Norwegian University of Life Sciences

Master's Thesis 2019 30 ECTS
Faculty of Chemistry, Biotechnology and Food Sciences

The chitinolytic system of *Enterococcus faecalis V583* and its putative role in virulence

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Acknowledgements

The research presented in this thesis was completed at the Faculty of Chemistry, Biotechnology, and Food Science at the Norwegian University of Life Sciences, supervised by Prof. Gustav Vaaje-Kolstad, Dr. Geir Mathiesen and Dr. Fatemeh Askarian.

I want to thank everyone who has helped me and contributed to this thesis. First, I would like to thank my main supervisor Prof. Gustav Vaaje-Kolstad. Your motivational words have inspired me to work hard and aim for the best possible thesis, and your feedback has always been full of useful details. I am grateful for all the conversations and discussions we have had regarding this thesis.

Secondly, I want to express my sincere gratitude to my co-supervisors Dr. Geir Mathiesen and Dr. Fatemeh Askarian for all the help and guidance with the experimental work. You have both been so dedicated and have kept my spirit up, despite all the challenges I have met with the experimental work. Your feedback and guidance have been invaluable to me, and you have always kept life in the lab positive and fun.

I would also like to thank the rest of the PEP group, and a special thanks to Dr. Magnus Øverlie Arntzen for your kind help with blood drawing. A big thank-you to all the helpful volunteers as well, for sharing your blood for my research.

Last but not least, I am beyond thankful for the support and encouragement provided by my family and friends throughout all five years of studying for this degree. You mean the world to me. Thank you from the bottom of my heart.

Ås, May 2019

Mariann Kristensen

Abstract

From a harmless gut commensal, *Enterococcus faecalis* has undergone a transition towards a multi-resistant nosocomial pathogen, posing a major threat to human health, particularly in immunocompromised individuals. The aim of the present study was to shed light on putative virulence properties of the proteins associated with the chitinolytic machinery of *E. faecalis V583*. The proteins of interest are a GH18 chitinase (*ef0361; Ef*Chi18A), an AA10 lytic polysaccharide monooxygenase (*ef0362*; *Ef*AA10A), a GH18 endo-β-N-acetylglucosaminidase (*ef2863*; *Ef*Endo18A), and a combined GH18 endo-N-glycosidase and GH20 hexosaminidase (*ef0114*; *Ef*EndoE). Although these proteins are presumed to be involved in chitin degradation, their enzymatic activities have also been linked to virulence in several other bacterial species.

The first part of the project was dedicated to investigating the potential roles of the putative chitinolytic enzymes in chitin hydrolysis. Gene-knockout strains were utilized for analysis of the separate proteins involved. Characterization of the wild type and gene deletion strains was based on analysis of morphology, growth rates on soluble and insoluble substrates, as well as enzymatic activity. Despite removal of complete sets of genes, the strains showed similar morphology and demonstrated similar growth rates on all the substrates tested. However, when cultivated on β-chitin the chitinase and LpmO did not give the bacteria a growth advantage and chitinase activity was not observed in the culture supernatants. N-acetylhexosaminidase activity was however detected when the bacteria were cultivated on β-chitin, suggesting possible involvement of *Ef*EndoE and/or *Ef*Endo18A in chitin metabolism, although this was not highly reflected in the growth experiments. The general observation was that *E. faecalis V583* grew minimally on β-chitin, suggesting other roles of the proteins than chitin degradation.

In the second part of the project, the role of the putative chitinolytic enzymes in virulence was investigated using *ex vivo* human serum and human whole blood assays. Transcriptional analysis by ddPCR showed upregulation of the *LpmO*-gene in presence of serum, compared to low expression in bacteriologic medium. The virulence-properties of the LpmO was further examined through serum killing assays, which showed resistance of *E. faecalis* towards the complement-mediated killing of serum, regardless of the presence or absence of the LpmO. In human whole blood however, killing of the bacteria was indeed observed. Thus, deletion of LpmO did not result in promotion of bacterial viability.

In conclusion, the chitinolytic machinery of *E. faecalis V583* was in this study found to show minimal activity towards chitin, in contrast to previously published studies. N-

acetylhexosaminidase activity was demonstrated, but no chitinase activity was observed. Interestingly, LpmO-expression was highly induced in presence of serum, but deletion of LpmO did not result in attenuation of bacterial survival in whole human blood or serum. The induced expression of LpmO observed in presence of serum and whole blood could be related to a stress-induced response. Further work and optimizations of the assays presented in this study are likely to contribute to a greater understanding of the role of these proteins in *E. faecalis* pathogenesis.

Sammendrag

Enterococcus faecalis har gått gjennom en forvandling fra en ufarlig tarm-kommensal til å bli et multiresistent, sykehusrelatert patogen, som utgjør en stor trussel mot menneskets helse, spesielt blant immunkompromitterte individer. Denne studien hadde som mål å avdekke putative virulens-egenskaper hos proteinene som er involvert i det kitinolytiske maskineriet hos *E. faecalis V583*. Proteinene av interesse er en GH18 kitinase (*ef0361*; *Ef*Chi18A), en AA10 lytisk polysakkarid monooksygenase (*ef0362*; *Ef*AA10A), en GH18 endo-β-N-acetylglukosaminidase (*ef2863*; *Ef*Endo18A), og en kombinert GH18 endo-N-glykosidase og GH20 heksosaminidase (*ef0114*; *Ef*EndoE). Selv om disse proteinene antas å være involvert i kitinnedbrytning, så har slike enzymaktiviteter blitt koblet til virulens for flere andre bakteriearter.

Prosjektets første del var dedikert til undersøkelse av potensielle roller for de antatte kitinolytiske enzymene innen kitinhydrolyse. Gen-delesjon-stammer ble brukt for analyse av de spesifikke proteinene involvert. Villtypen og delesjonsstammene ble karakterisert basert på morfologi, veksthastighet på løselige og uløselige substrater, samt enzymatisk aktivitet. Til tross for at komplette gener ble fjernet, viste stammene lik morfologi og relativt like vekstrater på alle de testede substratene. Kitinase- og LpmO-proteinene gav imidlertid ikke bakteriene noen vekstfordel i β-kitin og kitinaseaktivitet ble ikke observert i kultur-supernatantene. N-acetylheksosaminidase-aktivitet ble imidlertid detektert da bakteriene ble dyrket på β-kitin, hvilket kan indikere at *Ef*EndoE og/eller *Ef*Endo18A er involvert i kitinmetabolisme, til tross for at vekstkurvene ikke reflekterte dette funnet. Generelt sett vokser *E. faecalis* minimalt på β-kitin, hvilket kan tyde på at disse proteinene har andre roller enn kitinnedbrytning.

I prosjektets andre del ble proteinenes antatte rolle innenfor virulens analysert gjennom *ex vivo* humanserum- og humanfullblods-assays. Transkripsjonsanalyse ved hjelp av ddPCR viste oppregulering av *LpmO*-genet i nærvær av serum, sammenlignet med lavt uttrykk i bakteriologisk medium. Virulensegenskapene til LpmO-proteinet ble videre undersøkt gjennom serum-assays, der *E. faecalis* viste resistens mot komplement-mediert lysis i serum, uavhengig av proteinets tilstedeværelse. I human-fullblod derimot, ble det observert kraftig reduksjon i bakterieantall. Delesjon av LpmO resulterte derfor ikke i økt bakteriell overlevelse.

Arbeidet i denne studien avdekket minimal aktivitet av det kitinolytiske maskineriet til *E. faecalis V583* i kitin, til tross for at tidligere studier har vist funksjonell aktivitet. N-acetylheksosaminidaseaktivitet ble demonstrert, men det ble ikke observert kitinaseaktivitet.

LpmO-ekspresjon ble sterkt indusert i serum, men delesjon av proteinet resulterte ikke i redusert bakteriell overlevelse i fullblod eller serum. Den induserte ekspresjonen av LpmO i nærvær av serum og fullblod kan ses i sammenheng med en stress-indusert respons. Videre arbeid og optimaliseringer av assayene presentert i denne studien vil trolig bidra til bedre forståelse av disse proteinenes rolle i *E. faecalis* patogenese.

Abbreviations

AA	Auxiliary Activity
AP	Alternative Pathway
bp	Base Pair
BSA	Bovine Serum Albumin
CAZy	Carbohydrate-Active Enzyme
CBM	Carbohydrate-Binding Module
cDNA	Complementary DNA
CP	Classical Pathway
CFU	Colony Forming Unit
CytD	Cytochalasin D
ddPCR	Droplet Digital PCR
dH_2O	Milli-Q® Sterile Water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsDNA	Double Stranded DNA
FBS	Fetal Bovine Serum
gDNA	Genomic DNA
GH	Glycoside Hydrolase
GlcNAc	N-acetyl-D-glucosamine
HI-serum	Heat Inactivated Serum
HSA	Human Serum Albumin
IgG	Immunoglobin G
IgM	Immunoglobin M
kb	Kilo-Base Pair
LP	Lectin Pathway
LpmO	Lytic Polysaccharide Monooxygenase
MAC	Membrane-Attack Complex
MASP	Mannose-Binding Lectin (MBL)-Associated Serine Protease
MBL	Mannose-Binding Lectin
MHC	Major-Histocompatibility-Complex

MIC	Minimum Inhibitory Concentration
MQ	Milli-Q® Sterile Water
mRNA	Messenger RNA
Nhs	Normal Human Serum
nm	Nanometers
OD_{600nm}/OD_{595nm}	Optical density measured at 600/595 nm
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern-recognition receptor
REK	Regional Committees for Medical and Health Research Ethics
RNA	Ribonucleic Acid
rpm	Revolutions per minute
TAE	Tris-Acetate-EDTA
UV	Ultraviolet
v/v	Volume/volume
WT	Wild type
w/v	Weight/volume

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1 Introduction

In recent years the Gram-positive bacteria *Enterococcus* has undergone a pronounced transition from a human gut commensal to a multidrug-resistant nosocomial opportunistic pathogen (Arias & Murray, 2012; Gilmore et al., 2013). Enterococcal infections are mainly caused by two species, *Enterococcus faecium* and *Enterococcus faecalis* (Agudelo Higuita & Huycke, 2014). In general, *E. faecalis* is part of the normal flora of the oral cavity and can also be found in the gastrointestinal tract, male urethra, and female vaginal tract of humans (Gentry-Weeks et al., 1999). However, the bacterium can also cause life-threatening infections such as bacteraemia and endocarditis (Arias & Murray, 2012), urinary tract infection, endophthalmitis, peritonitis and wound infections (Garsin et al., 2014; Kau et al., 2005) – typically in association with hospitalization. Infections with *E. faecalis* can be especially challenging to treat because of their frequent resistance to multiple antibiotics. The increased use of antibiotics has resulted in development of antibiotic-resistant strains which facilitates the pathogenicity of the bacteria. This gives the resistant bacteria an advantage in environments with antibiotics, for instance in hospitals, which may allow them to outcompete other species that would normally keep enterococci under control (Garsin et al., 2014). The need for alternative therapeutics is increasing, and in this respect increasing the knowledge of the virulence properties of these bacteria is important. With regards to this, both identification of proteins that are differentially expressed in response to environmental factors, and targeted studies of potential important virulence determinants can be useful. Such studies may provide insight in mechanisms behind how *E. faecalis* survive and persist during infections.

1.1 Enterococci

The genus Enterococcus consists of Gram-positive cocci that occur as single cells, in pairs or in short chains. They are facultatively anaerobic, but can tolerate oxygen in lower dosage. Their metabolism is homofermentative, which means they produce lactic acid by substrate level phosphorylation of pyruvate as a product of glucose fermentation. Most enterococci lack Kreb's cycle and a respiratory chain, but *E. faecalis* is an exception since exogenous hemin can be used to produce d, b, and o type cytochromes (Pritchard & Wimpenny, 1978; Ritchey & Seeley, 1974). *E. faecalis* therefore has a functioning cytochrome-like respiration, generating a proton motive force (PMF) that utilizes oxidative phosphorylation (Ritchey & Seeley, 1976). Enterococci are chemo-organotrophs, which means they obtain energy and electrons from

oxidizing organic compounds as electron donors in their environment. Most Enterococci can tolerate a wide variety of growth conditions, as they can grow between temperatures of 10°C to 45°C and in the presence of up to 6.5% NaCl (Hardie & Whiley, 1997). In addition, they can also grow under hypotonic, hypertonic, acidic and alkaline environments, making them very robust (Huycke et al., 1998).

There are over 40 recognized species of Enterococcus that can be found mainly on mucosal surfaces of human and animals, but some are also found in soil, water, on plants, in dairy products and in other foods (Hardie & Whiley, 1997; Jett et al., 1994). Enterococci normally inhabit the bowel and are found in the intestine of nearly all animals (Huycke et al., 1998). The concentration of enterococci in stool samples collected from humans, are normally 10^8 CFU per gram (Rice et al., 1995). When found outdoors, on vegetation or in water, this is normally a result of contamination from animal excrement or untreated sewage (Jett et al., 1994).

1.2 *Enterococcus faecalis* and infection of the human host

Enterococci have emerged as a major cause of hospital-acquired infections and are especially dangerous to immunocompromised patients such as elderly, organ and bone marrow transplant patients and cancer patients. *E. faecalis* normally coexist with their host in a commensal relationship as part of the normal flora, but the opportunistic nature of this bacterium can cause disruption of the commensal mode and lead to severe infections. Historically, *E. faecalis* accounted for 80 to 90% of the clinical enterococcal isolates, whereas only 5 to 10% were *E. faecium* (Treitman et al., 2005). Presently, *E. faecium* has risen to be the cause of more than 30% of the enterococcal infections (Top et al., 2007). The infectious "mode" of *E. faecalis* is, amongst other properties, characterized by expression of a variety of virulence factors.

Virulence factors are molecules produced by a pathogen that aid colonization, invasion and immunosuppression of the hosts (Vu & Carvalho, 2011). Virulence factors often contribute to disease, and *E. faecalis* produce factors that promote adherence to host tissues, invasion and abscess formation, modulation of host inflammatory responses, and potentially secretion of toxins (Jett et al., 1994).

Many of the genes encoding the enterococcal virulence factors or antibiotic resistance are located on conjugative plasmids or encoded within transposons. This makes such genes easily transferable, not only amongst Enterococci, but also with other bacteria, such as *Staphylococcus aureus* (Palmer et al., 2010; Ray et al., 2003; Sung & Lindsay, 2007). The form of gene transfer

used by Enterococci to spread DNA containing e.g. virulence factors, is called conjugation. This gene transfer mechanism transfers genetic material "horizontally" between bacterial cells by direct contact or by creating a pore or a bridge-like connection between the cells.

Although many virulence determinants are described in literature, their exact mechanisms of *E. faecalis'* virulence are still not well understood. In this study, we propose that the proteins associated with the chitinolytic system of *Enterococcus faecalis V583* express putative virulence properties. *E. faecalis* V583 is a clinical isolate that originates from a patient suffering from persistent blood stream infection. It was the first Vancomycin-resistant clinical isolate reported in the United States (Vebo et al., 2009) and the first *E. faecalis* strain to be sequenced (Paulsen et al., 2003). Twenty-five percent of its genome consists of mobile elements and/or exogenously acquired DNA, including integrated phage regions, insertion elements, transposons, a pathogenicity island and integrated plasmid genes (Bohle, 2011).

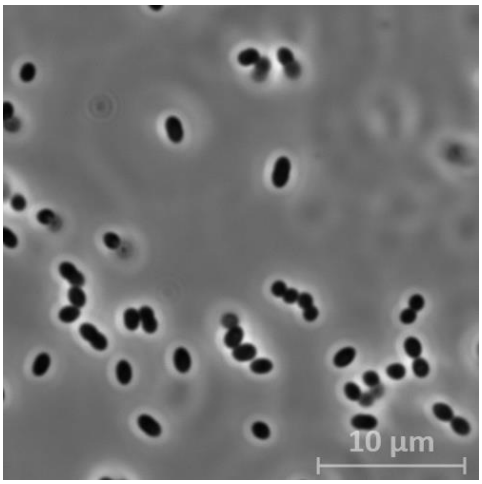

Figure 1.2.1.1 Morphology of *Enterococcus faecalis V583*, the clinical strain included in this study. The figure was obtained through microscopy (100x) of the bacteria.

1.3 The chitinolytic system of Enterococcus faecalis V583

1.3.1 Chitin and chitinases

Chitin is an insoluble linear β-1, 4-linked polymer of N-acetyl-D-glucosamine (GlcNAc), that is widely distributed in nature and is one of the most abundant biomasses present on Earth. Chitin participates in both the carbon-cycle and the nitrogen-cycle. Chitin is utilized as a structural component and builds up the cell walls of fungi and algae, the exoskeletons of insects and the shells of crabs and shrimps (Bhattacharya et al., 2007). The main role of chitin is to protect the organism or cell from harsh environmental conditions, such as chemical and mechanical stress (Gooday, 1990). Depending on the arrangement of the individual GlcNAc-

chains, chitin can be characterized as three forms. The most common form, α-chitin, is composed of antiparallel chains and this arrangement allows for many strong hydrogen-bonds, resulting in a densely packed, rigid and stable structure (Minke & Blackwell, 1978). The β-chitin form consists of parallel GlcNAc-chains, resulting in a looser packing compared to α-chitin due to a bigger proportion of hydrogen-bonds with water (Merzendorfer & Zimoch, 2003). The third form is ϒ-chitin, which is the least abundant form. Here the GlcNAc-chains are ordered in an alternating manner, with two parallel chains followed by one single anti-parallel chain (Gooday, 1990). Although chitin is produced in large amounts, has a rigid structure and is insoluble, chitin does not accumulate in most ecosystems (Horn et al., 2006). This suggests that nature has effective methods for chitin degradation.

Several enzymes can digest chitin, such as chitinases, N-acetyl-hexosaminidases and lytic polysaccharide monooxygenases. Chitinases are hydrolytic enzymes called glycoside hydrolases (GH) that catalyse the hydrolytic degradation of chitin. Chitinases belong to two protein families; glycoside hydrolase (GH) families 18 and 19 as defined by the Carbohydrate Active Enzyme database (CAZy; (Lombard et al., 2014)). In general, chitinases can be divided into two categories based on their mechanisms of chitin hydrolysis; exochitinases and endochitinases. Exochitinases cleave glycosidic bonds from the non-reducing ends of the polysaccharide chain, whereas endochitinases hydrolyse glycosidic linkages at random positions of the chitin chain (Gooday, 1990; Horn et al., 2006).

Chitinases are present amongst a variety of different organisms, such as viruses, fungi, insects (Merzendorfer & Zimoch, 2003), plants, yeasts, mammals and a wide range of bacteria (Hamid et al., 2013), including *E. faecalis* (Leisner et al., 2009). Chitinases have several different physiological functions amongst organisms, as different organisms produce the enzyme for different purposes. In plants, chitinases have a defensive role against infection by chitin-coated organisms. Other organisms with cell walls built from chitin require chitinases to degrade and modify their cell walls as they grow. Chitin is also a carbon-source for many organisms, and chitinases are needed in order to process and digest chitin to derive energy.

The genome of *Enterococcus faecalis V583* encodes several proteins involved in a chitinolytic system, which the bacteria may utilize to degrade and metabolize chitin. These proteins include a family GH18 chitinase (*ef0361*; *Ef*Chi18A), a family GH18 endo-β-N-acetylglucosaminidase (*ef2863*; *Ef*Endo18A), a family AA10 (formerly CBM33) lytic chitin monooxygenase (*ef0362*; *Ef*AA10A), and an endo-N-glycosidase with two enzymatic domains combining two glycoside hydrolase activities; family GH18 and GH20 (*ef0114*; *Ef*EndoE). These proteins are all secreted

as part of a chitinolytic machinery (Bohle et al., 2011; Vaaje-Kolstad et al., 2012). Studies reported in the literature have shown that the chitinase is most efficient in degrading β-chitin and has functional properties that corresponds to an endochitinase (Vaaje-Kolstad et al., 2012). Combination of the chitinase (*Ef*Chi18A) and the lytic chitin monooxygenase (*Ef*AA10A) enhances the degradation of crystalline chitin, especially seen with β-chitin as substrate (Vaaje-Kolstad et al., 2012).

1.3.2 Family 18 of Glycoside hydrolases

Glycoside hydrolases are enzymes that catalyse the hydrolysis of O-, N- and S-linked glycosides, resulting in cleavage of glycosidic bonds. Catalysis of glycosidic bonds occurs by a nucleophilic substitution at the anomeric carbon, and results in depolymerization of polysaccharides (Bohle, 2011).

