in order to gain insight into how they might react in a mouse model (La Rosa et al., 2015). None of our severe alteration mutant strains seemed to be previously isolated from either wounds or mice. However, we did find an article listing isolation spots for all of our strains and an accompanying test for their survival in *Caenorhabditis elegans* (La Rosa et al., 2015). Their survival in *C. elegans* seems to be linked to their virulence as mammalian pathogens, because pathogenic *E. faecalis* outperforms probiotic strains of *E. faecalis* in the survival rate in *C. elegans* (Garsin et al., 2001; La Rosa et al., 2015). Therefore, we chose LMG_3592, which was isolated from blood, LMG_3560, which was isolated from faeces, and LMG_3569, which was isolated from urine, in order to get a wider isolation spectrum. Also, all of these strains have shown high virulence in *C. elegans*, suggesting that they, to some degree, have some virulence promoting properties compared to our registered probiotic strains (La Rosa et al.,

2015). Finally, we picked one of each of the mutations so that we had tested all the types of mutations in our library.

5.2 Animal model

For our skin wound model, we decided to use a revised version of the experiment described by Chong and co-workers (Chong et al., 2017), using the same wound creation and inoculation dose of bacteria, hoping the disease would progress in the same manner in our strains.

However, few of our visual infection characteristics seemed to match Chong and co-workers' (Chong et al., 2017) observations. Our most prevalent type I characteristics resulted in visual characteristics somewhere in between the low tier infection and the high tier infection that Chong experienced, where the high tier is what we were expecting based on the initial inoculum. While Chong tested several bacterial strains, which were all isolated from clinical isolates, our bacterial strains had previously been isolated from blood, faeces, and urine. Therefore, we argue that because we used the same inoculum dose, our observation of reduced infection may be due to lower overall virulence of the strains in skin wounds.

Although we managed to obtain a relatively stable amount of bacteria in each wound, regardless of mutant or wildtype, we were not able to obtain the amount found in Chong and co-workers' study, who observed significantly higher values close to 10^5 bacteria in contrast to our $5x10^3$ (Chong et al., 2017). However, in their study, they worked with specific virulent strains that likely have increased the colonisation, as illustrated by the higher amount of bacteria and higher visual infection obtained with the same inoculation dose (Chong et al., 2017). Therefore, the variations between our results and Chong's results probably stem from an overall lower virulence in our bacterial strains.

However, two mice in our study showed visual characteristics and bacterial numbers more in line with Chong's observations. Although the reason for this remains unknown and might just be due to natural variability in the mice, it seems reasonable to assume that these two outliers do not represent the normal host reaction when inoculated with 10^6 of our bacteria. However, we cannot discount that this increase could be because of a collaboration effect between two bacteria, as *Enterococcus* is known to do (Ch'ng et al., 2018). However, this is mere speculation because our control group and CFU method were not designed to give any confirmative contamination results. Because of the narrow range of BHA, we cannot exclude the growth of most bacteria.

The model developed for this study was not designed to allow for the control and registration of unwanted bacteria growing in the wound. Therefore, we will not be able to identify whether we have a multi-species infection, because most bacteria will fail to grow on the medium used for

counting, and multi-species-*Enterococcus* infections will just be registered as one. Also, our control group was designed only to be able to pick up the background noise of *Enterococcus* contaminants, meaning it will not allow for the identification of other species.

This lack of contamination control is in contrast to studies like Chong that use elaborate methods to obtain data on whether the wound was contaminated by other bacteria. Contamination control is important to avoid factors that can affect the results and measures are made to be able to remove contaminants from comparisons. Thus, we are aware that consistent detection and removal of contamination is typically done. However, this experiment was designed as a pilot, and therefore, contained a substantially lower number of mice. Removing any mice from the comparison would decrease any comparison capabilities, which is why we did not include any significant detection method.

In addition, because of our small number of mice, we could not just remove the few known contaminants that grew on the plates, because when cross-referencing with the visuals of the control group, it is clear that our control showed visual contaminants and proliferation but did not show any CFU on our agar plates. This means that if our mutant and wildtype are contaminated with the same ratio as the control, it would not be unreasonable that a portion of the other groups are also contaminated with bacteria that we are not able to pick up on the BEA plates. This left us with no choice but to neglect all types of contaminants when comparing the wildtype to the mutants.

The control in our experiment did not show much growth, which was to be expected. However, we did have contaminants in two mice that showed approximately the same amount of bacteria, indicating that these two controls had been infected with a strain of *Enterococcus*. Because of the sheer amount of bacteria in the wound, we can most likely say this is not one of our bacteria because it is highly unlikely that the wound was inoculated with a high enough amount of CFU to reach this level, as established by Chong(Chong et al., 2017). This infection is, therefore, likely to be caused by a highly virulent strain of *Enterococcus* for a mouse wound because it went from a theoretically low inoculum to a high CFU per wound in three days.

Although experiencing a lower inflammation level (by visual inspection) and a lower number of CFU per wound than previously established models, we still believe this would not affect the results of our model's ability to differentiate between our mutants and wildtype strains. We argue that this model still stresses the bacteria enough by the presence of the immune system, that if

there is a difference in these bacteria's stress responses towards immune stresses, our model would be able to pick it up. As a result, in our mice, there is no meaningful difference in the survival rate or the proliferation of the different strains. Although we are working with a small dataset, we still believe there is no difference in the survivability of our isolates compared to the wild type. If the gene had an essential role in the proliferation of this niche, the removal of the gene should cause the difference in CFU to be much more significant than what we have observed in this study.