The GH18 family includes glycoside hydrolases that are catalytically active chitinases, inactive chitinase-like proteins and endo-β-N-acetylglucosaminidases. These enzymes perform catalysis through a double-displacement reaction with neighbouring group participation (Honda et al., 2000; Terwisscha van Scheltinga et al., 1995; Tews et al., 1997). The catalytic nucleophile utilized in the reaction is not enzyme-derived. Instead, and less commonly, the N-acetamido carbonyl oxygen of the substrate acts as a nucleophile. A carboxylate group of aspartic acid assists the nucleophile and deprotonates the N-acetamido nitrogen. A second catalytic residue, glutamic acid, acts as a catalytic acid to protonate the glycosidic oxygen, which together with the nucleophilic attack by the N-acetamido group results in cleavage of the glycosidic bond, as the leaving group departs. This generates an oxazolinium ion intermediate. Subsequently, the former catalytic acid will now act as a base and deprotonate the nucleophilic water molecule, which in turn will hydrolyse the oxazolinium intermediate of the reaction (Davies, 2013; van Aalten et al., 2001). The reaction is shown in **Figure 1.3.2.1**.

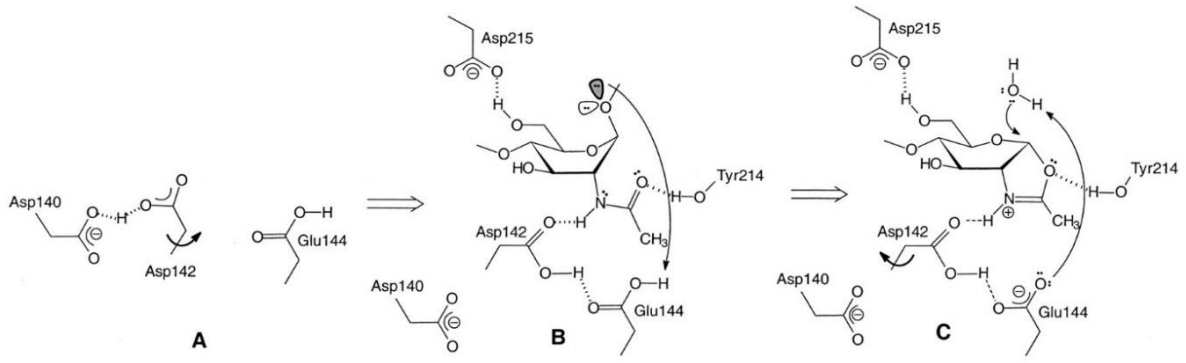

Figure 1.3.2.1 The mechanism of neighbouring group participation. Figure obtained from (van Aalten et al., 2001)

Enzymes of the GH18 family are characterized by a conserved amino acid sequence motif D-X-X-X-D-X-D-X-E (Hamid et al., 2013). The catalytic aspartic acid (D) and glutamic acid (E) is found in the D-X-E part of the motif and are placed on top of the three-dimensional protein structure that is called a TIM-barrel. This structure is made up of eight parallel β-strands and eight α-helices, in an alternating manner, resulting in a $(\beta\alpha)_8$ catalytic domain (Davies, 2013).

The present study includes three family GH18 enzymes expressed by *E. faecalis V583*. The chitinase *Ef*Chi18A belongs to the GH18 family and has functional characteristics of an endochitinase (Vaaje-Kolstad et al., 2012). The second GH18 protein expressed by *E. faecalis V583* is the endo-beta-N-acetylglucosaminidase *Ef*Endo18A. In addition to these, the bacterium produces a third GH18 enzyme called *Ef*EndoE, which is part of an endo-N-glycosidase with two enzymatic domains. This enzyme is unique in that it combines two glycoside hydrolase activities; one family GH18 and one GH20 domain.

Family GH20 consists of enzymes called hexosaminidases or chitobiosidases, that catalyse the removal of N-acetyl-D-glucosamine (GlcNAc) or N-acetyl-D-galactosamine (GalNAc) from various glycans, glycolipids and glycoproteins (Liu et al., 2018). GH18 and GH20 enzymes use similar catalytic mechanisms for substrate hydrolysis, as both families involve a mechanism of neighbouring group participation and formation of the oxazolinium ion intermediate (Greig, 2013).

1.3.3 Family AA10 of Lytic polysaccharide mononoxygenases (formerly CBM33)

Lytic polysaccharide monooxygenases (LpmOs) are copper-dependent enzymes that catalyse oxidative degradation of polysaccharides. The enzyme cleaves glycosidic linkages oxidatively in recalcitrant polysaccharides, including cellulose, chitin and starch (Frandsen & Lo Leggio, 2016). LpmOs act synergistically with the classical hydrolytic enzymes, such as chitinases, resulting in effective depolymerization of insoluble substrates.

The synergic properties of LpmOs in chitin degradation was first demonstrated for the family AA10 protein CBP21 in the chitin-degrading bacterium *Serratia marcescens*, and the three-dimensional structure of CBP21 was also the first structure to be solved of a family AA10 LpmO (Vaaje-Kolstad et al., 2005). Five years later, Vaaje-Kolstad et al. demonstrated that LpmOs in fact were redox enzymes that cleaved polysaccharide chains by an oxidative mechanism (Vaaje-Kolstad et al., 2010). Despite variations in the amino acid sequence, LpmOs share the same fold and architecture in the active site (Johansen, 2016). LpmOs have a common immunoglobulin-like β-sandwich core, made up of two β-sheets in an antiparallel arrangement

where each β-sheet typically consists of eight to ten β-strands (Beeson et al., 2015; Hemsworth et al., 2013). The active site contains one single copper-atom that is coordinated by two copper-binding histidine-residues in a histidine-brace conformation. This conformation involves three N-ligands; the N-terminal histidine coordinates the copper with its amino group and its sidechain, and the other histidine residue stabilizes the copper-atom with its sidechain (Beeson et al., 2015; Hemsworth et al., 2013). The catalytic site is located on the surface of the protein, unlike other enzymes which most often bind the substrate either in a cleft or a tunnel (Hemsworth et al., 2015).

The activity of LpmOs depend on several factors, such as the cofactor copper, as well as molecular oxygen and electron supply. The oxidative reaction is catalysed by the presence of electrons, which reduce the copper-atom in the active site. The electrons originate from external electron donors, such as ascorbate (Frandsen & Lo Leggio, 2016). The reduced copper will in turn activate the co-substrate of the reaction which can be either O_2 or H_2O_2 (the latter seems to be the preferred co-substrate; (Bissaro et al., 2017)), that through an unknown mechanism causes hydroxylation of either the C1 or C4 carbon of the glycosidic bond and concomitant cleavage of glycosidic bond and breakage of polysaccharide chains. Oxidation of the C1-carbon will generate an aldonic acid in the reducing end, whereas oxidation of the C4 carbon will produce a ketoaldose in the none-reducing end of the molecule (Hemsworth et al., 2015). Formation of new ends at the cleaved polysaccharide enable binding of other enzymes, such as glycoside hydrolases, for continuous depolymerization of the polysaccharide. Thus, the LpmOs work synergistically as they can attack and break linkages that other enzymes cannot access.

The database of Carbohydrate-Active Enzymes (CAZy) has organized the LpmOs in four auxiliary activity (AA) families called AA9, AA10, AA11 and AA13. The AA9 family, formerly classified as glycoside hydrolase family 61 (GH61), is mostly composed of cellulose-degrading enzymes in fungi. The AA11 family contains chitin-active LpmOs, while the LpmOs in family AA13 mainly degrade starch. The AA10 family, formerly named Carbohydrate-binding module family 33 (CBM33), consists of both chitin- and cellulose active LpmOs (Frandsen & Lo Leggio, 2016; Hemsworth et al., 2015). These proteins are most commonly found in bacteria and viruses and are special in that they normally occur as single domain proteins (Bohle, 2011). The LpmO of interest in this study, *Ef*AA10A, belongs to this family and has a typical β-sandwich-like three-dimensional structure (**Figure 1.3.3.1)**.

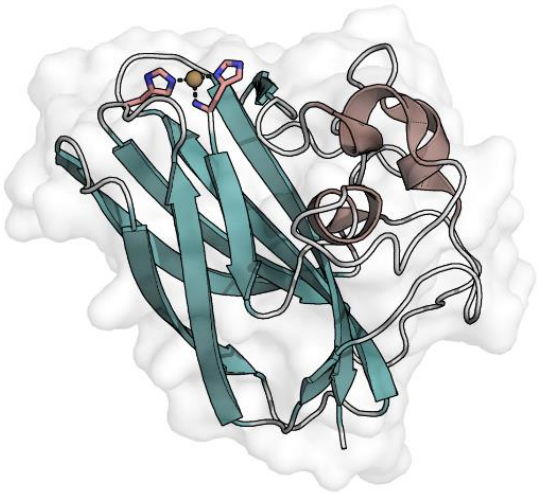

Figure 1.3.3.1 Crystal structure of EfAA10A. The *E. faecalis V583* LpmO, also called EF0362, belongs to protein family AA10. The enzyme has Uniprot-ID Q838S1 and PDB-ID 4ALC. The β-sheets are coloured in teal, α-helices in salmon-pink, whilst the connecting loops are white. The active site is shown with a histidine-bound copper atom. The figure was made using PyMol.

1.3.4 Chitinolytic systems and their role in virulence

Interestingly, chitinases and lytic chitin monooxygenases have been linked to virulence in several studies and seem to play an important role for bacterial survival in the host cells (Frederiksen et al., 2013; Tran et al., 2011). Such properties suggest the potential of these enzymes to modify the host immune responses.

Although the LpmOs have a clear role in degradation of polysaccharides, several studies reported in the literature have suggested a more complex role for these enzymes than just binding to chitin for nutrients (Frederiksen et al., 2013). An additional role within virulence and binding to host cells has been proposed for the AA10 chitin binding proteins, as they may bind other GlcNAc-residues such as those present on the surfaces of intestinal mucins and epithelial cells. Colonization and bacterial attachment to the host cells is an essential step for establishment of infection. AA10 LpmOs expressed in *Lactobacillus plantarum* (Sánchez et al., 2011) and *Vibrio cholerae* (Bhowmick et al., 2008; Kirn et al., 2005) have been reported to enhance the bacterial colonization of the intestine through binding to mucin. Also, it has been shown that the AA10 chitin binding protein of *Listeria monocytogenes* contributes to bloodstream infection in mice (Chaudhuri et al., 2010).

For *E. faecalis V583*, several of the proteins involved in the chitinolytic machinery of the bacterium have been linked to virulence. Studies have reported that two of the enzymes involved, the chitinase *Ef*Chi18A (*ef0361)* and the chitin-binding module (CBM) *Ef*AA10A (*ef0362)* of *E. faecalis V583*, both are upregulated in the presence of urine and blood, which

may indicate a role in virulence (Vebo et al., 2009; Vebo et al., 2010). Here it has been suggested that the upregulation in urine is part of an initial step of adherence to uroepithelial cells.

*Ef*EndoE (EF0114) with GH18 endo-N-glycosidase- and a GH20 hexosaminidase-activity, is also suggested to be an interesting factor involved in virulence. The endoglycosidase EndoS expressed in *Streptococcus pyogenes* can cleave the N-linked glycans from immunoglobin G (IgG), resulting in inhibition of the immunoglobin-mediated opsonophagocytosis and increased survival of *S. pyogenes* in blood (Collin et al., 2002). *E. faecalis* possesses a very similar endoglycosidase named EndoE which also shows activity on human antibodies. EndoE from *E. faecalis HER1044* corresponds to EF0114 of *E. faecalis V583* with a sequence identity of 99% and is reported to release glycans from the immunoglobin IgG in the same manner as EndoS (Collin & Fischetti, 2004).

The family GH18 endo-beta-N-acetylglucosaminidase *Ef*Endo18A (*ef2863*) is known to hydrolyse the N-linked glycans of glycoproteins, which potentially enables *E. faecalis V583* to deglycosylate host glycoproteins. Many of the proteins involved in adaptive and innate immunity are glycosylated, as the glycosylations are important for stability and recognition (Rudd et al., 2001). Such proteins include key molecules involved in antigen recognition, and proteins covering the epithelial cell surface and mucus layer (Bohle, 2011). Ability of *E. faecalis V583* to deglycosylate glycoproteins could change the function of the proteins, which may help the bacteria evade the immune response (Frederiksen et al., 2013).

1.4 The host immune responses

In order to investigate the role of virulence factors in infection, it is important to have a good understanding of host mechanisms that may be targeted by the virulence factors.

1.4.1 Blood and serum

The blood is composed of plasma and blood cells. Plasma is mainly water, but it also contains many important substances such as proteins (albumin, globulins, fibrinogens, clotting factors, antibodies, enzymes, and hormones), glucose, and lipids like fatty acids and cholesterol (Dean, 2005). There are three types of blood cells; erythrocytes that transport oxygen to the tissues, leukocytes comprising the immune cells, and thrombocytes which are the coagulating components of blood (clotting factor).

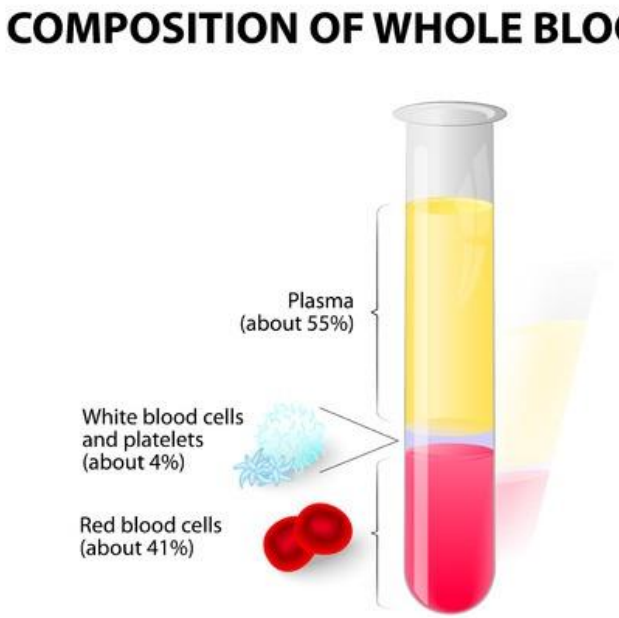

Figure 1.4.1.1 The components of blood. Blood will, if left to stand, separate into three layers as the denser components, the erythrocytes, sink to the bottom of the tube and the plasma remains at the top. Figure obtained from (D'Onofrio, 2016)

The leukocytes, also known as the white blood cells, can be divided into two types. Granulocytes are characterized by the presence of granules in their cytoplasm and a multilobed nuclei, which is usually lobed into three segments. Due to this feature, they are often called polymorphonuclear leukocytes (PML). There are three main types of granulocytes, which are the neutrophils, basophils and eosinophils. The second type of leukocytes are the mononuclear cells, which can be further grouped into lymphocytes and monocytes. Lymphocytes are round cells containing a single large, round nucleus. They consist of natural killer cells, T-cells and B-cells. Natural killer cells are effectors of the innate immunity. T-cells, called helper T-cells, secrete chemicals that recruit other immune cells and help coordinate their attack (Dean, 2005). B-cells are a component of the adaptive immune system and secrete antibodies. The other subgroup, the monocytes, are immune cells that can differentiate into macrophages or dendritic cells. Monocytes are produced by the bone marrow and circulate in the blood and spleen for about one to three days, before they typically move into tissues throughout the body where they develop into macrophages and dendritic cells (Chiu & Bharat, 2016). In addition to the leukocytes, blood also consist of complement proteins (see **section 1.4.3** for more details).

Whole blood is the term for the blood in its natural form, with all its components present and evenly distributed. Blood plasma is the yellow fluid that separates from the erythrocytes, leukocytes and thrombocytes upon centrifugation. Plasma thereby contains proteins, including albumins, globulins and fibrinogen, immunoglobins, complement factors, electrolytes, as well

as hormones, enzymes and vitamins in smaller amounts. Removal of the coagulation-component, fibrinogen, results in a suspension called blood serum (Mathew J, 2019).

1.4.2 Innate immunity

The immune system is commonly divided into the innate immune system, which we are born with, and adaptive immunity, which we acquire. The adaptive components, naïve B- and T-cells, are specialized cells that induce clonal expansion of lymphocytes in response to infection. Here, clones are produced and differentiated into effector cells, which in turn can eradicate the pathogen (Medzhitov & Janeway, 2000). Acquired immunity also creates immunological memory after an initial response to a specific pathogen and leads to an enhanced response to subsequent encounters with that pathogen. Although the adaptive immune system is very effective, production of the specific antibodies takes time. The innate immune system therefore plays an important role in the early recognition of a pathogen, as it is activated immediately upon infection. The strategy of the innate immune system is to focus on a few, highly conserved structures present in large groups of microorganisms rather than to recognize every possible antigen. These structures are collectively called pathogen-associated molecular patterns (PAMPs) and the receptors of the innate immune system that recognize such structures are called pattern-recognition receptors (PRRs) (Medzhitov & Janeway, 2000).

PRRs are present on many effector cells of the innate immune system, such as macrophages and dendritic cells. Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are examples of PRRs and play a key role in innate immunity. Recognition of a PAMP by the PRR will trigger an immunologic response. PRRs can be divided in three functional groups; secreted, endocytic, and signalling pattern-recognition receptors (Medzhitov & Janeway, 2000).

Secreted pattern-recognition molecules function as opsonins by binding to microorganisms and flagging them for recognition by the complement system and phagocytes (Medzhitov & Janeway, 2000). An example of a secreted PRR is the mannan-binding lectin, which is part of the lectin pathway of the complement system and will be described in the next section.

Endocytic pattern-recognition receptors occur on the surface of phagocytes. When the pathogen is identified by the receptor, it is taken up and delivered to lysosomes where the pathogen is eradicated. Endocytic PRRs thereby mediate phagocytosis by macrophages. Pathogen-derived proteins processed by the phagocyte will thereafter be transported to the surface where the peptide is presented on major-histocompatibility-complex (MHC) molecules on the surface of the macrophage. T-cells will notice the bound peptides and kill the infected cell.

The third class of PRRs are the signalling receptors involved in cell activation. Upon binding to PAMPs, signal-transduction pathways are activated that will induce expression of several immune-response genes, including inflammatory cytokines.

1.4.3 The complement system

The complement system is part of the innate immunity but can also be recruited by antibodies generated by the adaptive immune system – thus the complement system works in both innate and adaptive immunity (Khanal, 2017).

The complement system is composed of a series of proteins (>30) that circulates in blood and tissue fluids (Ricklin et al., 2010). When encountering a pathogen, the system initiates its response immediately. Activation of the proteins will lead to a cascade of reactions on the surface of pathogens. The activation of one protein enzymatically cleaves and thereby activates the next protein in the cascade, resulting in a cascade of further cleavages. As a result, phagocytes are stimulated to clear foreign pathogens, immunogenic particles or damaged cells, inflammation occurs to attract additional phagocytes, and the cell-lysing membrane attack complex (MAC) is activated (Laarman et al., 2010).

The complement activation occurs through three biochemical pathways; the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) (Khanal, 2017), which are schematically presented in **Figure 1.4.3.1**. The early steps of the complement system vary in these different pathways, but all pathways result in formation of the enzyme-complex C3 convertase, which cleaves C3 into C3a, a anaphylatoxin promoting inflammation, and the opsonin C3b. C3b will in turn bind to the serine protease C5 convertase, which cleaves C5 into C5a and C5b. C5a, an anaphylatoxin, will attract neutrophils to the site of infection, whereas C5b will then induce the lytic pathway as it associates with C6 and C7 (Laarman et al., 2010). This complex becomes inserted in the cell membrane where it interacts with C8 and C9 to form a lytic pore; the membrane-attack complex (Muller-Eberhard, 1985). As a result, the intracellular contents of the microbe leak out and the cell cannot maintain its osmotic stability, leading to lysis due to influx of water and loss of electrolytes.

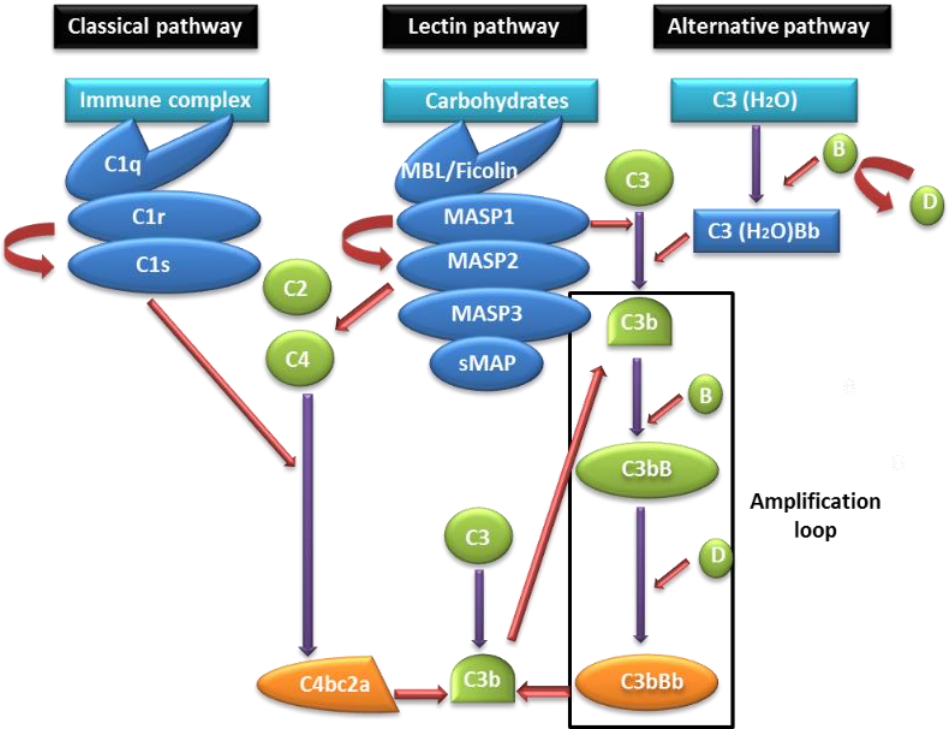

Figure 1.4.3.1 The three activating pathways of the complement system, including the classical (CP), alternative (AP) and lectin pathway (LP). Adapted from (Askarian, 2014), doctoral thesis.

The initiation of the different pathways is somewhat different. The CP is triggered by an antibody-antigen interaction on the surface of the microbe and is therefore often referred to as "antibody-dependent" (Ricklin et al., 2010). This immune complex, with bacterium-bound immunoglobin M or G (IgM/IgG), will in turn activate the C1-complex upon binding (Laarman et al., 2010). C1 is a large, multimeric protein complex composed of one subunit of C1q, a recognition protein, and two subunits each of C1r and C1s, which are serine proteases. The complex binds to the Fc region of the antigen-bound immunoglobin via the C1q molecule, leading to a consecutive activation of C1r and C1s. Activated C1s cleaves C4 and C2 molecules, resulting in generation of the serine protease C3 convertase (C4b2a) (Ricklin et al., 2010).

Initiation of the LP is dependent on the circulating mannose-binding lectins (MBL) and ficolins, which bind to mannose residues on the glycoproteins or carbohydrates on the microbial surface. Upon binding, MBL and ficolin assemble with MBL-associated serine proteases (MASPs), which share structural similarity with C1r/C1s (Ricklin et al., 2010). These include MASP1, MASP2, MASP3 and the small MBL-associated protein (sMAP). This complex activates C4 and C2 by cleavage, which in turn form the C3 convertase (C4b2a) (Khanal, 2017). In addition, MASP1 can cleave the C3 molecule directly which will further amplify the complement response (Endo et al., 2011).

The CP and LP are amplified by the AP. A small fraction of the C3 molecules are hydrolysed to $C3_{H2O}$, exposing new binding sites (Ricklin et al., 2010). This allows for binding of factor B (fB) to surface-bound C3b and subsequent cleavage by factor D (fD) (Laarman et al., 2010). AP thereby forms an additional C3 convertase (C3bBb) that can activate more C3, hence the pathway is often called the "amplification loop".

In other words, the complement system is a highly regulated surveillance system of the human body, that enables the recognition, tagging and eradication of microbial intruders and stimulates downstream immune responses.

1.4.4 Phagocytes

Macrophages and dendritic cells, both monocytes, together with the neutrophils are phagocytic cells. These cells are responsible of eliminating microorganisms and of presenting them to cells of the adaptive immune system. Neutrophils comprise up to 70% of the leukocyte numbers in the blood and are the main phagocytes (Actor, 2012).

Neutrophils are short-lived and are pre-programmed to undergo spontaneous apoptosis after release into circulation (reviewed in (Eisenreich et al., 2017)), while macrophages are long-lived migratory cells potentially involved in dissemination of the pathogen and formation of sites of infection through circulation to deeper tissues (Petti & Fowler, 2003). Indeed, several pathogens including *E. faecalis* can persist inside macrophages (Gentry-Weeks et al., 1999), despite trafficking of the bacterium into mature phagolysosomes (Tranchemontagne et al., 2016). This intracellular lifestyle provides protection against the host immune responses, hinders the efficacy of antimicrobial therapy and enhances *E. faecalis* persistence in hosts.

The movement of phagocytes towards the source of invasion is directed by extracellular gradients of diffusible chemicals; a mechanism called chemotaxis (Jin et al., 2008). The gradient is mainly caused by a set of short peptides called chemoattractant cytokines, chemokines, in which bind to G-protein-coupled receptors (GPCRs) on the surface of phagocytes. Binding activates a signalling network that leads to chemotaxis (Jin et al., 2008). Chemoattractants are produced either through activated host cells, e.g. chemokines or cytokines, or through complement-derived activation products, such as anaphylatoxins C3a and C5a (Askarian, 2014).

The process of phagocytosis is initiated via receptor signalling or the binding of host opsonins on the pathogen. Opsonin-coated microorganisms attach to specific receptors on the surface of the phagocyte. The best characterized and maybe most important opsonic phagocytic receptors

are the Fc receptors (FcR) and the complement receptors (CR) (Rosales & Uribe-Querol, 2017). Upon binding to these receptors, the cell membrane of the phagocyte extends around the microbe, eventually enveloping it through endocytosis which results in formation of a phagosome. Following internalization, the phagosome matures through fusion with lysosomes to form a phagolysosome, in which the pathogen is degraded. The phagolysosome contains different antimicrobial and degradative agents and enzymes, such as lysosomal hydrolases, reactive oxygen radicals, proteolytic and hydrolytic enzymes (glycosidases, lipases, DNAses and proteases), and nitrogen intermediates. Acidification of the vacuole also contributes to the destruction of the pathogen (Yates et al., 2005).

Following phagocytosis, certain proteins of the ingested microbe is brought back to the phagocyte's surface to be presented as phagosome-antigens for initiation of adaptive immunity. This is mainly seen in dendritic cells, where the antigenic peptides are presented to T-cells (Pauwels et al., 2017).

1.5 Aim of this study

Chitinolytic systems have been proposed and reported to play important roles within virulence. The most natural role of such systems would be food scavenging and degradation of polysaccharides to make nutrients more accessible for the microbe. However, some studies have indeed shown additional roles of these enzymes like adherence to host cells and persistence of the pathogen during infection.

The aim of the present study was to elucidate the role of the chitinolytic system in *Enterococcus faecalis V583* virulence using an *ex vivo* whole human blood model. Gene regulation studies of *E. faecalis V583* have indicated that the genes encoding the chitinase, *ef0361*, and the lytic chitin monooxygenase, *ef0362*, are co-upregulated under stress and during host-microbe interactions, which shows their putative function other than nutrient acquisition. Both enzymes are upregulated in response to bile stress (Solheim et al., 2007) and in the presence of urine (Vebo et al., 2010). Even more interestingly, both genes have been shown to be upregulated when grown in horse blood (Vebo et al., 2009). These findings will be further investigated in this study, but analyses will be conducted in the more relevant experimental setting (human blood).

The genes of interest in this study are *ef0361*, encoding the chitinase *Ef*Chi18A, the lytic chitin monooxygenase-encoding *ef0362* (*Ef*AA10A), *ef2863* which encodes the GH18 endo-beta-N-

acetylglucosaminidase *Ef*Endo18A, and lastly *ef0114,* in which combines GH18 endo-N-glycosidase and GH20 hexosaminidase (*Ef*EndoE). To evaluate the separate involvement and effect of these genes, gene-knockout strains were utilized. Four gene-knockout strains were included, consisting of one double gene-knockout strain of *ef2863* and *ef0114*, two single gene-knockout strains of *ef0361* and *ef0362*, as well as an additional double gene-knockout strain lacking both *ef0361* and *ef0362*. An overview of the strains included in the study are presented in **table 2.4.1.**

The first part of this study involved the verification and characterization of the gene-knockout strains. The enterococcal strains were grown on different substrates, both soluble and insoluble, to characterize their growth. Further characterization was conducted by assessing morphological differences between the strains using light microscopy. Lastly, the enzymatic activity with regards to the chitinase and hexosaminidase of the bacterium, were analysed for all strains.

The second part of this study evaluated survival of the different strains in human blood and serum *ex vivo*. In addition, gene expression of the lytic chitin monooxygenase *ef0362* was analysed upon stimulation in serum.

2 Materials

2.1 Laboratory equipment and materials

Table 2.1.1 shows an overview of the laboratory equipment and materials used throughout this study.

Table 2.1.1 Laboratory equipment and materials. The table lists the equipment and materials used throughout this study, along with the relevant supplier.

Category	Equipment	Supplier
Appliances	Bunsen Burner, Fireboy	Integra Biosciences
	Freezer, -20°C	Liebherr
	Freezer, -80°C	Sanyo
	Incubator, New Brunswick™ Scientific Innova® 44, 37°C	Eppendorf
	Incubator, 37°C	Termaks
	Incubator, MIDI 40 CO2 Incubator, 37°C	Thermo Fisher Scientific
	Incubator, Minitron, 37°C	Infors HT
	Laminar Flow Workbench, Safe 2020	VWR
	Liquid Aspiration System, Vacusafe Comfort	Integra Biosciences
	Microwave oven, MD142	Whirlpool
	Milli-Q® Direct Water Purification System, Direct 16	Merck Millipore
	Refrigerator, 4°C	Siemens
	Vacuum Hand Operator, Vacuboy	Integra Biosciences
	Waterbath, 37°C and 56°C	Julabo
Instruments	Automated Cell Counter, Countess™ II	Thermo Fisher Scientific
	Cell Density Meter, Ultrospec 10	Biochrom
	CertoClav	OneMed
	FastPrep®-24 Tissue and Cell Homogenizer	MP Biomedicals
	Hoefer DQ 300 Fluorometer	Harvard Bioscience Hoefer
	Microscope, ICC50 W	Leica
	Multiskan™ FC Microplate Photometer	Thermo Fisher Scientific
	NanoDrop™ UV-Vis spectrophotometer	Thermo Fisher Scientific
	Programmable Rotator Multi RS-60	BioSan

	Qubit™ fluorometer	Invitrogen
	Spectrophotometer, BioPhotometer D30	Eppendorf
	Thermal cycler, SensoQuest Labcycler	SensoQuest GmbH
	Thermal cycler, SimpliAmp	Thermo Fisher Scientific
	ThermoMixer™ C	Eppendorf
Gel Equipment	8 Well Comb	Bio-Rad
	15 Well Comb	Bio-Rad
	Benchtop UV Transilluminator	UVP
	Gel Imaging System, Gel Doc™ EZ	Bio-Rad
	Gel Casting Tray	Bio-Rad
	Mini-Sub® Cell GT Horizontal Electrophoresis System	Bio-Rad
	Power Supply, PowerPac™ Basic	Bio-Rad
	Scalpel, Stainless Steel, Surgical Blade No. 10	Swann-Morton
Centrifuges	Centrifuge, Allegra X-30R	Beckman Coulter™
	Centrifuge, Heraeus™ Multifuge X1R	Thermo Fisher Scientific
	Microcentrifuge, Centrifuge 5418 R	Eppendorf
	Microcentrifuge, Heraeus™ Pico™ 21	Thermo Fisher Scientific
	Microcentrifuge, Mini Star	VWR
Tubes, Vials and Plates	Cellstar® Tubes, 50 ml	Greiner Bio-One
	Cellstar® Tubes, 15 ml	Greiner Bio-One
	CryoPure Tube, 1.8 ml	Sarstedt
	Eppendorf Tubes, 1.5 ml	Axygen
	Falcon™ Tissue Culture Treated Flasks, Canted Neck, 250 ml	Corning
	FastPrep® Tubes and Blue Caps	MP Biomedicals
	Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates	Thermo Fisher Scientific
	PCR® tubes, 0.2 ml	Axygen
	Petri dishes 9 cm	Heger
	Protein LoBind Tube 2.0 ml	Eppendorf

	Sarstedt Tubes, 13 ml	Sarstedt
	Qubit™ assay tubes	Invitrogen
Assorted Equipment	Automated Pipettes	Thermo Fisher Scientific
	Countess™ Cell Counting Chamber Slides	Invitrogen
	Cover Slips 24x32 mm and 24x60 mm	Thermo Scientific
	Disposable Cuvettes, PS 1.5 ml Semimicro	Brand®
	Eclipse™ Needle	BD
	Glass beads	Sigma-Aldrich
	Glassware	Duran Group
	Hirudin Blood tube, 3 ml	Roche
	Inoculation Loops, 1 µl white	Sarstedt
	Lysing Matrix B	MP Biomedicals
	Magnet, Teflon Stirring Bar	SP Scienceware
	Magnetic Stirrer, RCT Basic	IKA®
	Microscope Slides	Thermo Scientific
	Nitrile gloves	VWR
	Parafilm® M	Bemis Company, Inc.
	Pipetboy Acu 2	Integra
	Pipette Refill Tips	VWR
	Scale, Entris Sartorius	VWR
	Scale, Serie GS	Kern
	Sealing tape, Nunc™	Thermo Scientific
	Serological Pipette, 5 ml, 10 ml, 25 ml	Sarstedt
	S-Monovette®-Needle 21Gx1	Sarstedt
	S-Monovette® 1.6 ml Hirudin	Sarstedt
	SureBeads™ Magnetic Rack	Bio-Rad
	Syringe 20 ml	BD Plastipak™
	Syringe Filtration Unit, Filtropur S 0.2 µm	Sarstedt
	Syringe Filtration Unit, Filtropur S 0.45 µm	Sarstedt
	Toothpicks	Playbox
	Vortex, MS 3 Basic	IKA®

2.2 Chemicals

Chemicals used in this study are shown in **table 2.2.1**.

Table 2.2.1 Chemicals. The table summarizes all the different chemicals used throughout this study, along with the relevant supplier.

Chemical	Supplier
2-Mercaptoethanol	Sigma-Aldrich
4-methylumbelliferone	Sigma-Aldrich
4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside hydrate	Sigma-Aldrich
4-methylumbelliferyl N-acetyl-β-D-glucosaminide	Sigma-Aldrich
50x TAE Electrophoresis Buffer, diluted to 1x TAE	Thermo Scientific
Agar Powder	VWR
Bacto™ Tryptone	Becton, Dickinson and Company
Bacto™ Yeast Extract	Becton, Dickinson and Company
β-chitin (extracted from squid pen, batch 20140101), processed to smaller particles with a diameter of 0.85 mm through mechanical crushing and milling	France Chitin
Brain-Heart Infusion (BHI)	Oxoid
Citric acid ($C_6H_8O_7$)	Sigma-Aldrich
D-(+)-Glucose solution, 100ml	Sigma-Aldrich
D-(+)-glucose anhydrous	VWR
Diacetyl-chitobiose (A2)	Megazyme
Dimethyl sulfoxide DMSO	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich
EDTA, 0.5 M	Invitrogen
Ethanol (C2H6O) absolute	VWR
Fetal Bovine Serum, 500 ml	Gibco Invitrogen
Fish peptone	Maritex AS
Gentamicin solution	Sigma-Aldrich
Glycerol (C3H8O3), 85%	Merck
Glycerol Phosphate disodium salt hydrate	Sigma-Aldrich

HEPES Buffer Solution 1 M, 100ml	Gibco Invitrogen
L-Ascorbic acid	Sigma-Aldrich
Magnesium sulfate	Sigma-Aldrich
Manganese sulfate tetrahydrate	Merck
N-acetyl-D-glucosamine (A1)	Sigma-Aldrich
Penicillin-Streptomycin 10,000 U/mL, 100ml	Gibco Invitrogen
peqGREEN DNA/RNA Dye	Peqlab
Phorbol 12-myristate 13-acetate (1 mg)	Sigma-Aldrich
Protein Assay Dye Reagent Concentrate	Bio-Rad
Qubit™ dsDNA BR Buffer	Invitrogen
RNaseZAP™	Sigma-Aldrich
RNAprotect™ Bacteria Reagent	Qiagen
Ringer tablets	Merck
RPMI Medium 1640 (1X) (with phenol red), 500 ml	Gibco Invitrogen
RPMI Medium 1640 (1X) (no phenol red), 500 ml	Gibco Invitrogen
Saponin	Sigma-Aldrich
SeaKem® LE Agarose	Lonza
Sodium Carbonate	Sigma-Aldrich
Sodium Chloride	VWR
Sodium Phosphate Dibasic (HNa_2O_4P)	Sigma-Aldrich
Sodium Pyruvate 100 mM, 50 ml	Gibco Invitrogen
Tris-HCl, 1 M, pH 8.0	Sigma-Aldrich
Trypan Blue Stain 0.4%	Invitrogen

2.3 Proteins and Enzymes

Table 2.3.1 lists the proteins and enzymes used throughout this study.

Table 2.3.1 Proteins and enzymes. The table contains all the proteins and enzymes used in this study, along with the respective supplier.

Protein/enzyme	Supplier
ChiB from *Serratia marcescens*	Kind gift from Dr. Mekasha, NMBU, Ås, NO
Red Taq DNA Polymerase Master Mix (2x)	VWR
iScript™ Reverse Transcription Supermix for RT-qPCR	Bio-Rad
Purified BSA 100x	New England Biolabs
Human Serum Albumin	Kind gift from Assoc. Professor Van Sorge, UMC, Utrecht, NL.

2.4 Bacterial strains

Table 2.4.1 contains all the bacterial strains involved in this study.

Table 2.4.1 Bacterial strains. The table shows all the bacterial strains of *Enterococcus faecalis* that were involved in this study.

Bacterial strain	Hereafter referred to as	Description
V583	WT	Wild type
V583ΔChi18A	ΔChi	Knockout of gene *ef0361* encoding the chitinase (*Ef*Chi18A)
V583ΔAA10A	ΔLpmO	Knockout of gene *ef0362* encoding the LpmO (*Ef*AA10A)
V583ΔChi18A-ΔAA10A	ΔChi-ΔLpmO	Double-knockout of genes *ef0361* and *ef0362* encoding the chitinase and LpmO
V583ΔEndoE-ΔEndo18A	ΔDeglycosidase	Double-knockout of genes *ef0114* and *ef2863*, encoding the combined endo-N-glycosidase and hexosaminidase (*Ef*EndoE), and the endo-beta-N-acetylglucosaminidase (*Ef*Endo18A)

2.5 Primers

Table 2.5.1 shows an overview of the primers used in this study.

Table 2.5.1 Primers. The table lists all primers utilized in this study by name, along with the area of usage and the primer sequences.

Primer	Usage	Sequence
0362_SekF	First colony PCR of WT, ΔChi, ΔLpmO and ΔChi-ΔLpmO	GCG ACT TCC TTT TTT GTA TGG AT
0361_SekR	First colony PCR of ΔChi, ΔLpmO and ΔChi-ΔLpmO	GAC AGG TGA GTA AAA TTT AAA AAG
Sek_0114F	Sequencing of ΔDeglycosidase	GCA AAT TCG TTA TTT TTT GTT ATT CTA TTG AT
Sek_0114R	Sequencing of ΔDeglycosidase	CGT GCC AGT CTT TAT AGA TAA CTC G
Sek_EF2863F	Sequencing of ΔDeglycosidase	AAT GAT AAC ATA TCT TTG TTA GCG CTT AC
Sek_EF2863R	Sequencing of ΔDeglycosidase	CCT TAA CAA CAC AAA ACA CTT TTT CA
0361_2018F	Second colony PCR of WT and ΔChi-ΔLpmO	GCG CAT ACA AAA TAA TTT TTT AGG AGG TTT TTT TCG TGA AAC GCG GCT ATC TAC AGA T
0361_2018R	Second colony PCR of WT and ΔChi-ΔLpmO	ACC GCG GTG GCG GCC GCT AAT TTT TTG ATT AAT TAA CTT ACT AAA AGT AAG T
0362_2F	Second and third colony PCR of WT and ΔChi-ΔLpmO	GTC CTG GCT TTT AGT TTT TAC CAA GCG ATT GAT GT
PCR_UP0362_F	Third colony PCR of WT and ΔChi-ΔLpmO	CAT GGA TAC AAC AAT GCT GAT AT
PCR_Down0361_R	Third colony PCR of WT and ΔChi-ΔLpmO	GCA GTG ACG TGG TGT AAA C

EF0361F	mRNA Expression Analysis by Reverse Transcription (RT) PCR	TCA TTA ATC CAT GGC ACG CG
EF0361R	mRNA Expression Analysis by Reverse Transcription (RT) PCR	AAC GTC CCA ATT TGC AGA CC
EF0362F	ddPCR of WT to quantify expression of *LpmO (ef0362)*	CTC ATG GTT ACG TAG CAA GTC C
EF0362R	ddPCR of WT to quantify expression of *LpmO (ef0362)*	AAA CCT GAG ACA CCT GCA CT
EF2863F	mRNA Expression Analysis by Reverse Transcription (RT) PCR	CCT GCT TCT CGT TTA GGT GC
EF2863R	mRNA Expression Analysis by Reverse Transcription (RT) PCR	TCG ACC ATA AAG TTC TTG TGT CG
EF0114F	mRNA Expression Analysis by Reverse Transcription (RT) PCR	AAG GAA TGG TGT TCA AAG TGG G
EF0114R	mRNA Expression Analysis by Reverse Transcription (RT) PCR	GCT CAT TGG CTT GGC TGA TC

A schematic overview of the binding regions of the primers to the genes of interest are presented in the following figures. **Figure 2.5.1** shows the primers targeting the genes *ef0361* and *ef0362* as they appear in the wild type (A), as well as their binding regions in the knock out strains ΔChi, ΔLpmO and ΔChi-ΔLpmO (B).