If the experiment had been successful in differentiating between the wildtype and mutant, we would have hoped to see no surviving bacteria from the mutant isolates. In such a case, four mice would have been a sufficient sample size to reveal a statistically significant difference between the mutant and wild-type strains. Given the results presented in this study, it is clear that the Eep truncation did not have the intended effect. Running a t-test did not show that there are any statistical significance to support that there is any significant difference (p:-50%) between our isolates and the wildtype.

However, if Eep has a role other than the stress response, our model would not be able to pick up any deviations in these interactions. The different roles of Eep could include multispecies interactions, sex pheromones (Varahan et al., 2014), or biofilm formation (Frank et al., 2012) with or without other species. Our model was designed to test single species interactions that aimed to test the theoretical stress response associated with Eep.

The fact that the animal trial experienced no deviation towards the mutant and wildtype, in the *in vivo* trial, did not come that unexpectedly. Before even trying out our mutants *in vivo*, we decided to test our mutants *in vitro*. We tried to develop an *in vitro* phenotype to ensure predictable results of the animal experiment and to ensure the non-functionality of the stress response of our truncated mutants.

Therefore, we developed and modified several protocols in order to obtain an *in vitro* phenotype. Our methods were designed to predict our final results by emulating the stress the bacteria might encounter in a pathogenetic niche. However, all such attempts failed to differentiate the mutant from the wild type. This lack of a stress phenotype is not what we expected, because other related studies have managed to confirm a stress phenotype, showing susceptibility towards heat, ethanol, oxidative stress, and lysozyme (Ovchinnikov et al., 2017; Varahan et al., 2013).

The lack of a stress phenotype in our strains may arise from two possibilities. First, the lack of a stress phenotype means it is highly likely that it is not the only factor controlling the response for these stresses in our strains. Alternatively, in these niches, or even these bacterial strains, it is not necessarily a part of the stress response but might have diverged to have additional functions, which is why it has been found to have a role in the cleavage of sex pheromones (Varahan et al., 2014) (Chandler & Dunny, 2008) and in biofilm formation (Frank et al., 2012). The second possibility is the way we created our mutants, which may have resulted in the insufficient truncation of the proteinase or mutations outside of *eep*. However, whether the lack in deviation between the mutant and wild type is because of insufficient truncation of the Eep or if some other processes control the stresses for these *E. faecalis* strains is an area of speculation. However, we still conducted the animal trials knowing that we had no *in vitro* phenotype. Yet, we were still hoping that the complexity of the murine environment would be able to differentiate between the mutant and wildtype.

5.3 Further research

For further research, we would suggest a controlled and complete deletion of the *eep* gene in our strains. Moreover, testing if this has a result on the *in vitro* phenotypes would help us determine if our results were because of insufficient truncation, other mutations, or Eep's role in the stress response. If it turns out that our truncation of Eep is breaking the site II proteinase, one might look into if the bacteria have other mutations by whole-genome-sequencing. It is an interesting thought that one mutation in RsiV is enough to make the entire system remain in a state that is always-on and expressing the Sigma factor (Benachour et al., 2005; Varahan et al., 2013), because this might be critical for treatment options.

Biofilm formation is, as mentioned, a significant virulence factor in *E. faecalis*. Unfortunately, we did not have the chance to test for biofilm *in vitro*; we did try to obtain *in vivo* samples for Electron Microscopy, but the results proved inconclusive. However, because biofilm genes vary from strain to strain, we would recommend only focusing on one strain in a more established experiment.

However, we do not see any reason to expand this pilot into a full experiment before many of the limitations have been fully elaborated. For the future, if the testing of this stress response is to be conducted in another animal model, it is deemed necessary to know whether the removal of Eep actually stops the ECF in our strains or if the model needs to be re-worked into a model capable of detecting interspecies reactions.

Conclusion

In this thesis, we isolated our mutant strains from naturally occurring bacteriocin resistant bacteria by exposing them to EJ97. This exposure allowed us to obtain several EJ97 bacteriocin resistant *E.faecalis*, presumably because of mutations in the Eep proteinase. Because of problems with the sequencing, we did not manage to get the whole sequence, giving us a limited sample size with nine severe-alteration mutants. All of these severe alterations resulted in the truncation of roughly half of the Eep proteinase, giving it a high possibility that these mutations would affect the transcribed protein to a degree that should alter the regulation of Eep.

However, none of our severe alteration mutations seemed to result in an *in vitro* phenotype, which previously has been observed in other strains. Although we had no *in vitro* phenotype, we still hoped that the complexity of the murine skin-wound model would be able to differentiate between the isolates and wildtype. Unfortunately, this does not seem to be the case for our bacteriocin-truncated-mutants, because our mutants failed to give different numbers of CFU both in the *in vitro* and the *in vivo* trials. The lack of differentiation in CFU indicated that if Eep had been successfully inhibited, the stress response, which Eep is believed to be associated with, does not show any virulence promoting properties or survival enhancing effect in any of our models. Thus, we have not been able to show that eep gene plays a vital role in the virulence of our *E.faecalis* stains.

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