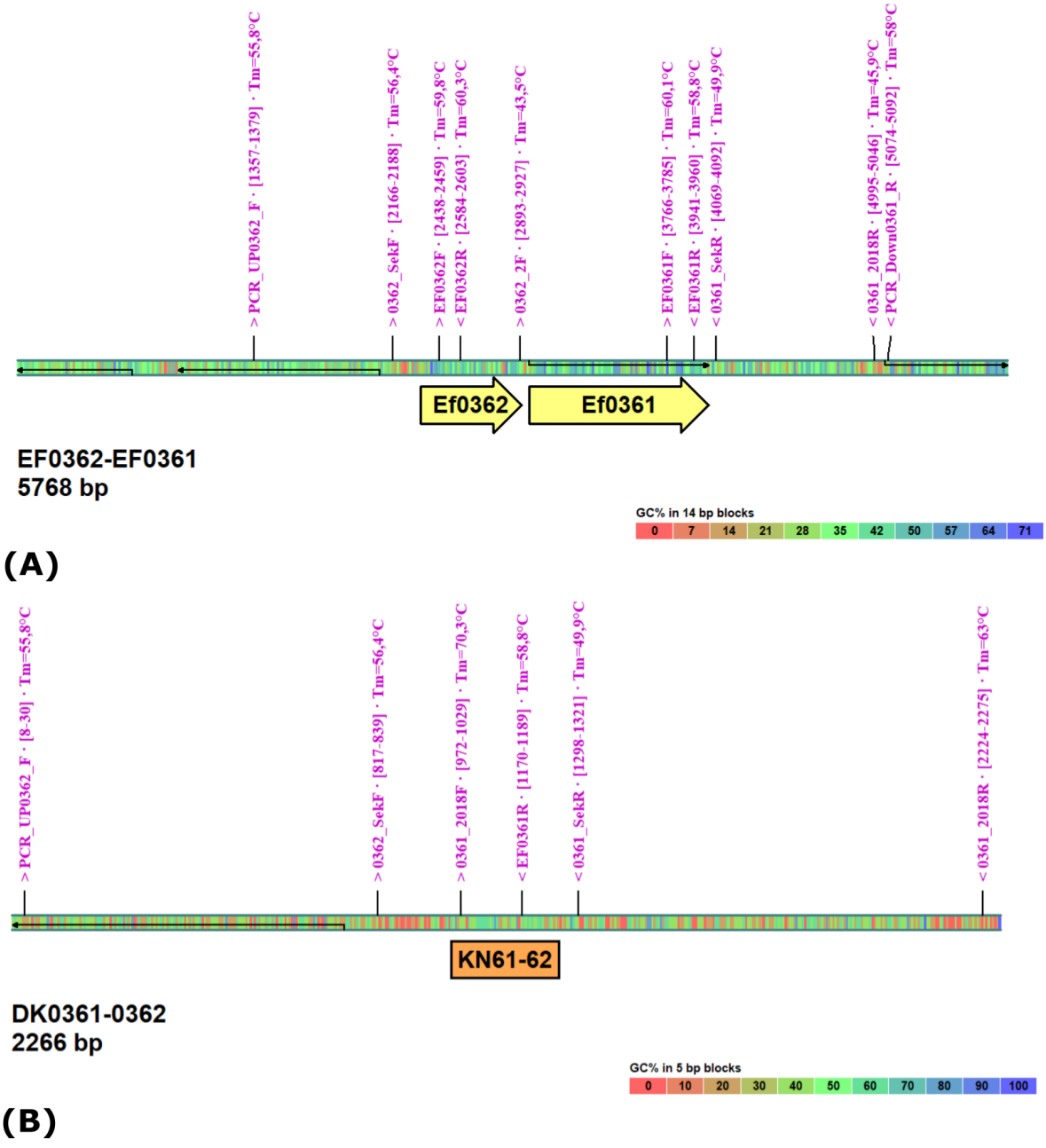

Figure 2.5.1 Schematic overview of the primer regions of *ef0361* and *ef0362* in the wild type (A), as well as the corresponding gene-knockouts (B).

Figure 2.5.2 shows the primers targeting the gene *ef0114* as it appears in the wild type (A), as well as the primers utilized for the corresponding knock out strain ΔDeglycosidase (B).

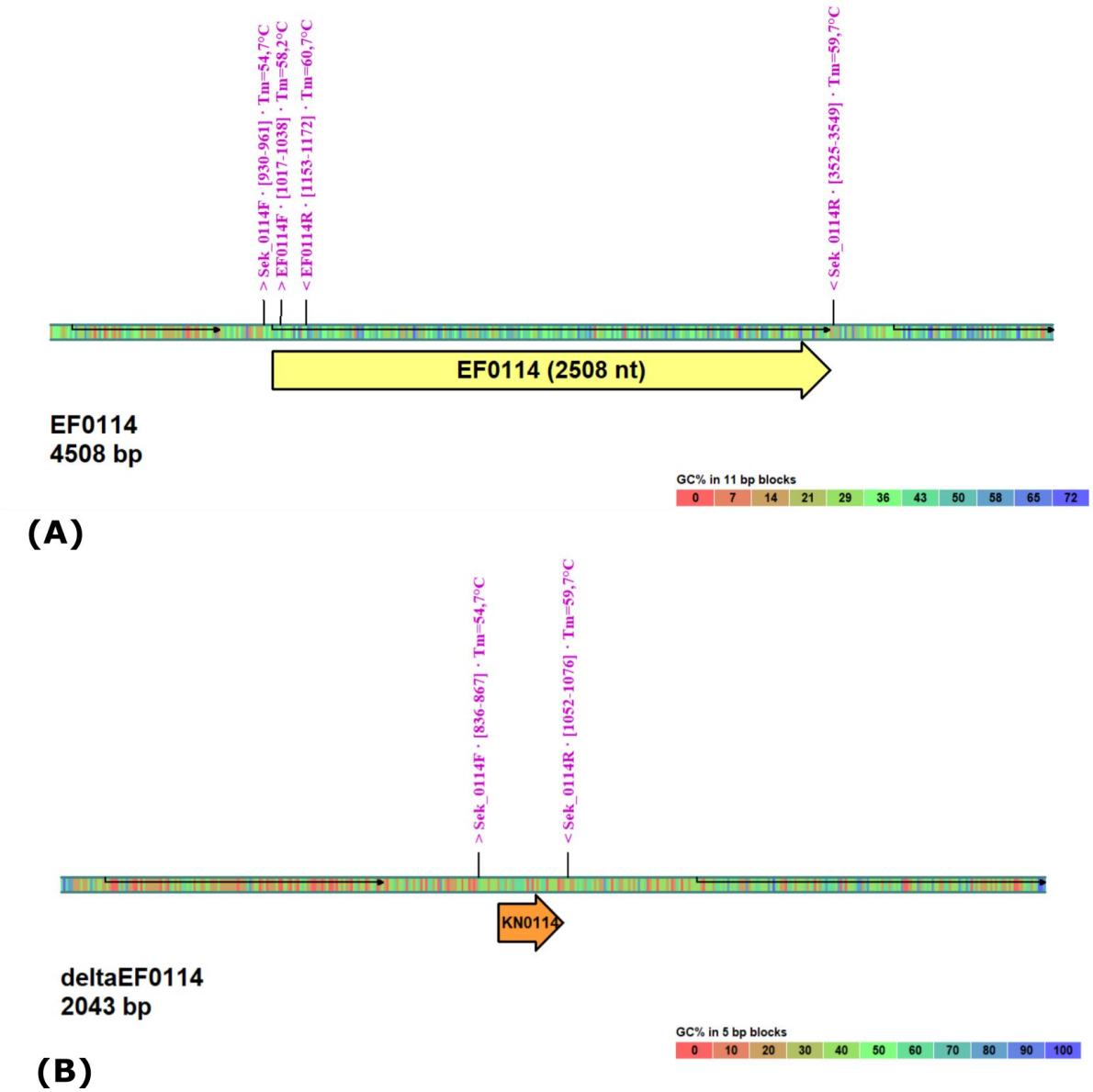

Figure 2.5.2 Schematic overview of the primer regions of *ef0114* in the wild type (A), as well as the corresponding gene-knockout (B).

Figure 2.5.3 shows the primers targeting the gene *ef2863* as it appears in the wild type (A), as well as the primers utilized for the corresponding knock out strain ΔDeglycosidase (B).

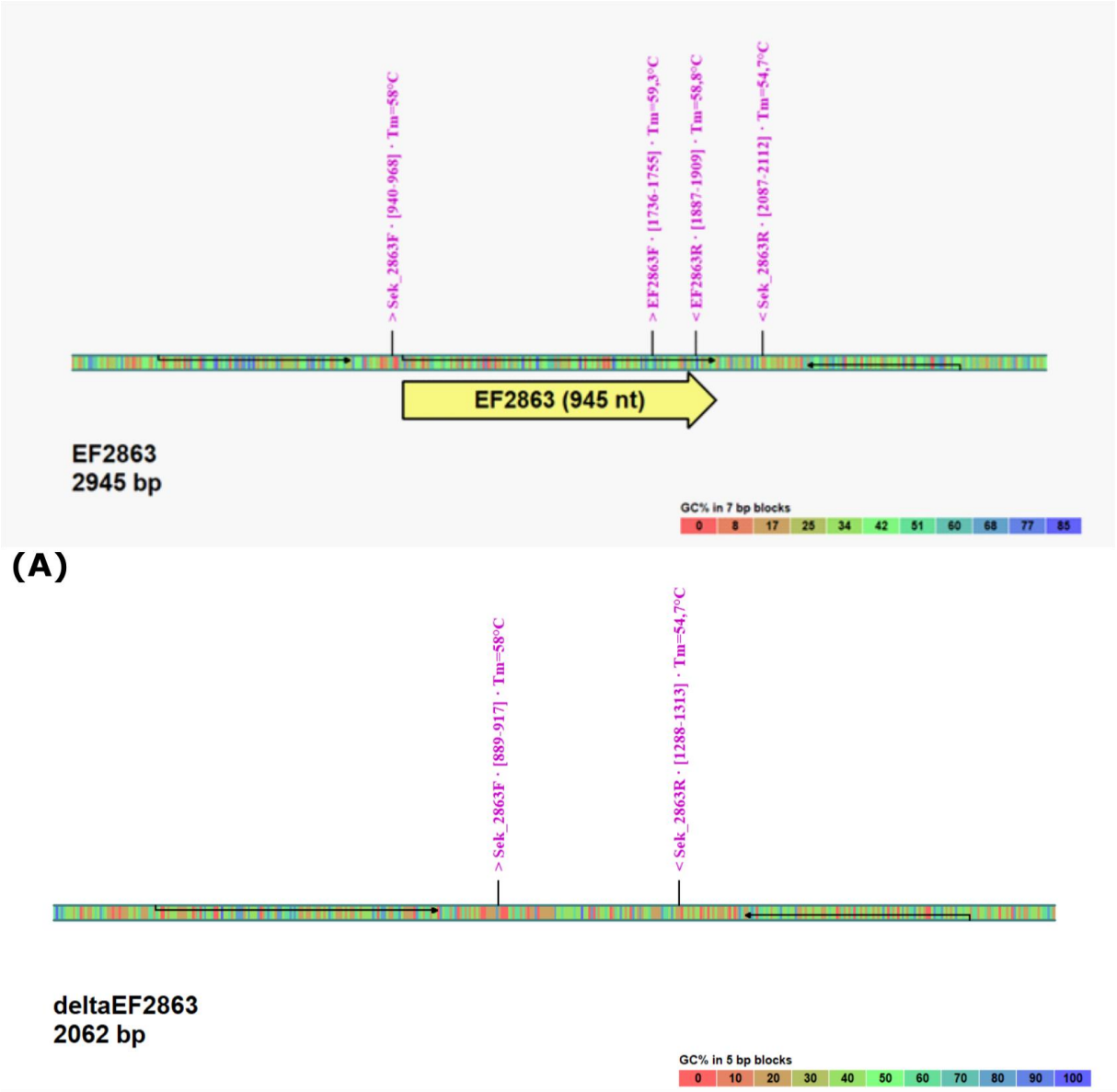

Figure 2.5.3 Schematic overview of the primer regions of *ef2863* in the wild type (A), as well as the corresponding gene-knockout (B).

2.6 Kits

Table 2.6.1 contains the kits that have been utilized throughout this study.

Table 2.6.1 Kits. The table shows all relevant kits for this study, along with the supplier and kit contents.

Kit	Supplier	Contents
Nucleospin® Gel and PCR Clean-up	Macherey-Nagel	Binding Buffer NTI Wash Buffer NT3 Elution Buffer NE NucleoSpin® Gel and PCR Clean-up Columns Collection Tubes (2 mL)
MagAttract® HMW DNA Kit (48)	Qiagen	MagAttract Suspension G Buffer ATL Buffer AL Buffer MB Buffer MW1 Buffer PE Buffer AE Proteinase K RNase A (100 mg/ml) Nuclease-Free Water
Heat&Run gDNA Removal Kit	ArcticZymes®	10x Reaction Buffer HL-dsDNase
RNeasy® Mini Kit	Qiagen	RNeasy Mini Spin Columns Collection Tubes (1.5 ml) Collection Tubes (2 ml) Buffer RLT Buffer RW1 Buffer RPE RNase-Free Water
Qubit™ dsDNA BR Assay Kit	Invitrogen	Qubit® dsDNA BR Reagent (Component A) Qubit® dsDNA BR Buffer (Component B)

	Qubit® dsDNA BR Standard #1 (Component C)
	Qubit® dsDNA BR Standard #2 (Component D)

2.7 Cultivation Media and Agar

All components and suppliers are listed in **section 2.2**.

2.7.1 LM17ent

Liquid medium:

- 1.25 g Maritex Fish Peptone
- 1.25 g Bacto yeast
- 0.25 g Ascorbic acid
- 0.125 g Magnesium sulfate
- 9.5 g Disodium glycerolphosphate
- 0.025 g Manganese sulfate

All components were dissolved in 500 ml dH_2O in a 500 ml flask. The medium was sterilized at 121°C for 15 minutes in a Certoclav, and then stored at room temperature.

When needed, LM17ent was supplemented with 0.4% (w/v) N-acetyl-D-glucosamine (GlcNAc), 0.4% (w/v) Diacetyl-chitobiose ($GlcNAc_2$) and 1% (w/v) β-chitin. GlcNAc and $GlcNAc_2$ were dissolved in LM17ent and the solution was sterile filtered prior to usage. β-chitin is however insoluble and was therefore added prior to autoclaving.

2.7.2 GLM17ent

Liquid medium:

- 1.25 g Maritex Fish Peptone
- 1.25 g Bacto Yeast Extract
- 0.25 g Ascorbic acid
- 0.125 g Magnesium sulfate
- 9.5 g Disodium glycerolphosphate
- 0.025 g Manganese sulfate
- 5 ml Glucose (40%)

All solid components were dissolved in 495 ml dH_2O in a 500 ml flask. 5 ml glucose (40%) was then added to a final concentration of 0.4% glucose. The solution was autoclaved at 121°C for 15 minutes and then stored at room temperature.

2.7.3 Lysogeny Broth (LB)

Liquid medium:

- 10 g Bacto-Tryptone
- 5 g Bacto-Yeast Extract
- 10 g NaCl

All solid components were dissolved in 1 l dH_2O. The solution was autoclaved at 121°C for 15 minutes and then stored at room temperature.

Agar plates:

15 g of agar powder was added to 1 l of the liquid LB-medium (1.5% (w/v) agar). The solution was autoclaved at 121°C for 15 minutes, and thereafter allowed to cool down to ~60°C. The medium was then poured into Heger plates and allowed to solidify under the flow hood, before further storage at 4°C.

2.7.4 Brain Heart Infusion (BHI)

Liquid medium:

BHI-medium was prepared according to supplier's instructions. 37 g of BHI powder was dissolved in 1 l dH_2O, followed by autoclaving at 121°C for 15 minutes. The medium was then stored at room temperature until use.

Agar plates:

15 g of agar powder was added to 1 l of the liquid BHI medium (1.5% (w/v) agar). The solution was autoclaved at 121°C for 15 minutes, and thereafter allowed to cool down to ~60°C. The medium was then poured into Heger plates and allowed to solidify under the flow hood, before storage at 4°C.

2.7.5 RPMI

Roswell Park Memorial Institute (RPMI) Medium 1640 (Thermo Fisher Scientific, USA) was used to provide more physiological relevant conditions for the bacteria, in the context of human infection. When needed, bacteria were cultivated in RPMI supplemented with 5% BHI (v/v) to support bacterial growth or 0.05% HSA (v/v) obtained from human blood.

2.8 Buffers and Solutions

All components and suppliers are listed in **section 2.2**.

2.8.1 4-methylumbelliferone (4-MU) solution

Stock solution (1 mM):

0.00991 g 4-methylumbelliferone was dissolved in 50 ml dH_2O. The solution was shaken well to avoid crystallization. The container was then covered with aluminium foil to protect the solution from light and stored at 4°C until use.

2.8.2 Citrate phosphate buffer, pH 6

Stock solution (50 mM):

17.9 ml 0.1 M Citric Acid

32.1 ml 0.2 M Dibasic Sodium Phosphate

Diluted in dH_2O to 100 ml

2.8.3 Carbonate buffer

Stock solution (0.2 M):

10.599 g Sodium Carbonate

Dissolved in 500 ml dH_2O

2.9 DNA

Table 2.9.1 shows the DNA used in this study.

Table 2.9.1 DNA. The table shows DNA used in this study, along with the relevant supplier.

DNA	Supplier
Quick-Load® 100 bp DNA ladder	New England Biolabs
Quick-Load® Purple 1 kb Plus DNA Ladder	New England Biolabs

2.10 Software and Online Resources

Table 2.10.1 gives an overview of the various software and online resources utilized throughout this study.

Table 2.10.1 Software and online resources. The table lists all software and online resources used in this study, along with the relevant supplier.

Software/Online Resource	Supplier
CLC DNA Main Workbench 7	Qiagen
GraphPad Prism 8	GraphPad Software
Image Lab™ Software	Bio-Rad
http://www.microbesonline.org/	The Virtual Institute for Microbial Stress and Survival sponsored by the US Department of Energy Genomics: GTL program.
pDRAW32 DNA-Analysis Software	AcaClone software
PyMOL 2.3	Schrödinger; Accessed via pymol.org

2.11 Antibiotics

Table 2.11.1 shows the various antibiotics that have been used in this study.

Table 2.11.1 Antibiotics. The table shows the antibiotics used, along with the relevant supplier.

Antibiotic	Supplier
Gentamicin, 50 mg/ml	Sigma-Aldrich
Rifampicin	Sigma-Aldrich
Spectinomycin dihydrochloride pentahydrate	Sigma-Aldrich
Tetracycline	Sigma-Aldrich

3 Methods

3.1 Cultivation of *Enterococcus faecalis* strains

Enterococcus faecalis is a Gram-positive, facultative anaerobe. The bacterium has rapid growth rate and an ability to grow both in presence and absence of oxygen, and both with and without agitation.

This study involved five different strains of *E. faecalis V583*; the wild type (WT) and the four gene knockout strains ΔChi, ΔLpmO, ΔChi-ΔLpmO and ΔDeglycosidase (see **table 2.4.1**). All five strains were grown in liquid and agar BHI or LB medium as a normal. LM17ent was used as the minimal growth medium.

All media and other reagents used for cultivation were autoclaved and/or filtered under sterile conditions using a sterile syringe and a Filtropur S 0.2 µm filtration unit. The bacterial cultures were made in sterile conditions using a laminar flow workbench to prevent contamination. Overnight cultures were made by inoculating 5 or 10 ml of BHI medium in a sterile culture tube with a single colony from a LB/BHI agar plate or directly from a glycerol stock using an inoculation loop (see **section 3.2**). The cultures were cultivated overnight at 37°C with agitation (230 rpm).

3.2 Glycerol stocks - Long term storage of bacteria

Bacteria on agar plates can be stored at 4°C for a few weeks, but when storing bacteria over longer periods of time, glycerol stocks should be made. Glycerol stocks of bacteria can be stored stably at -80°C for many years. The addition of glycerol will preserve the wildtype and gene knock out strains of *E. faecalis*, as glycerol prevents damage of cell membranes and keeps the bacteria stable and alive when frozen.

Glycerol stocks were made by adding 1 ml of bacterial overnight culture and 300 µl glycerol (85%) to a cryogen vial, and the content was mixed by turning the tube a few times. The glycerol stocks were stored at -80°C.

The glycerol stocks were used to inoculate new overnight cultures when needed throughout this study. The bacteria were transferred from glycerol stocks to new culture tubes with media using an inoculation loop. The culture tubes were then incubated at 37°C with agitation (230 rpm).

The glycerol stocks were also used to grow colonies on agar plates, to be used in further experiments in this study. Plating of the bacterial strains was done by streaking the glycerol stock-bacteria on the plate using an inoculation loop. Streaking was performed with the

quadrant method, also called four-streak method, to ensure isolated, single-growing colonies on the plate.

3.3 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a biotechnological method used to selectively amplify DNA-sequences. The method generates millions of identical copies of one original segment of DNA, and requires a thermostable DNA polymerase, the DNA template, forward and reverse primers, and deoxynucleotide triphosphates (dNTPs) to synthesize complementary DNA. The DNA sequence to be amplified can be unknown, but the method prerequisites that around 20 base pairs on each side of the DNA-segment are known. These regions are called the primer regions and synthetic oligonucleotides called primers are designed to match these regions. During a standard qualitative PCR, all components are mixed in PCR tubes which in turn are placed in a thermocycler. This machine has a heating block that can be set to different temperatures and is preprogramed to run the three essential PCR steps, where the temperature and duration of each step is varied accordingly.

The first step of the PCR is the heating of the reaction to separate the double stranded template DNA into single strands of DNA. This is called the melting or denaturation step and results in single stranded DNA carrying the target sequence to be amplified. In the second step, the temperature is lowered to enable hybridization of the forward and reverse primers to their complementary regions flanking the target DNA. The binding of the primers to the template DNA strands is called annealing. The last and final step, known as elongation, requires an increase in temperature again to activate the thermostable DNA polymerase. The enzyme will bind to the 3'-OH end of the primers and replicate along the target sequence by adding dNTPs to the continually growing strand. These three PCR steps combine to one cycle, and PCRs are normally run for 25-35 cycles depending on the amount of DNA needed. For every cycle the number of target DNA molecules is doubled. The PCR is an exponential reaction, where all newly made DNA-strands will work as templates for the next round of amplification, generating millions of copies.

In this study, PCR was used for several purposes:

- Colony PCR to confirm the bacterial knock out strains (**Section 3.3.1**).
- mRNA Expression Analysis by Reverse Transcription (RT) PCR (**Section 3.13.6**).

3.3.1 Colony PCR

In order to verify the establishment of gene-targeted deletions in the isogenic mutant strains of *E. faecalis V583*, colony PCR was performed. Colonies of the different strains were grown on LB agar plates, and the PCRs were carried out with these colonies as template.

Colony PCR is an efficient method to confirm the presence or absence of a partial or complete gene in DNA constructs. The colony PCR involves an initial heating-lysis step to rupture the bacterial membrane and release the DNA into solution, so the DNA can serve as template for the reaction. The PCR products can thereafter be visualized on an agarose gel (**Section 3.4**) to ensure that the fragments are of correct size and the knockout-strains lack the genes of interest, by comparison to a DNA-ladder with fragments of known sizes.

Materials:

- PCR tubes, 0.2 ml
- Red Taq DNA Polymerase Master Mix (2x)
  - Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 3.0 mM $MgCl_2$, 0.2% Tween® 20
  - 0.4 µM of each dNTP
  - 0.2 units/µl VWR Taq polymerase
  - Inert dye and stabilizer
- Forward and reverse primers (**Table 2.5.1**)
- Bacterial colony
- dH_2O
- Microwave
- Microcentrifuge

Method:

PCRs were prepared according to **table 3.3.1.1.** The PCRs were performed in a Thermal cycler using the conditions described in **table 3.3.1.2.**

Preparing the template DNA:

Single bacterial colonies were picked with a sterile toothpick and were deposited at the bottom of 0.2 ml PCR tubes. The PCR tubes were then run for 60 seconds in the microwave on full effect (900 W), to lyse the bacteria due to their thick cell wall. The PCR tubes were then put on ice to cool down, and the other reaction components were added to the tubes containing the

template DNA. The Red Taq DNA polymerase Master Mix (2x) was added lastly. All reaction mixtures were made on ice and were made in accordance with **table 3.3.1.1.**

Table 3.3.1.1 PCR components for colony PCR to verify the gene knockout strains.

Component	Volume (µl)
Red Taq DNA Polymerase Master Mix (2x)	25
10 µM Forward Primer	1
10 µM Reverse Primer	1
dH_2O	23
Template DNA	-

Due to the Red Taq DNA Polymerase and the primers being frozen, they were defrosted, vortexed and spun down before usage. All reaction mixtures were mixed well by pipetting and finally spun down with the microcentrifuge.

Table 3.3.1.2 PCR conditions for colony PCR to confirm gene knockout strains.

Step	Number of cycles	Temperature (°C)	Time (mm:ss)
Initial Denaturation	1	95	02:00
Melting	35	95	00:30
Annealing	35	50	00:30
Elongation	35	72	02:00
Final Extension	1	72	05:00

PCR-products were thereafter visualized using agarose gel electrophoresis (**Section 3.4**).

3.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA fragments based on their relative size. Agarose is a linear polysaccharide made up of repeating units of agarobiose. The agarose powder is dissolved in Tris-Acetate-EDTA (TAE) buffer, and after heating and subsequent cooling the agarobiose chains bind non-covalently to form a gel matrix with pores of a certain size. The size of the pores depends on the concentrations of agarose added, and all gels in this study are made as 1.2% agarose gels. When DNA samples are run on such gels, smaller DNA-fragments will encounter less resistance due to the gel pores and will thus travel farther through the gel than larger DNA-fragments. This principle allows for separation of DNA fragments based on their size.

A prerequisite for the migration of DNA-fragments through the gel, is the presence of an electric field with an anode and a cathode end. To achieve this, a current is applied throughout the gel chamber. Since DNA is negatively charged, due to the phosphate backbone of its structure, the DNA-molecules will migrate from the negatively charged cathode towards the positively charged anode.

To visualize the DNA-fragments that have travelled through the gel, both the gel and the samples must be stained with a visualizing dye. For the gel, peqGREEN dye is added before the gel solidifies, whereas the samples are added a gel loading dye. In this study, the PCR products from colony PCR were already stained, as there is a red dye present in the Red Taq DNA Polymerase Master Mix (**Section 3.3.1**).

DNA ladders composed of DNA-fragments of known sizes in bp are run alongside the samples to determine the sizes of the different DNA-fragments. The ladders used in this study are the Quick-Load® Purple 1 kb Plus DNA Ladder and the Quick-Load® 100 bp DNA Ladder, depending on the expected sizes of the DNA-fragments.

Materials:

1.2 % Agarose Gel

- 6 g agarose
- 500 ml 1X Tris-Acetate-EDTA (TAE) Buffer
- 2.5 µl peqGREEN dye

Running buffer

- Tris-Acetate-EDTA (TAE) Buffer (1X)

DNA ladder

- Quick-Load® 100 bp DNA Ladder
- Quick-Load® Purple 1 kb Plus DNA Ladder

Method:

Preparing the 1.2% agarose:

1.2% agarose was prepared as a stock solution. 6 g of agarose were added to 500 ml 1X TAE buffer, and the solution was autoclaved at 121°C for 15 minutes. The solution was then stored at 56°C until usage. A new solution should be made when the colour of the agarose-solution starts to turn yellow.

Preparing the gel electrophoresis chamber and loading the samples:

The gel was prepared by mixing 55 ml agarose and 2.5 ml peqGREEN in a beaker, which in turn was poured into the gel casting tray. An 8-well comb or 15-well comb was placed, and the gel was left to solidify for 20-30 minutes. Once the gel was solid, the well comb was gently pulled out, leaving the gel ready for sample application. The casting tray containing the gel was transferred to a Mini-Sub® Cell GT Horizontal Electrophoresis System and the chamber was filled with 1X TAE buffer. The ladders and samples were loaded into the wells on the gel. Depending on the size of the wells (8-well comb or 15-well comb), either 30 or 50 µl of sample and 10 or 20 µl of ladder was added.

Running the gel and visualizing the DNA bands:

The PowerPac™ Basic power supply was turned on, and the gel was run at 90V for 30-60 minutes. Once the run was completed, the DNA bands were visualized using a Benchtop UV Transilluminator. Finally, gel imagining was done with a UV Sample Tray and a Gel Doc™ EZ Imager. Gel images were generated using the Image Lab™ Software.

3.5 Extraction and purification of DNA fragments from agarose gel

Extraction and purification of DNA fragments from the agarose gel is a preliminary step before DNA sequencing. In the present study, DNA sequencing of the ΔDeglycosidase was performed to further verify that the genes of interest are correctly knocked out in the gene deletion strain.

Following agarose gel electrophoresis, the DNA fragments of interest were isolated from the gel using the Nucleospin® Gel and PCR Clean-up Kit. This was done following the manufacturer's protocol, "Protocol 5.2 DNA extraction from agarose gels", from the "User manual" of the kit.

Materials:

- 1.2% agarose gel with DNA-fragments of interest
- Scalpel
- Benchtop UV Transilluminator
- Eppendorf tubes, 1.5 ml
- Scale, Entris Sartorius
- Nucleospin® Gel and PCR Clean-up Kit
- Microcentrifuge, Heraeus™ Pico™ 21

Method:

Extracting DNA-fragments from the 1.2% agarose gel:

After the gel was removed from the gel electrophoresis chamber (**Section 3.4**), the gel was placed on the Benchtop UV Transilluminator. The DNA bands were then visualized by applying UV light for as short periods of time as possible, to reduce the risk of damaging the DNA, and the bands were gently excised from the gel using a clean scalpel.

Purification of the DNA-fragments:

Three and three gel slices of the same DNA-fragment were placed in pre-weighed 1.5 ml Eppendorf tubes. The tubes were weighed to determine the mass of each gel slice. Following the Nucleospin® Gel and PCR Clean-up Kit Protocol, the DNA was extracted and purified accordingly. In the final elution step, the DNA was eluted in 20 µl Buffer NE that was preheated to 60°C.

All centrifugation steps were performed in a Heraeus™ Pico™ 21 Microcentrifuge.

3.6 Measurement of dsDNA concentration

3.6.1 Fluorometric quantitation with Qubit™

Following the extraction and purification of the DNA-fragments (**Section 3.5**), the concentrations of the eluted DNA were measured using the Qubit™ fluorometer.

Qubit™ is a fluorescence-based quantitation method where the dsDNA to be measured binds to a target-specific dye. This dye emits fluorescence when bound to dsDNA, which can be measured with the Qubit™ fluorometer, allowing for selective measurements of dsDNA concentrations and reduced effects of contaminants such as RNA and proteins.

Materials:

- Purified DNA
- Qubit™ fluorometer
- Qubit™ assay tubes
- Qubit™ dsDNA BR Buffer
- Qubit™ dsDNA BR Assay Kit

Method:

1. A working solution of the Qubit™ dsDNA BR Reagent was prepared by diluting it 1:200 in the Qubit™ dsDNA BR Buffer. The tube was mixed by vortexing 2-3 seconds, carefully to avoid creating any bubbles. Always make enough working solution for one more tube than needed.
2. Two µl DNA sample was mixed with 198 µl working solution in a Qubit™ assay tube, the solution was incubated for two minutes at room temperature, and the DNA concentration was finally measured using the Qubit™ fluorometer.

The fluorometer was pre-calibrated with Qubit® dsDNA BR Standard #1 and #2 with the known concentrations 0 and 100 ng/µl.

3.6.2 Spectrophotometric quantitation with Eppendorf D30 BioPhotometer® (A_{260})

Another method of measuring dsDNA concentration, based on spectrophotometry, exploits the fact that DNA absorbs light at 260 nm. The dsDNA concentration in a given sample is determined by measuring its UV absorbance at 260 nm (A_{260}). Based on the Beer-Lambert law, the absorbance can be translated into dsDNA concentration measured in µg/ml. The Beer-Lambert Law, **Equation 3.6.2.1**, relates the absorbance (A) of a sample to the path length (b) through which the UV light travels and to the concentration of the sample (c).

Equation 3.6.2.1 Beer-Lambert Law.

$$A = \varepsilon * b * c$$

ε is the extinction coefficient and at 260 nm, the average extinction coefficient of double-stranded DNA is 0.02 $(\mu g/mL)^{-1}$ cm^{-1}. This value relates the measured absorbance to the concentration of DNA in µg/mL.

In this study, the path length b was always 1 mm - the length of the µCuvette.

Materials:

- Microvolume measuring cell, Eppendorf µCuvette
- Nuclease-free water
- DNA-sample
- Eppendorf D30 BioPhotometer®

Method:

1. The Eppendorf D30 BioPhotometer® was set to blank by adding two µl nuclease-free water to the µCuvette and inserting it in the instrument.

2. Samples were measured in the same manner, by adding two µl sample to the measuring cell and inserting it in the instrument to read the dsDNA-concentration of the sample.
3. A built-in program calculates the dsDNA concentration (µg/ml) of the given sample.

This method was used to measure the amount of eluted DNA prior to whole genome sequencing of the ΔChi-ΔLpmO strain (**Appendix B**).

3.7 DNA Sequencing

Following extraction and purification (**Section 3.5**), as well as determination of the dsDNA concentration (**Section 3.6**), the two hypothesised knockout regions of the genes *ef0114* and *ef2863* in strain ΔDeglycosidase were to be verified through sequencing.

Samples were prepared for sequencing by combining purified template DNA, forward or reverse primers, and dH_2O in 1.5 ml Eppendorf tubes. Sequencing reactions were prepared as shown in **table 3.7.1**.

Table 3.7.1 Components for GATC sequencing (Eurofins Scientific, France) of mutant gene knockouts of *ef0114* and *ef2863*.

Component	Volume (µl)
Template DNA	4.5
Forward or Reverse Primer (10 µM)	2.5
dH_2O	4

Total sample amounts in each tube were 11 µl. Forward and reverse sequencing reactions were performed using the Sek_0114F and Sek_0114R primers that anneal to each side of *ef0114*, and Sek_EF2863F and Sek_EF2863R which bind to each side of *ef2863* (See **table 2.5.1**).

The concentrations of DNA measured with the Qubit™ fluorometer (**Section 3.6.1**) were used to determine the volumes of purified DNA to be added to each reaction to achieve a final amount of 400 ng DNA. The calculations were based on the following formula:

$$Volume\ of\ purified\ DNA\ to\ be\ added = \frac{400\ ng}{Concentration\ of\ purified\ DNA\ (\frac{\mu g}{ml})}$$

The sequencing was performed by Eurofins Scientific, using their GATC services. The sequencing results were analysed in CLC Genomics Workbench.

3.8 Characterization of growth

As part of the characterization of the four strains of *Enterococcus faecalis* (see **table 2.4.1**), their growth on different substrates were analysed. Growth curves were made to evaluate any differences between the strains.

3.8.1 Growth on soluble substrates

Growth of the different strains were tested using soluble substrates in a microtiter plate assay. Five different soluble substrates with different carbon sources were tested, presented in **table 3.8.1.1**.

Table 3.8.1.1 Soluble substrates used in growth analysis.

Substrate	Description
GLM17ent	LM17ent supplemented with 0.4% Glucose
LM17ent + GlcNAc	LM17ent supplemented with 0.4% N-acetyl-D-glucosamine
LM17ent + $GlcNAc_2$	LM17ent supplemented with 0.4% Diacetyl-chitobiose
BHI	Positive control
LM17ent	Negative control

Materials:

- Sarstedt Tubes, 13 ml
- GLM17ent-medium
- Overnight cultures of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- LM17ent-medium supplemented with 0.4% GlcNAc
- LM17ent-medium supplemented with 0.4% $GlcNAc_2$
- BHI-medium
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- Microtiter plate
- Sealing tape
- Multiskan™ FC Microplate Photometer

Method:

1. Overnight cultures of all four strains were prepared in 10 ml GLM17ent at 37°C with agitation (230 rpm).
2. The following day, overnight cultures were diluted to $OD_{600nm} \approx 0.15$ in 5 ml prewarmed tubes containing each of the five different medias (**table 3.8.1.1**). Controls were included without addition of bacteria.
3. 200 µl of the diluted bacterial cultures were transferred to a microtiter plate in three parallels. The control samples were also transferred with three parallels per control medium.
4. The microtiter plate was sealed and analysed in a plate reader on the following program; incubation at 37°C with continuous background shaking, with absorbance measurements (OD_{595nm}) every 15 minutes for a total period of 24 hours. The program was turned off when the curves flattened out and the bacterial strains had reached late stationary phase.

The assay was repeated three times, resulting in three biological parallels of the growth curves.

3.8.2 Growth on insoluble substrates

Due to the known chitinase activity of the bacterium, growth on the insoluble substrate β-chitin was characterized for all four strains. The growth was observed through absorbance measurements and by plating and calculating CFU/ml. The bacterial growth was analysed both with and without agitation.

3.8.2.1 Growth on β-chitin

Materials:

- Sarstedt tubes, 13 ml
- GLM17ent-medium
- Overnight cultures of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R
- 1X PBS
- LM17ent-medium
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- 250 ml Erlenmeyer flasks

- LM17ent-medium supplemented with 1% β-chitin
- Incubator, Minitron, 37°C
- Microtiter plate
- Ringer solution
- BHI-plates
- Incubator, Termaks, 37°C

Method:

1. Overnight cultures of all four strains were prepared and grown overnight in 10 ml GLM17ent at 37°C with agitation (230 rpm).
2. The following day, five sterile Erlenmeyer flasks with 50 ml 1% β-chitin-medium were prewarmed at 37°C; one flask for each of the four strains and a fifth as a medium-control without addition of bacteria.
3. The overnight cultures were centrifuged at 4255xg for 15 minutes at room temperature, washed once in 10 ml 1X PBS, before the bacterial pellets were resuspended in 1 mL LM17ent.
4. Thereafter, the bacterial suspensions were transferred to each of their respective flask with 1% β-chitin-medium, diluting the bacteria to a final OD_{600nm} of approximately 0.15.

   The absorbance was challenging to measure in the media, due to the β-chitin-particles which interfered with the light through the sample. To solve this problem, four new culture tubes were prepared with 5 ml LM17ent-medium. The bacterial suspensions were instead of being added directly to the β-chitin-media, first adjusted to the correct OD_{600nm} in these tubes. Ten times the volumes required to reach $OD_{600nm} \approx 0.15$ in the culture tubes were then transferred to the flasks with the 1% β-chitin-medium.

5. The bacterial cultures in the Erlenmeyer flasks were placed in an incubator at 37°C. The assay was performed both with agitation (140 rpm) as well as under static conditions.
6. Samples were collected from the bacterial cultures once a day over 7 days to monitor the growth. This was done by absorbance measurements and enumeration by plating the cultures onto BHI-plates for calculation of CFU/ml.

Measuring the absorbance:

Absorbance measurements were challenging due to the interfering β-chitin particles in the cultures. The flasks were therefore held stably in a diagonal position for a certain amount of time (see **Appendix C** for optimization of sinking times). OD_{600nm} was then measured with the cell density meter, and immediately after (without moving the flask) 150 µl bacterial suspension was transferred to a the first well of a microtiter plate for estimations of CFU/ml.

Multi-dilution plating:

150 µl bacterial sample was transferred to the first well in the microtiter plate, and serial dilutions were made by adding 180 µl Ringer solution to the remaining wells and transferring 20 µl bacterial suspension from the first well to the next and so on. The dilutions to be plated were transferred to the BHI plates through a method called "multi-dilution plating". Using a multichannel pipet, 20 µl of the 10^{-3} to 10^{-7} dilutions were transferred to the same BHI plate and the droplets were allowed to run down in stripes. It is important that none of the droplets run into one another and that they are allowed to dry. Once the plates were dry, they were incubated at 37°C overnight. Plating was performed in two or three parallels.

The next day the dilutions with numbers between 20 and 200 colonies on the plates were counted. Based on these numbers, CFU/ml were calculated with the following formula:

$$\frac{CFU}{ml} = Number\ of\ colonies * dilution\ factor * 50$$

3.8.3 Growth on BHI in culture tubes

Due to future assays in this study requiring growth curves for the strains on BHI in culture tubes, the bacterial strains were not only grown on BHI in microtiter plates as described in **section 3.8.1**, but also in culture tubes. Growth was monitored under incubations both with and without agitation.

Materials:

- Sarstedt Tubes, 13 ml
- BHI-medium
- Overnight cultures of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- Centrifuge, Allegra X-30R
- 1X PBS
- Ultrospec 10 Cell Density Meter

- Disposable cuvettes
- Incubator, Minitron, 37°C

Method:

1. Overnight cultures of all four strains were prepared and grown overnight in 10 ml GLM17ent at 37°C with agitation (230 rpm).
2. The overnight cultures were centrifuged at 4255xg for 10 minutes, and the bacterial pellets were washed once in 10 ml 1X PBS. Following the wash step, the pellets were resuspended in 1 ml prewarmed BHI.
3. The bacterial suspensions were transferred to new tubes with 10 ml prewarmed media, diluting the bacteria to a final OD_{600nm} of approximately 0.15.
4. The tubes were incubated further at 37°C, and the absorbance (OD_{600nm}) was measured every hour until (late) stationary phase was reached.

3.9 Enzyme activity assay

Enzymatic activity can be measured in culture supernatants by a fluorometric enzyme assay, based on the hydrolysis of 4-methylumbelliferone-containing substrates. The 4-MU-unit will be cleaved off by the enzyme if present, and the amount of released 4-MU can be measured quantitatively using a fluorometer.

In the present study, chitinase- and hexosaminidase-activity was measured in culture supernatants of all the strains of *E. faecalis* (see **table 2.4.1**) over a period of seven days, where the bacteria had been cultivated statically on the minimal medium LM17ent supplemented with 1% β-chitin. Chitinase activity was determined using the $GlcNAc_3$ analogue 4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside (4-MU-$GlcNAc_2$) as substrate, and N-acetylhexosaminidase activity was determined using the $GlcNAc_2$ analogue 4-methylumbelliferyl-β-D-N-acetylglucosamine (4-MU-GlcNAc) as substrate.

Materials:

- Eppendorf tubes, 1.5 ml
- Culture supernatants of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- Microcentrifuge, Heraeus™ Pico™ 21
- Assay tubes, 13 ml, with lids
- Citrate Phosphate Buffer, pH 6.0, 50 mM
- Bovine Serum Albumin, 1.0 mg/ml
- Substrates; 4-MU-GlcNAc/4-MU-$GlcNAc_2$, 500 µM
- Water bath, 37°C
- 4-MU solution, 1.0 µM
- Carbonate buffer (0.2 M Na_2CO_3)
- Fluorometer
- ChiB from *Serratia marcescens*

Table 3.9.1. Reaction mixtures for measuring enzymatic activity of the wild type and gene deletion strains.

Component	Volume (µl)	Final concentration
Citrate Phosphate Buffer, 50 µM, pH 6	30	30 µM
Bovine Serum Albumin, 1.0 mg/ml	5	0.1 mg/ml
4-MU-GlcNAc/4-MU-$GlcNAc_2$, 500 µM	5	50 µM

Method:

1. Triplicates of 40 µl-reaction mixtures were prepared according to **table 3.9.1** in assay tubes with lids. The glass tubes were then prewarmed in a 37°C water bath.
2. Samples were collected from the bacterial strains to be analysed, and the cultures were spun down at 21.1xg for four minutes in Eppendorf tubes using a centrifuge to collect supernatants.
3. The reactions were started by adding ten µl of the supernatants to the reaction mixtures, vortexing the tubes, and then quickly putting them back in the water bath. Each reaction was started with a one-minute interval.
4. After exactly ten minutes, the reactions were stopped by adding 1.95 ml Carbonate buffer (0.2 M Na_2CO_3). Immediately after, the total sample volume (two ml) was transferred to the cuvette to measure the amount of released 4-MU using a fluorometer. The cuvette was rinsed with Carbonate buffer between each reading.

Calibration of the fluorometer:

The fluorometer was calibrated to show a 500 ± 10 reading when measuring a 50 nM solution of 4-MU. The calibration solution was made by mixing 1.9 ml 0.2 M Na_2CO_3 with 100 µl 1 µM 4-MU-solution. Based on this, each measured unit of the fluorometer will correspond to 0.1 nM 4-MU.

Positive control:

The assay included the positive control ChiB; chitinase B expressed in *Serratia marcescens*. The enzyme catalyses hydrolysis of the β-1,4 glycosidic linkages in chitin and belongs to the GH18 family. The positive control used in this assay had a measured protein concentration of 3.008 [±0.3803] mg/ml. Ten µl of ChiB was added to three assay tubes containing 40 µl reaction mixture in the same manner as the other samples with a one-minute interval. Each reaction was stopped ten minutes later by adding 1.95 ml 0.2 M Carbonate buffer, and the 4-MU-release was measured accordingly.

3.10 Determination of protein concentration

The protein concentration of a sample can be measured spectrophotometrically using a variety of methods. One such method is the Bradford-method that utilizes the acidic Coomassie® Brilliant Blue G-250 dye to determine the protein concentration of the given sample. The acidic dye will bind to basic and aromatic amino acid residues in the sample, particularly to arginine-residues, and development of colour is dependent on the various concentrations of protein. The absorbance maximum for an acidic solution of the dye shifts from 465 nm to 595 nm upon protein binding, thus the protein concentration can be determined by measuring the absorbance at 595 nm with a spectrophotometer.

The measured absorbance (A_{595nm}) can be translated into protein concentration (µg/ml) by pre-calibration of the spectrophotometer with standards of known concentrations. In the present study, the instrument was pre-calibrated with six standards ranging from 1 µg/ml to 25 µg/ml, which resulted in a standard curve with a coefficient of determination (R^2) equal to 0.9971.

Materials:

- Eppendorf tubes, 1.5 ml
- Buffer: Tris-HCl, pH 8.0, 20 mM
- Protein sample
- Protein Assay Dye Reagent Concentrate
- Vortex
- Eppendorf D30 BioPhotometer®

Method:

1. The assay was set up in triplicates. Four Eppendorf tubes were therefore prepared, including one tube for the blank and three tubes for the sample to be measured.
2. The blank was added 800 µl buffer (Tris-HCl pH 8.0), and the sample tubes were added 798 µl buffer and 2 µl sample.
3. With an interval of 30 second, each tube was added 200 µl of dye reagent and vortexed.
4. The tubes were incubated at room temperature for 5 minutes, and the absorbance was then measured at 595 nm.

5. A built-in program translates the A_{595nm} to protein concentration based on the standard curve, thus the instrument gives the protein concentration as its output. Since the protein sample is diluted, the final formula for calculating the protein concentration (µg/ml) is:

$$\frac{\text{Protein concentration } \left(\frac{\mu g}{ml}\right)}{Dilution\ factor} = \frac{\text{Protein concentration } \left(\frac{\mu g}{ml}\right)}{\frac{2}{800}} = Final\ protein\ concentration\ \left(\frac{\mu g}{ml}\right)$$

This method was used to measure the protein concentration of the positive control, ChiB, used in the enzyme activity assays in **section 3.9**.

3.11 Light microscopy

As part of the characterization of the different strains included in this study (see **table 2.4.1**), light microscopy was performed to look for any morphological variations between the mutants. The microscopy of the bacteria was conducted with a Leica ICC50 W microscope.

Materials:

- Sarstedt tubes, 13 ml
- Overnight cultures of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- GLM17ent-medium
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Microscope Slides
- Cover Slips 24x32 mm
- Microscope, Leica ICC50 W

Method:

1. Overnight cultures of the wild type and deletion strains were prepared in culture tubes with 5 ml GLM17ent. The tubes were incubated overnight at 37°C with agitation (230 rpm).
2. The next day, 500 µl overnight culture was transferred to an Eppendorf tube and was centrifuged at 5000xg for 1 minute to harvest the cells. The pellet was thereafter washed once in 500 µl 1X PBS and resuspended in 500 µl of PBS.
3. Ten µl of washed bacterial suspension was transferred to a microscope slide and was covered with a cover slip. The bacteria were then observed under the microscope (100x).

3.12 Estimation of the bacterial number at a certain OD

When working with bacteria, knowledge of the approximate number of the added bacteria is important. Estimates of the bacterial number were needed in several assays of this study, such as:

- Minimum Inhibitory Concentration-assay (**Appendix A, section 7.1.3**)
- Internalization assay (**Appendix A, section 7.1.4**)
- Whole blood survival assay (**Section 3.14**)

Thus, the number of bacteria at stationary phase of growth was determined for the wild type, by diluting the bacteria to $OD_{600nm} \approx 0.4$ and calculating CFU/ml.

Materials:

- Sarstedt Tubes, 13 ml
- BHI-medium
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- 1X PBS
- Centrifuge, Allegra X-30R
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- Microtiter plates
- BHI-plates
- Incubator, Termaks, 37°C

Method:

1. Bacteria were grown overnight in culture tubes containing 5 ml and 10 ml BHI, at 37°C with agitation (230 rpm). The overnight cultures were prepared at three different timepoints to check for differences in growth, and the time points were around 8-9 am, 12 pm and 15-16 pm.
2. The next day, the overnight cultures were washed two times in 1X PBS by centrifugation at 4255xg for 10-15 minutes at 24°C. The wash steps were performed by resuspending the bacterial pellets in the same volume 1X PBS as original amount of media in the tube (5 or 10 ml). After the last wash, the bacterial pellets were resuspended in 1 ml 1X PBS.
3. Thereafter, the bacteria were transferred to another set of tubes containing 5-6 ml 1X PBS until reaching to $OD_{600nm} \approx 0.4$.

4. Serial dilutions were made in microtiter plates in 1X PBS for plating on BHI plates, in order to calculate CFU/ml. See **section 3.8.2.1** for a description of the multi-dilution plating. The plates were incubated at 37°C overnight.
5. The colonies were enumerated the next day and CFU/ml were calculated, as described in **section 3.8.2.1** under "Multi-dilution plating".

3.13 Transcriptional analysis of *ef0361, ef0362, ef0114* and *ef2863* genes using ddPCR

Transcriptional analysis studies the activity of the formation of a functional gene product from its coding gene. In the present study, transcriptional analyses were performed to evaluate expression of the genes ef*0361, ef0362, ef0114* and *ef2863* under bacteriologic (different phases of bacterial growth) and under host mimicking conditions (10% pooled human serum). Samples were harvested from both exponential and stationary growth phases and from samples exposed to serum as described in the following section.

3.13.1 Harvest cells for RNA isolation

Materials:

- Sarstedt tubes, 13 ml
- BHI-medium
- Overnight culture of the wild type
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- LM17ent-medium
- Pooled normal human serum (nhs)
- RNAprotect™ Bacteria Reagent
- Vortex
- Centrifuge, Allegra X-30R

Method:

The samples were harvested as follows:

1. Two to three single colonies of *E. faecalis V583* wild type were inoculated in 5 ml BHI at 37°C with agitation (230 rpm).
2. The next day, to evaluate the transcriptional analysis under bacteriologic and host mimicking conditions, 100 µl overnight culture was transferred to two tubes containing

5 ml BHI and minimal medium (LM17ent), respectively. The media was preheated to room temperature and the tubes were incubated at 37°C in ambient air with agitation (230 rpm).

3. To evaluate the expression of the genes under bacteriologic conditions using BHI, bacterial samples were harvested at OD_{600nm} = 0.6-0.7 (exponential phase) and over 1.5 (stationary phase).
4. To assess the expression of the genes in presence of 10% serum, bacteria were grown to OD_{600nm} = 0.7 (mid log). Thereafter, pooled normal human serum (nhs) was added to the bacterial culture at the final volume of 10% (v/v). Bacterial culture without supplementation of nhs served as controls and were added LM17ent-medium instead of nhs in equal amounts. All samples were harvested 30 minutes post-exposure to nhs.
5. Samples were taken by transferring 2 ml of the bacterial cultures to new tubes and RNAprotect™ Bacteria Reagent was added to the sample at the final volume of 1:3 ratio (bacteria:RNAprotect™ Bacteria Reagent). The tubes were vortexed immediately for 5 seconds and incubated for at least 5 minutes at room temperature.
6. The cells were then harvested by centrifugation at 4255xg for 10 minutes at 4°C. The supernatants were decanted off and discarded, and residual supernatant was removed by inverting the tube on a paper towel for 10 seconds and gently dabbing the inverted tube. The bacterial pellets were frozen at -80°C until RNA extraction.

3.13.2 Isolation of RNA

RNA is easily degraded by RNases in the environment. Isolation of RNA should therefore be conducted under the laminar flow hood where all surfaces and equipment are cleaned with RNaseZAP™, a cleaning agent for removing RNases.

RNA was isolated from the harvested samples using the RNeasy® Mini Kit. The isolation was done following the manufacturer's protocols for "Mechanical Disruption of Bacteria" and "Purification of total RNA from bacteria using the RNeasy® Mini Kit".

Materials:

- RNaseZAP™
- Centrifuge, Allegra X-30R
- FastPrep®-24 Tissue and Cell Homogenizer
- FastPrep® tubes
- Lysing Matrix B (RNase free)

- RNeasy® Mini Kit
- Microcentrifuge, Heraeus™ Pico™ 21

Method:

The protocol "Mechanical Disruption of Bacteria" was followed. In order to perform mechanical disruption, 10 µl β-mercaptoethanol was added per 1 ml Buffer RLT, and the harvested cell pellets were resuspended in 700 µl Buffer RLT. The cells were mechanically lysed using Lysing Matrix B and were run with the FastPrep®-24 Tissue and Cell Homogenizer (MP Biomedicals) at 6.5 m/s for 30 seconds three times, with a one-minute interval between each run.

Following mechanical disruption, the RNA was extracted following the protocol "Purification of total RNA from bacteria using the RNeasy® Mini Kit". In the final elution step, the RNA was eluted in 35 µl RNase-free water.

3.13.3 RNA concentration and quality analysis

The RNA concentration and quality were evaluated using NanoDrop™ UV-Vis spectrophotometer, which provides the quantity yield and the amount of contamination. DNA, RNA and protein concentrations of a sample can be determined through absorbance measurements at the wavelengths 260 nm for nucleic acids, including dsDNA, ssDNA and RNA, and 280 nm for proteins. Concentrations are then calculated based on the absorbance reading and a conversion factor, where an A_{260nm} of 1.0 equals 40 µg/ml RNA. Absorbance measurements at 230 nm are used to determine the amount of contaminants in the sample. Nucleic acid purity can then be calculated as the ratio of absorbance of the nucleic acid to the absorbance contributed by the contaminants; A_{260nm}/A_{230nm}. Protein contamination is calculated similarly, but as the ratio between nucleic acid and protein (A_{260nm}/A_{280nm}). Typical requirements for the ratios of purity are $A_{260nm}/A_{230nm} > 1.7$, and $A_{260nm}/A_{280nm} = 1.8\text{-}2.2$ (Wieczorek D., 2012).

Materials:

- NanoDrop™ UV-Vis spectrophotometer
- RNase-free water
- Solution containing RNA

Method:

1. The instrument was set to blank by loading 1-2 µl RNase-free water.

2. Subsequently, 1-2 µl of the isolated RNA-samples were loaded onto the instrument and the sample concentrations were read. The NanoDrop™ presents the quantity yield and the percentage of contamination with proton (A_{260nm}/A_{280nm} and A_{260nm}/A_{280nm}) as its output.

3.13.4 DNase treatment for removal of genomic DNA

The genomic DNA (gDNA) in the isolated RNA samples will affect the downstream transcriptional analyses. Thus, the isolated RNA was treated with the Heat&Run gDNA removal kit (ArcticZymes, Norway) according to the manufacturer's procedure as follow:

Materials:

- 10x Reaction Buffer
- HL-dsDNase
- ThermoMixer
- Solution containing RNA

Method:

1. Ten µl of the solution containing isolated RNA (see **section 3.13.2**) was transferred to an empty RNase free Eppendorf tube on ice. A control was also included using ten µl RNase-free water.
2. One µl of 10x Reaction Buffer was added for each ten µl of RNA.
3. One µl HL-dsDNase was added and the suspension was gently mixed.
4. The mixture was incubated at 37°C for 10 minutes, and then for 5 minutes at 58°C to inactivate the enzyme.

The RNA-concentration was re-measured after this treatment using NanoDrop, as described above (**section 3.13.3**). The control sample was used as a blank to set the instrument to zero.

3.13.5 Reverse transcription

Due to the highly unstable nature of RNA, it is reverse transcribed to cDNA before further analysis. Reverse transcription was performed using the iScript™ Reverse Transcription Supermix (Bio-Rad, USA). The Supermix contains all the components necessary for the reaction including RNase H+ Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, $MgCl_2$ and stabilizers. Any DNA contamination was ruled out through minus reverse transcriptase (-RT, iScript No-RT).

Materials:

- PCR tubes, 0.2 ml
- Box with ice
- iScript™ Reverse Transcription Supermix
- iScript™ No-RT Supermix
- Nuclease-free water
- Thermal cycler

Method:

Table 3.13.5.1 Reaction set up for reverse transcription of RNA to cDNA.

Materials	Volume per reaction (µl)
iScript RT Supermix	4
RNA template (50 ng/µl)	2
Nuclease free water	14

The components (**table 3.13.5.1**) were mixed in 0.2 ml PCR tubes on ice and incubated in a thermal cycler under the following program (**table 3.13.5.2**).

Table 3.13.5.2 Reaction protocol for synthesis of RNA to cDNA.

Step	Temperature (°C)	Time (mm:ss)
Priming	25	05:00
Reverse transcription	46	20:00
RT inactivation	95	1

The resulting cDNA samples were stored at -20°C.

3.13.6 mRNA Expression Analysis by Reverse Transcription (RT) PCR

To evaluate gene expression of the genes *ef0361, ef0362, ef0114* and *ef2863* in different growth phases and in presence of serum, PCR was performed using primers for the genes of interest (**table 3.13.6.1**).

Table 3.13.6.1 Overview of the primers utilized in the mRNA expression analysis by reverse transcription of the genes *ef0361, ef0362, ef0114* and *ef2863*.

Gene of interest	Primer mix
ef0361	EF0361F and EF0361R
ef0362	EF0362F and EF0362R
ef0114	EF0114F and EF0114R
ef2863	EF2863F and EF2863R

The 5'-3' sequences are provided in **table 2.5.1**.

Materials:

- cDNA template
- Nuclease-free water
- 8-strip PCR tubes
- Red Taq DNA Polymerase Master Mix
- Forward and revers primers (10µM)
- Thermal cycler

Method:

The cDNA samples were diluted 10x by adding 5 µl cDNA to 45 µl nuclease free water in order to reach the final concentration of 2.5 ng/µl.

Table 3.13.6.2 Reaction set up for PCR amplification of the genes of interest.

Component	Volume per reaction (µl)
Red Taq DNA Polymerase Master Mix	12.5
Forward Primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5
cDNA template (2.5 ng/µl)	11.5

The components (**table 3.13.6.2**) were assembled in 8-strip PCR tubes, mixed, and the strips were then placed in a thermal cycler. The PCR was carried out following the program shown in **table 3.13.6.3**.

Table 3.13.6.3 PCR conditions for confirmation of gene expression.

Step	Number of cycles	Temperature (°C)	Time (mm:ss)
Initial Denaturation	1	95	02:00
Melting	30	95	00:30
Annealing	30	55	00:30
Elongation	30	72	00:30
Final Extension	1	72	07:00

The PCR-products were thereafter visualized on a 1.2 % agarose gel (**Section 3.4**).

3.13.7 Droplet Digital PCR

Droplet digital PCR (ddPCR) is a method where nucleic acid concentration can be determined quantitatively using gene specific primers. ddPCR is a digital PCR method that calculates the absolute concentration of a given sample without the need for external standard curves or endogenous controls, unlike other quantitative PCR methods such as Real-Time PCR (RT PCR). Any presence of PCR inhibitors will be diluted down, making this method very precise and sensitive.

The sample of interest is diluted and partitioned into many discrete fractions, specifically into 20 000 micellar droplets, so that each droplet ideally contains either zero or one (or maximum a few) copies of the template and so that each template molecule can be amplified individually. Thus, instead of one PCR per sample, there will be as many individual PCRs as there are droplets. Positive droplets contain the target sequence, whereas negative droplets lack the template and will not be detected. Detection is based on fluorescence and involves the non-specific dsDNA binding dye EvaGreen® (**figure 3.13.7.1**). EvaGreen® is an intercalating dye which is non-fluorescent in its free form, but as it binds to dsDNA the conformation changes to its active form and it emits fluorescence (Biotium, 2019).

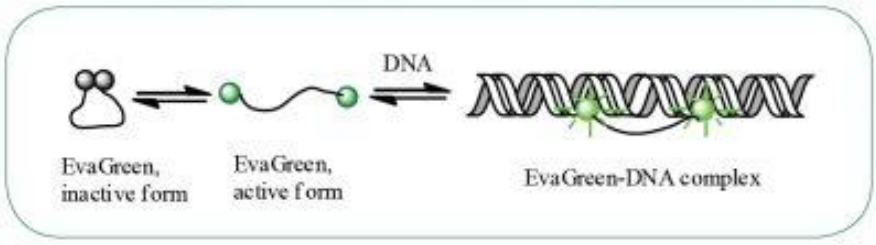

Figure 3.13.7.1 Mechanism of dsDNA binding dye EvaGreen®. In the absence of DNA, the dye holds a looped conformation that is inactive in DNA-binding. When DNA is available, the looped conformation of the dye opens to its active form, allowing emittance of fluorescence upon binding to dsDNA. Figure obtained from Biotium (2019).

The critical step in ddPCR is the sample partitioning prior to the amplification by PCR. The sample containing target molecules, gene specific primers and PCR mix is mixed with the droplet generator oil in a droplet generator device. Due to the hydrophobic properties of oil, the sample will disperse as it is mixed with the oil, resulting in equally nanolitre sized droplets that are uniform in both size and volume (**figure 3.13.7.2**). The templates in the droplets are then amplified by qualitative PCR and will bind to the dsDNA dye present in the reaction mix. Following amplification, each sample is then analysed by a droplet reader. The total number of droplets in the sample are counted, as well as the number of positive droplets generating fluorescent signals due to presence of template.

These numbers are used to calculate the absolute template concentrations, and these calculations rely on the statistical Poisson model. During droplet generation, the template molecules are distributed randomly in the droplets. Due to this random partitioning, the fluorescence data after amplification are well fit by a Poisson distribution. The Poisson distribution is used to determine the number of template molecules in a droplet, and by that the template concentrations in the original sample (Bio-Rad, 2019).

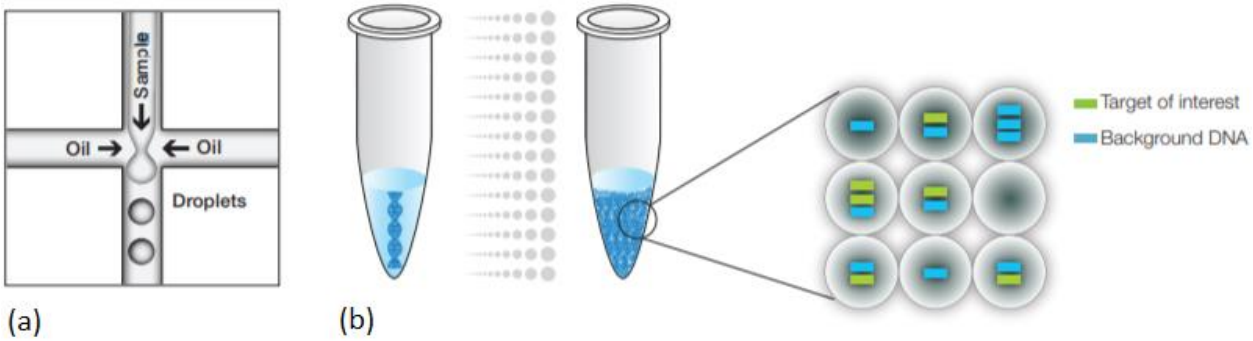

Figure 3.13.7.2 Droplet Digital PCR is a method based on water-oil emulsion droplet technology. (a) In the droplet generator, the sample is split up into 20 000 equally sized droplets based on the behaviour of water in oil. (b) For every single droplet of the sample, there will be an equal number of separate PCRs. Each droplet is counted individually and is scored as positive or negative based on the fluorescence detection. Figure obtained from Bio-Rad Laboratories (2019).

Materials:

- Template cDNA
- Nuclease-free water
- 8-strip PCR tubes
- 2x QX200 ddPCR EvaGreen Supermix
- Forward and reverse primers (2 µM)
- DG8™ cartridges
- Droplet generation oil for EvaGreen
- QX200 Droplet Generator
- Thermal cycler
- QX200 Droplet Reader
- QuantaSoft Software

Method:

1. Before setting up an assay, a test run should be performed to optimize the Poisson analysis. The test run should be performed with dilutions of the cDNA ranging from 10x to 100 000x to determine the dilution in which returns the most accurate analysis (ratio between positive and total amount of droplets, see https://www.bio-rad.com/en-no/applications-technologies/absolute-quantification-pcr-targets-with-droplet-digital-pcr-system?ID=MDV359ESH#poisson)
2. The cDNA samples generated through reverse transcription were diluted to the appropriate dilutions. In this study, all samples were diluted 10x and 100x. The assay was set up in triplicates, with three tubes per sample.
3. The EvaGreen® ddPCR mix was prepared as shown in **table 3.13.7.1** and twenty µl was transferred to each tube of the 8-strip PCR tube. When assembling the reaction mix, it is important to avoid making bubbles as this will inhibit the droplet generation in the droplet generator.

Table 3.13.7.1 Reaction set up for EvaGreen® ddPCR.

Component	Volume per reaction (µl)	Final concentration
2x QX200 ddPCR EvaGreen® Supermix	11	1x
2 µM Forward primer	1	100 nM
2µM Reverse primer	1	100 nM
RNase/DNase-free water	7	-
cDNA template	2	100 ng

4. Two µl of the cDNA templates were added to the 20 µl reaction mixes. Three tubes were kept as controls and instead 2 µl dH_2O were added to these.
5. Twenty µl of each sample reaction mixture was loaded into the sample wells of the DG8™ cartridge, followed by 70 µl of Droplet generation oil for EvaGreen® into the oil wells.
6. The DG8™ cartridge was placed in the QX200 Droplet Generator and the droplet generation started. When the droplets were made, 40 µl of the droplet-suspensions were transferred to individual wells in a 96-well PCR-plate. The droplet-suspensions were pipetted slowly when transferred to the PCR-plate to avoid breaking the droplets.
7. The PCR-plate was sealed with foil by the PX1 plate sealer.
8. The sealed 96-well PCR-plate was loaded onto a thermal cycler and PCR was carried out following the program in **table 3.13.7.2**.

Table 3.13.7.2. Reaction protocol for EvaGreen ddPCR.

Step	Number of cycles	Temperature (°C)	Time (mm:ss)
Enzyme activation	1	95	01:00
Denaturation	40	95	00:30
Annealing/extension	40	60	01:00
Signal stabilization	1	4	05:00
	1	90	05:00
Hold	1	4	Infinite

9. The PCR-plate was loaded onto the QX200 Droplet Reader for analysis of the droplets.
10. The results were analysed using the Quantasoft Software. The QuanaSoft Software reports the concentrations as copies/µl, where copies refer to the target molecule. In this study, 2 µl were used as template for the PCR and the total volume loaded into the wells

of the DG8™ Droplet Generator Cartridge was 20 µl. Copies/20 µl are therefore divided by two to obtain copies/µl. This number is then multiplied by the dilution factor, which was 10x or 100x in the present study (10x was the dilution that resulted in the most accurate analysis). As 100 ng RNA was reverse transcribed into cDNA and was used as template in this study, the final formula for calculating copies/ng RNA is:

$$\frac{\frac{Copies}{\mu l} * Dilution\ factor}{Amount\ of\ revers\ transcripted\ RNA\ (ng)} = \frac{\frac{Copies}{\mu l} * 10}{100\ ng} = \frac{Copies}{ng\ RNA}$$

3.14 Whole Blood Survival Assay

Survival in blood was analysed for all four *E. faecalis* strains (see **table 2.4.1**) using an *ex vivo* whole human blood model. The assays were performed using bacteria either in stationary phase or exponential phase of growth, to ensure the expression of the genes of interest. The assay was set up in duplicates but repeated at least three times for each bacterial growth phase using blood from different healthy donors. The blood donors were independent, representing both genders and different age groups. Hirudin was used as an anticoagulant to preserve the complement activity.

Materials:

- Sarstedt tubes, 13 ml
- BHI-medium
- Overnight cultures of WT, ΔChi, ΔLpmO and ΔDeglycosidase
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R
- 1X PBS
- RPMI 1640-medium supplemented with 0.5% HSA (RPMI/HSA)
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- Box with ice
- Protein LoBind tubes, 2.0 ml
- Microcentrifuge, Mini Star
- Incubator, Minitron, 37°C
- Programmable Rotator, Multi RS-60

- 0.3% Saponin in MQ
- Vortex
- Microtiter plate
- BHI-plates
- Incubator, Termaks, 37°C

Equipment for blood drawing:

- Eclipse™ Needle
- Hirudin Blood tube, 3 ml
- S-Monovette®-Needle 21Gx1
- S-Monovette® 1.6 ml Hirudin

Method:

1. The bacterial strains were grown overnight in culture tubes containing 10 ml BHI, at 37°C with agitation (230 rpm).
2. The following day, the bacteria were re-grown to exponential phase by diluting the overnight culture to approximately 1:100 through addition of 200 µl overnight culture bacteria to 10 ml preheated BHI. The bacteria were grown until OD 0.6-0.7 (approximately 2 hours) to reach mid exponential phase. Overnight culture of bacteria was used directly when the assay should be performed using bacteria at stationary phase of growth.
3. The bacteria in stationary phase and exponential phase of growth were centrifuged at 4255xg for 15 minutes at 24°C and were washed two times with 1X PBS. After the last centrifugation, the bacterial pellets were resuspended in 1 ml RPMI supplemented with 0.05% human serum albumin (RPMI/HSA).
4. Thereafter, the bacteria were re-diluted in another set of tubes containing 2 ml RPMI/HSA until reaching to OD_{600nm} 0.4.
5. The blood assays were performed using different numbers of bacteria. When required, the bacterial stock was diluted further (10x or 100x in RPMI/HSA). The bacteria were kept on ice.
6. Freshly isolated human blood obtained from healthy individuals was used in the assays.
7. The assay was performed in siliconized protein low binding tubes, to abolish possibility of bacterial binding to the plastic. The tubes were prepared as follows;
    1. Addition of 20 µl buffer (RPMI/HSA)

2. Addition of 20 µl bacteria

   Upon addition of buffer and bacteria, the tubes were centrifuged using a microcentrifuge for one second.

3. Addition of 160 µl fresh blood. Wide orifice pipet tips were used for addition of blood in order to reduce the chance of mechanical disturbance.

8. The tubes were spun down for one second again and were then incubated at 37°C on a rotator to ensure free movement of the assay components. The program of the rotator is described in **table 3.14.1.**

Table 3.14.1 Settings of the programmable rotator used for incubation of the whole blood assay.

Orbital (rpm)	Reciprocal (deg)	Vibro/Pause (deg)
5	73	2

9. The sample tubes were collected one- and three-hours post inoculation, spun down for one second and kept in ice.
10. 800 µl of ice cold milli-Q supplemented with 0.3% saponin was then added to the samples in order to lyse the immune cells and release any bacteria that might have survived inside the immune cells. Ice cold milli-Q water alone is not efficient enough to lyse immune cells, but supplementation of 0.3% saponin facilitates an efficient lysis. The tubes were vortexed on full speed for five seconds to further lyse all cells, and were kept on ice for five minutes, before being vortexed for five seconds once again.
11. After pipetting the sample solution up and down, serial dilutions (10^0 to 10^{-4}) were made in microtiter plates in 1X PBS for plating on BHI plates. Plating was performed in accordance with "multi-dilution plating"-method described in **section 3.8.2.1**. The plates were incubated at 37°C overnight.
    1. To calculate the exact number of the bacteria added to the assay and later use them to calculate percentage of viable bacteria after passing through the blood, another set of tubes were prepared as follows, which referred as control tubes:
        1. Addition of 180 µl buffer (RPMI/HSA)
        2. Addition of 20 µl bacteria (with 10x the concentration needed)
        3. Addition of 800 µl ice cold MQ supplemented with 0.3% saponin (to make equivalent dilution of both blood-treated and non-blood treated bacteria)
    2. The components were mixed well and were immediately serial diluted and plated.

3. The serial dilutions made ranged from 10^0 to 10^{-6} and were prepared in 1X PBS in microtiter plates. The bacteria were plated on BHI plates by "multi-dilution plating" (see **section 3.8.2.1**) and the plates were incubated overnight at 37°C.

12. The colonies were enumerated the next day. The percentages of survival relative to the inoculum were calculated using the following formula:

$$\% \text{ of survival relative to the inoculum} = \frac{\frac{CFU}{ml} of\ blood\ tube}{\frac{CFU}{ml} of\ control\ tube} * 100$$

Ethical aspects

Fresh human blood analysis will be carried out in accordance with the ethical principles of the Helsinki Declaration and approval of Regional Ethic Committee (REK) (REK project number REK 2018-1586). A written informed consent will be provided to blood donors and the protection of confidentiality and privacy will be secured for human samples.

3.15 Serum assay

Bacterial viability in normal human serum was analysed for the wild type and ΔLpmO. Normal human serum (nhs) was derived from whole blood from healthy human volunteers (n>5) and processed to serum by pooling the liquid portion of coagulated whole blood. The assay was set up in triplicates. No-serum controls were included, as well as heat-inactivated (HI) serum (incubated 30 minutes at 56°C) as an additional control. In addition to this, the assay was performed for one hour and three hours post inoculation.

Materials:

- Sarstedt tubes, 13 ml
- BHI-medium
- Overnight cultures of the wild type and ΔLpmO
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R
- 1X PBS
- RPMI 1640-medium supplemented with 0.5% HSA (RPMI/HSA)
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- Box with ice
- Protein LoBind tubes, 2.0 ml

- Pooled normal human serum (nhs)
- Water bath, 56°C
- Microcentrifuge, Mini Star
- Incubator, Minitron, 37°C
- Programmable Rotator, Multi RS-60
- Microtiter plate
- BHI-plates
- Incubator, Termaks, 37°C

Method:

1. The *E. faecalis* wild type and ΔLpmO strains were grown overnight in cultures tubes with 10 ml BHI at 37°C with agitation (230 rpm).
2. The assay was performed with bacteria at stationary phase of growth. The following day, the overnight cultures were therefore centrifuged at 4255xg for 15 minutes at 24°C and were washed two times in 1X PBS. After the last centrifugation, the bacterial pellets were resuspended in 1 ml RPMI supplemented with 0.05% HSA (RPMI/HSA).
3. Thereafter, the bacteria were re-diluted in another set of tubes containing 2 ml RPMI/HSA until reaching to OD_{600nm} 0.4. The bacteria were then diluted 2x in RPMI/HSA and kept on ice until further use.
4. To inactivate the pooled normal human serum (nhs), it was incubated at 56°C in a water bath for 30 minutes and thereafter kept on ice until further use.
5. The assay was performed in siliconized protein low binding tubes to abolish possibility of bacterial binding to the plastic. The tubes were prepared as follows:
    1. Addition of 160 µl buffer (RPMI/HSA)
    2. Addition of 20 µl bacteria

    Upon addition of buffer and bacteria, the tubes were centrifuged using a microcentrifuge for one second.

    3. Addition of 20 µl nhs or HI nhs

    The tubes were spun down for one second again and were then incubated at 37°C on a rotator to ensure free moving of the assay components. The program of the rotator is described in **table 3.14.1.**
6. The sample tubes were collected one- and three-hours post inoculation and were spun down for one second to gather the content.

7. After pipetting up and down, the serial dilutions (10^0 to 10^{-5}) were made in microtiter plates in 1X PBS for plating on BHI plates. Plating was performed in accordance with the "multi-dilution plating"-method described in **section 3.8.2.1.** The plates were incubated at 37°C overnight.
    1. To calculate the exact number of bacteria added to the assay and later use them to calculate percentages of viable bacteria after passing through serum, another set of tubes were prepared as follows, which referred as control tubes:
        1. Addition of 180 µl buffer (RPMI/HSA)
        2. Addition of 20 µl bacteria
    2. The components were mixed well and were immediately serial diluted and plated. The serial dilutions made ranged from 10^{-1} to 10^{-6} and were prepared in 1X PBS in microtiter plates. The bacteria were plated on BHI plates by "multi-dilution plating" (see **section 3.8.2.1**) and the plates were incubated overnight at 37°C.
8. The colonies were enumerated the next day. The percentages of survival relative to the inoculum were calculated using the following formula:

$$\% \text{ of survival relative to the inoculum} = \frac{\frac{CFU}{ml} of\ nhs\ tube\ or\ HI\ serum\ tube}{\frac{CFU}{ml} of\ control\ tube} * 100$$

4 Results

4.1 Verification of bacterial gene knockout strains

The *E. faecalis* gene knockout strains included in the present study had previously been constructed by Dr. Ingrid Lea Karlskås as part of her doctoral thesis (Karlskås, 2014) and were readily provided for this study. The double knockout strain ΔChi-ΔLpmO was however not successfully constructed by Dr. Karlskås, but was later constructed and verified through sequencing by Dr. Zhian Salehian and Dr. Geir Mathiesen.

To confirm the genomic deletion in the *E. faecalis* variants (ΔChi-ΔLpmO, ΔChi and ΔLpmO), DNA isolated from the mutants were analysed using PCR with specific primers targeting internal or external regions of the deleted genes. Using primer set 0362_SekF and 0361_SekR, the expected size of the obtained fragments from WT, ΔChi-ΔLpmO, ΔChi and ΔLpmO were estimated to 1926, 504, 1284 and 1443 bp, respectively (see **figure 2.5.1** for more information on the confirmation strategy). The PCR products of the amplified regions were analysed using 1.2% agarose gel electrophoresis, which confirmed the absence of the deleted genes (**figure 4.1.1**). The sizes of the obtained bands were compared with the Quick-Load® Purple 1 kb Plus DNA ladder. Genomic DNA obtained from the wild type strain (WT) was included as a control.

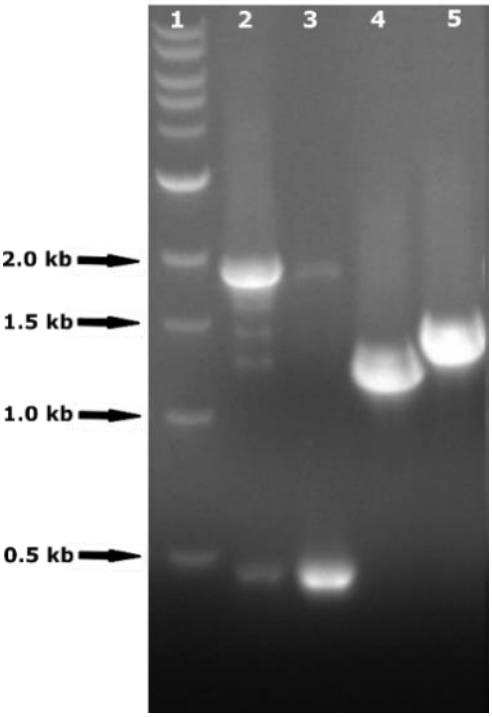

Figure 4.1.1 Verification of *E. faecalis* genomic deletions including the wild type, ΔChi-ΔLpmO, ΔChi and ΔLpmO. Lane 1 shows a 1 kb ladder; lane 2 contains the wild type; lane 3 contains the ΔChi-ΔLpmO; lane 4 contains the ΔChi; lane 5 contains the ΔLpmO. Expected PCR-fragment sizes for the respective deletion mutants are 1926, 504, 1284 and 1443 bp.

It should be noted that an extra band of around 2000 bp was obtained for the ΔChi-ΔLpmO sample (**figure 4.1.1**, lane 3), which corresponds to the expected size of the wild type (**figure 4.1.1**, lane 2).

Due to inconclusive results for whether the two genes of ΔChi-ΔLpmO were correctly knocked out, additional control PCRs were carried out with different primer pairs (**figure 4.1.2**).

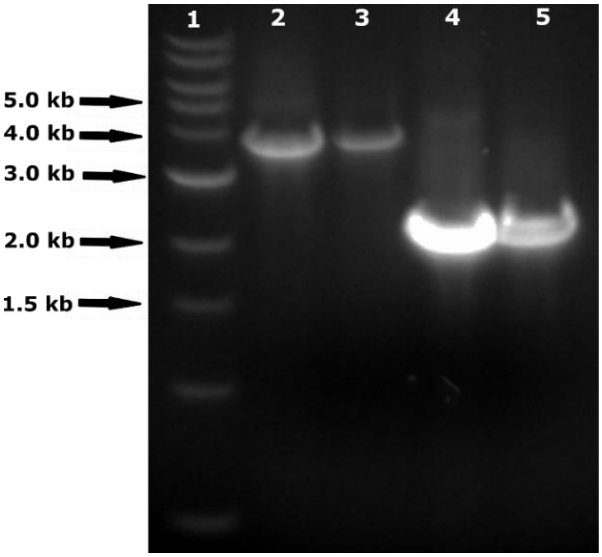

Figure 4.1.2 Verification of *E. faecalis* genomic deletions including the wild type and ΔChi-ΔLpmO. Lane 1 shows a 1 kb ladder; lane 2 contains the wild type generated by primer pair PCR_UP0362_F and PCR_Down0361_R; lane 3 contains the ΔChi-ΔLpmO generated by primer pair PCR_UP0362_F and PCR_Down0361_R; lane 4 contains the wild type generated by primer pair 0362_2F and PCR_Down0361_R; lane 5 contains the ΔChi-ΔLpmO generated by primer pair 0362_2F and PCR_Down0361_R. Expected PCR-fragment sizes are 3735, 2313, 2199 bp and no product expected.

Using primer set PCR_UP0362_F and PCR_Down0361_R, PCR products were obtained with sizes ~3.7 kb for the wild type (**figure 4.1.2**, lane 2; expected size 3735 bp) and ~3.8 kb for ΔChi-ΔLpmO (**figure 4.1.2**, lane 3; expected size 2313 bp). Using a second set of control primers, 0362_2F and PCR_Down0361_R, the sizes obtained for the PCR products were ~2.1 kb for the wild type (**figure 4.1.2**, lane 4; expected size 2199 bp) and ~2.2 kb for ΔChi-ΔLpmO (**figure 4.1.2**, lane 5; no product expected).

Based on the PCR-based gene deletion verification strategy presented above, it is clear that the ΔChi-ΔLpmO deletion strain is incorrect. Due to the unexpected sizes of the PCR products, whole genome sequencing of the strain is planned (described in **Appendix B**), but at the time of writing, not yet performed. Thus, no further analysis of the ΔChi-ΔLpmO strain was performed.

In addition to the gene deletion strains mentioned so far, genomic deletions of ΔDeglycosidase were also verified through PCR and subsequent analysis using 1.2% agarose gel electrophoresis. Two sets of specific primer pairs were used to target external regions of the deleted genes *ef0114* and *ef2863*. Using primer set Sek_0114F and Sek_0114R, the expected size of the obtained fragments from WT and ΔDeglycosidase were estimated to 2620 and 240 bp, respectively. The second primer set, Sek_2863F and Sek_2863R, were expected to yield fragments of the sizes 1172 bp for the WT and 424 bp for ΔDeglycosidase (see **figure 2.5.2** and **figure 2.5.3** for more information on the confirmation strategy). The PCR products of the amplified regions confirmed the absence of the deleted genes (**figure 4.1.3**). The sizes of the obtained bands were compared with the Quick-Load® Purple 1 kb Plus DNA ladder and the Quick-Load® 100 bp DNA ladder. Genomic DNA obtained from the wild type strain (WT) was included as a control.

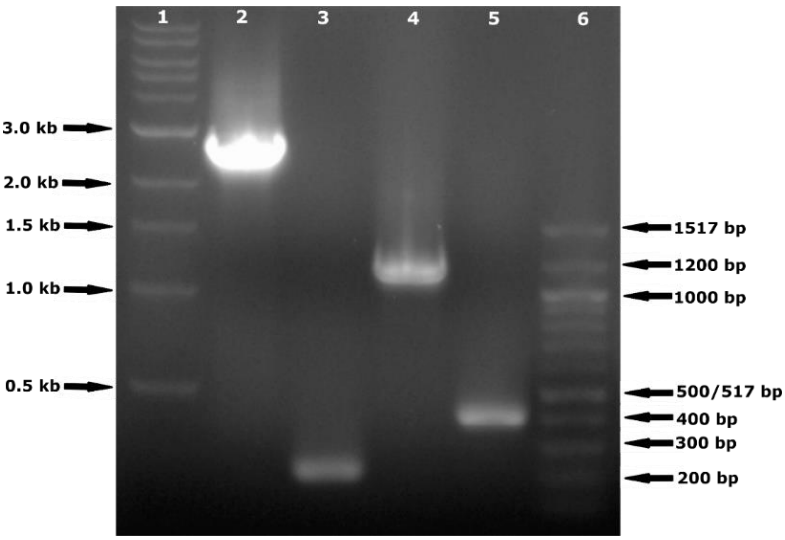

Figure 4.1.3 Verification of *E. faecalis* genomic deletions including the wild type and ΔDeglycosidase. Lane 1 contains a 1 kb ladder; lane 2 contains the wild type and lane 3 contains the ΔDeglycosidase, both generated by primer pair Sek0114F and Sek0114R; lane 4 contains the wild type and lane 5 contains the ΔDeglycosidase, both generated by primer pair Sek2863F and Sek2863R; lane 6 shows a 100 bp ladder. Expected PCR-fragment sizes are 2620, 240, 1172 and 424 bp.

To further verify accurate deletion of *ef0114* and *ef2863*, the obtained fragments of the ΔDeglycosidase were excised from the gel and purified for sequencing. The sequencing results confirmed a correct knockout of the genes *ef0114* and *ef2863* (results not shown). Verification by sequencing was only conducted on the ΔDeglycosidase, as it had previously been performed on the other knockout strains.

4.2 Characterization of the bacterial strains

Once the gene knockout strains were confirmed correct, the wild type and deletion strains were characterized by microscopy, growth abilities on different substrates, and their enzymatic chitinase and N-acetylhexosaminidase activity.

4.2.1 Light microscopy

The morphology of the wild type and the gene deletion strains was analysed using light microscopy (**figure 4.2.1.1)**.

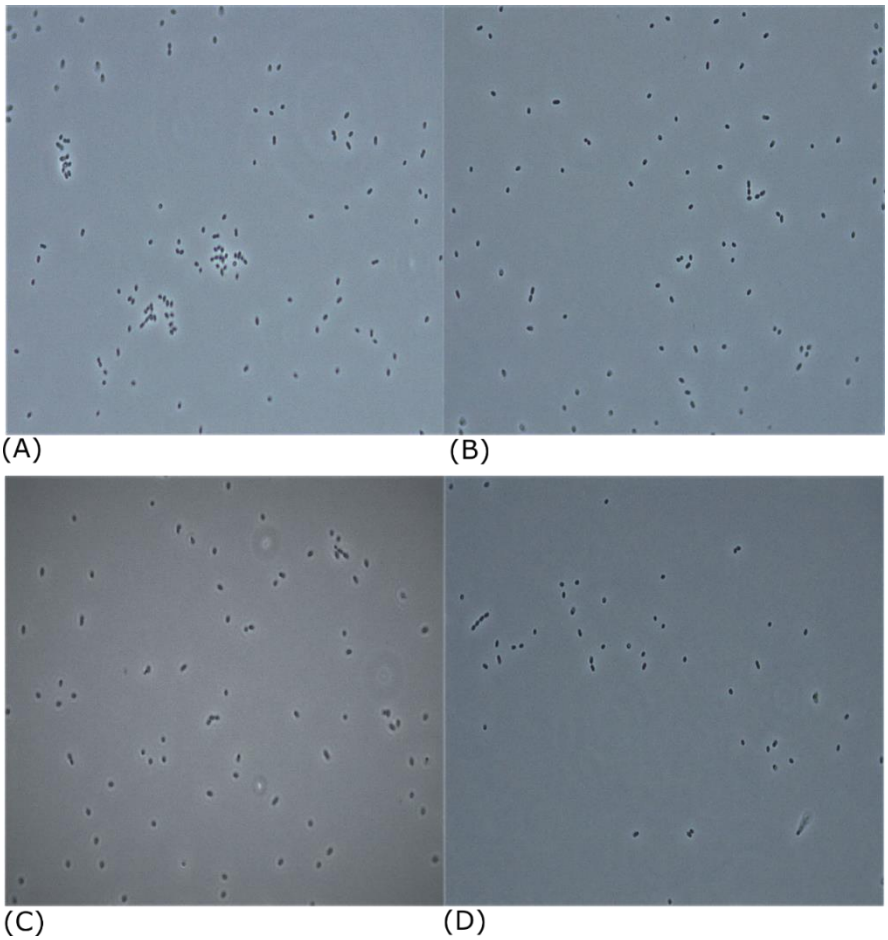

Figure 4.2.1.1 Light microscopy of the wild type (A), ΔChi (B), ΔLpmO (C) and ΔDeglycosidase (D). The morphology of all four strains is visualized in the figure at 100x magnification.

All strains showed the same typical ovoid, cocci-like form of Enterococci, mostly as single cells. There are no apparent differences in morphology between the wild type and the knockout strains.

4.2.2 Growth curves

4.2.2.1 Soluble substrates

The wild type and gene deletion strains were grown in five different soluble substrates in microtiter plates, and the growth was monitored by absorbance measurements every 15 minutes.

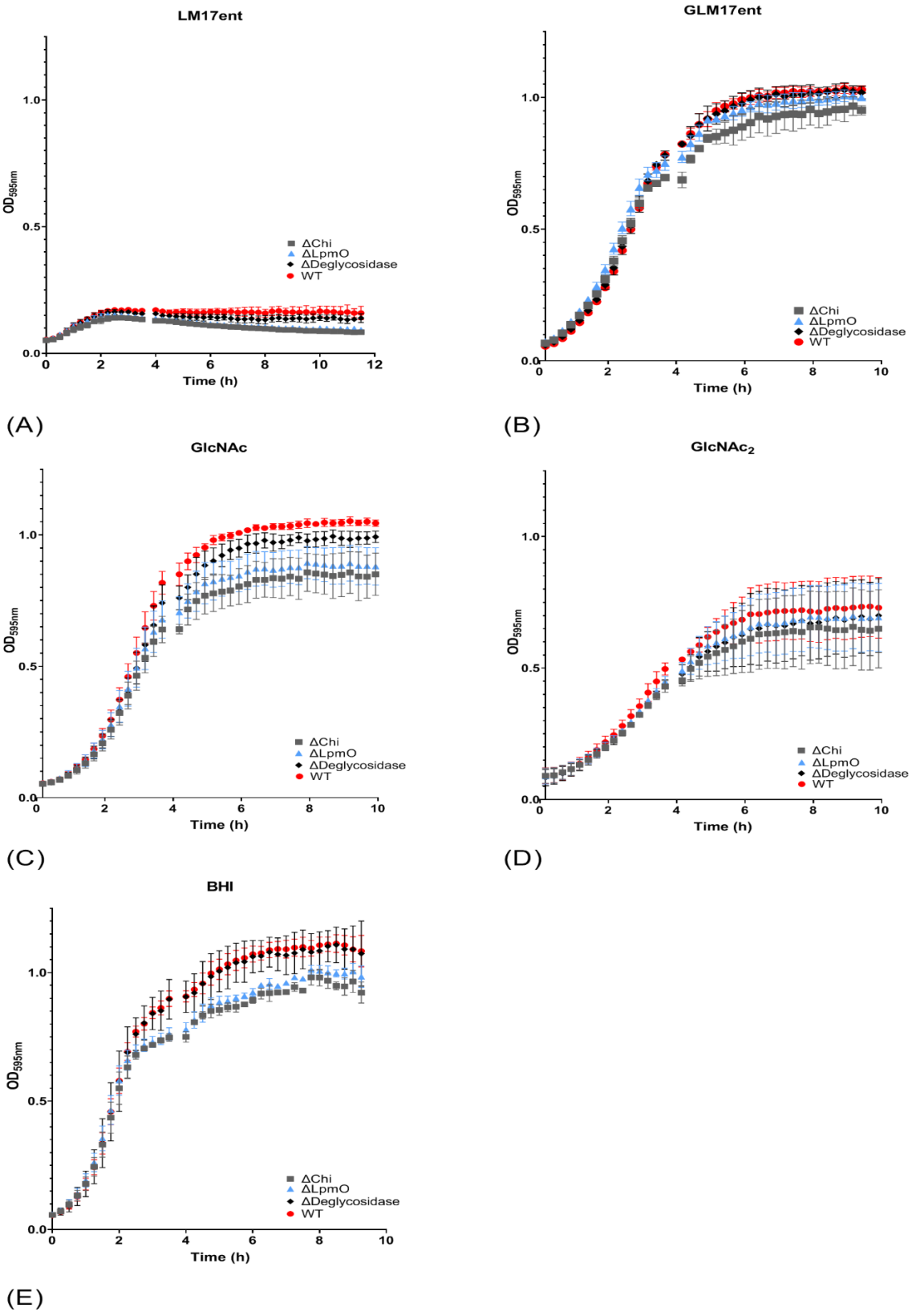

Figure 4.2.2.1.1 Growth curves for ΔChi, ΔLpmO, ΔChi-ΔLpmO, ΔDeglycosidase and the wild type on the soluble substrates LM17ent (A), GLM17ent (B), LM17ent supplemented with 0.4% GlcNAc (C), LM17ent supplemented with 0.4% $GlcNAc_2$ (D) and BHI (E). The growth is monitored by absorbance measurements at OD_{595nm} with three biological replicates and two technical replicates. Standard errors of the mean are included as error bars in the figure.

Figure 4.2.2.1.1 shows the growth rates of the four bacterial strains on each of the five medias tested. The minimal medium LM17ent (**figure 4.2.2.1.1**, panel A) served as a control since it does not contain any additional carbon source. All the bacterial strains demonstrated minimal growth on this substrate, although the wild type showed a slightly higher growth rate than the other strains. Stationary phases of growth were reached only after about two hours, compared to nearly six hours for the other medias tested.

As expected, all the strains showed the highest growth rate when cultivated in BHI (**figure 4.2.2.1.1**, panel E), which served as a positive control for the growth assay. The wild type and ΔDeglycosidase demonstrated the highest growth rates and seemed to grow equally well in BHI, while ΔChi and ΔLpmO reached slightly lower cell densities.

The bacterial strains also exhibited high growth rates on LM17ent supplemented with 0.4% glucose (**figure 4.2.2.1.1**, panel B; GLM17ent) and LM17ent supplemented with 0.4% GlcNAc (**figure 4.2.2.1.1**, panel C; GlcNAc). Interestingly, an observable trend in both medias was the demonstration of highest growth rate of the wild type, while the ΔChi-strain reached the lowest cell density. All four bacterial strains showed similar growth rates when grown in GLM17ent, although ΔChi reached slightly lower OD_{595nm} measurements compared to the other strains. When grown in GlcNAc however, greater differences in growth rates were demonstrated. The wild type showed the highest growth rate, while ΔChi exhibited the slowest rate.

Generally, the bacterial strains showed slightly slower growth on LM17ent supplemented with 0.4% $GlcNAc_2$ (**figure 4.2.2.1.1**, panel D; $GlcNAc_2$), compared to BHI, GLM17ent and GlcNAc. The wild type reached an OD_{595nm} of 0.7 in $GlcNAc_2$, in comparison to values around 1.0 in the other three medias. The same growth tendencies seen with the other medias tested were also present here, as the wild type demonstrated the highest growth rate and ΔChi showed the lowest growth rate of the bacterial strains – albeit the differences are rather small.

In summary, the growth assay performed with the different soluble substrates generally showed small growth differences of the bacterial strains, regardless of the gene-deletions.

4.2.2.2 β-chitin

Growth on LM17ent supplemented with 1% β-chitin was monitored for all the bacterial strains through absorbance measurements, as well as multi-dilution plating followed by calculations of CFU/ml. The growth assays were difficult to perform, as extensive optimizations of the assay were needed in order to receive reliable results. As absorbance measurements was one of the methods used to follow the growth, the β-chitin particles were required to sink to the bottom of

the flasks to prevent interference with the measurements. The time required for the absorbance measurements to stabilize was determined to be minimum 10 minutes (data shown in **Appendix C**). The results presented are therefore those assays performed with a sinking time of 10 minutes.

Initially, the bacteria were incubated with agitation (140 rpm) and the assay was performed in three consecutive parallels. Due to different time readings of the parallels, the results of each parallel must be presented separately as mean values cannot be calculated. The absorbance measurements and calculated CFU/ml from one of the parallels are presented in **figure 4.2.2.2.1.**

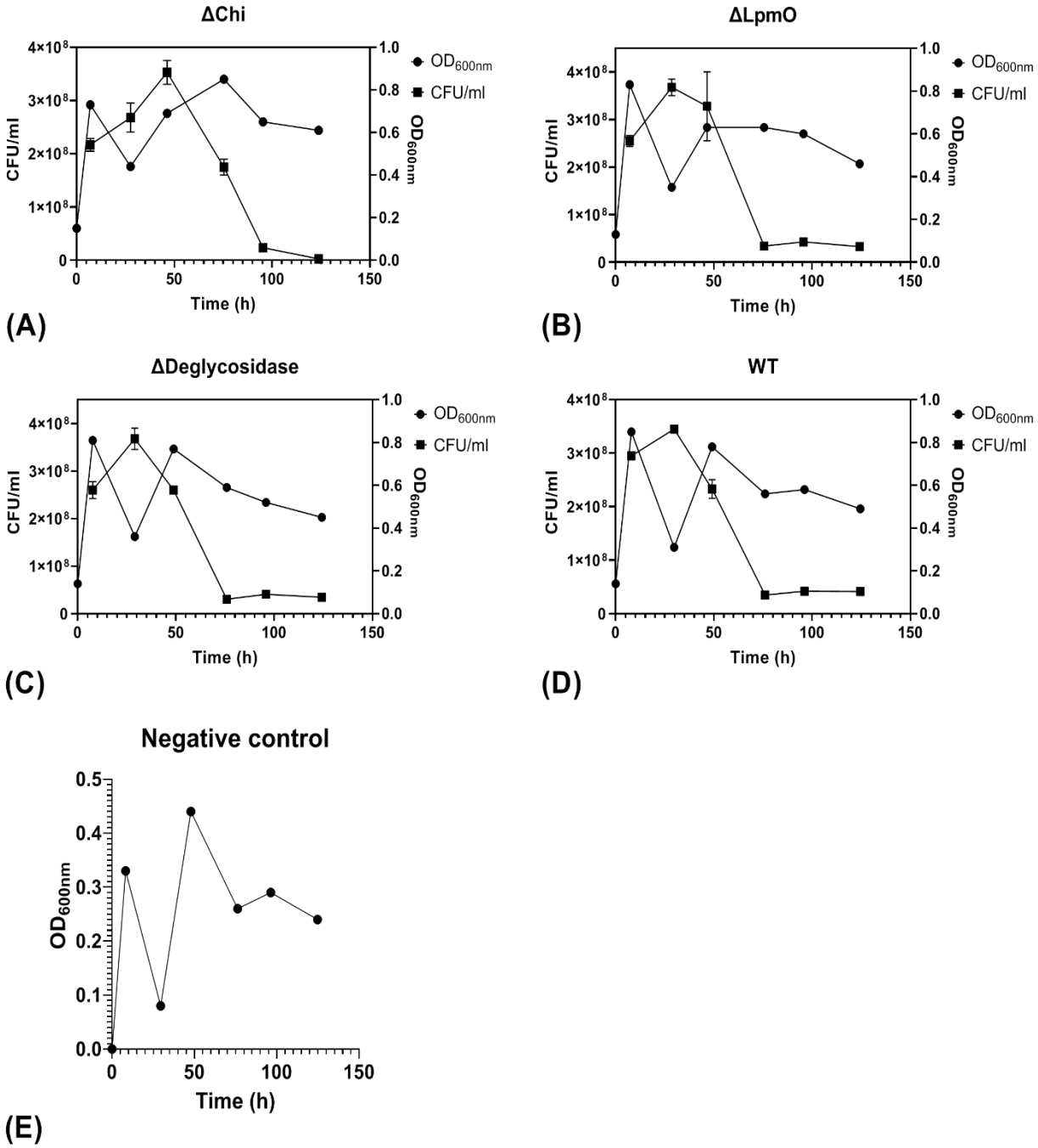

Figure 4.2.2.2.1 Growth of *E. faecalis* strains in 1% β-chitin with agitation. Growth was analysed by measurements of OD_{600nm} and CFU/ml of ΔChi (A), ΔLpmO (B), ΔDeglycosidase (C), the wild type (D), and the negative control containing 1% β-chitin medium only (E), when grown on 1% β-chitin over a period of up to 146 hours. The figure presents the third biological parallel of the growth-assay and the calculated CFU/ml are based on three technical replicates.

The absorbance measurements vary considerably (**figure 4.2.2.2.1**), even though the chitin in the bacterial cultures have been allowed to sediment for 10 minutes prior to sample-harvesting. The absorbance measurements performed on the negative control (**figure 4.2.2.2.1**, panel E) are highly unstable and could indicate presence of bacteria. Growth in the negative control was

however controlled by plating, which revealed no bacterial growth throughout the whole incubation period. As a result, the absorbance measurements have been excluded from the rest of the results. The number of colony-forming units per millilitre will however be presented as the measurement of growth on the substrate. The CFU/ml increased during the first 30-40 hours, but then decreased in all the bacterial cultures (**figure 4.2.2.2.1**), suggesting poor growth on β-chitin.

There are great variations between the three biological parallels of the growth assay (**figure 4.2.2.2.2**). Therefore, comparison of the parallels is not feasible, as reproducibility is not achieved. However, the common trend is decreasing bacterial numbers and generally poor growth on β-chitin. Standard errors of the means are shown as error bars and clearly reflect the instability of the growth assay.

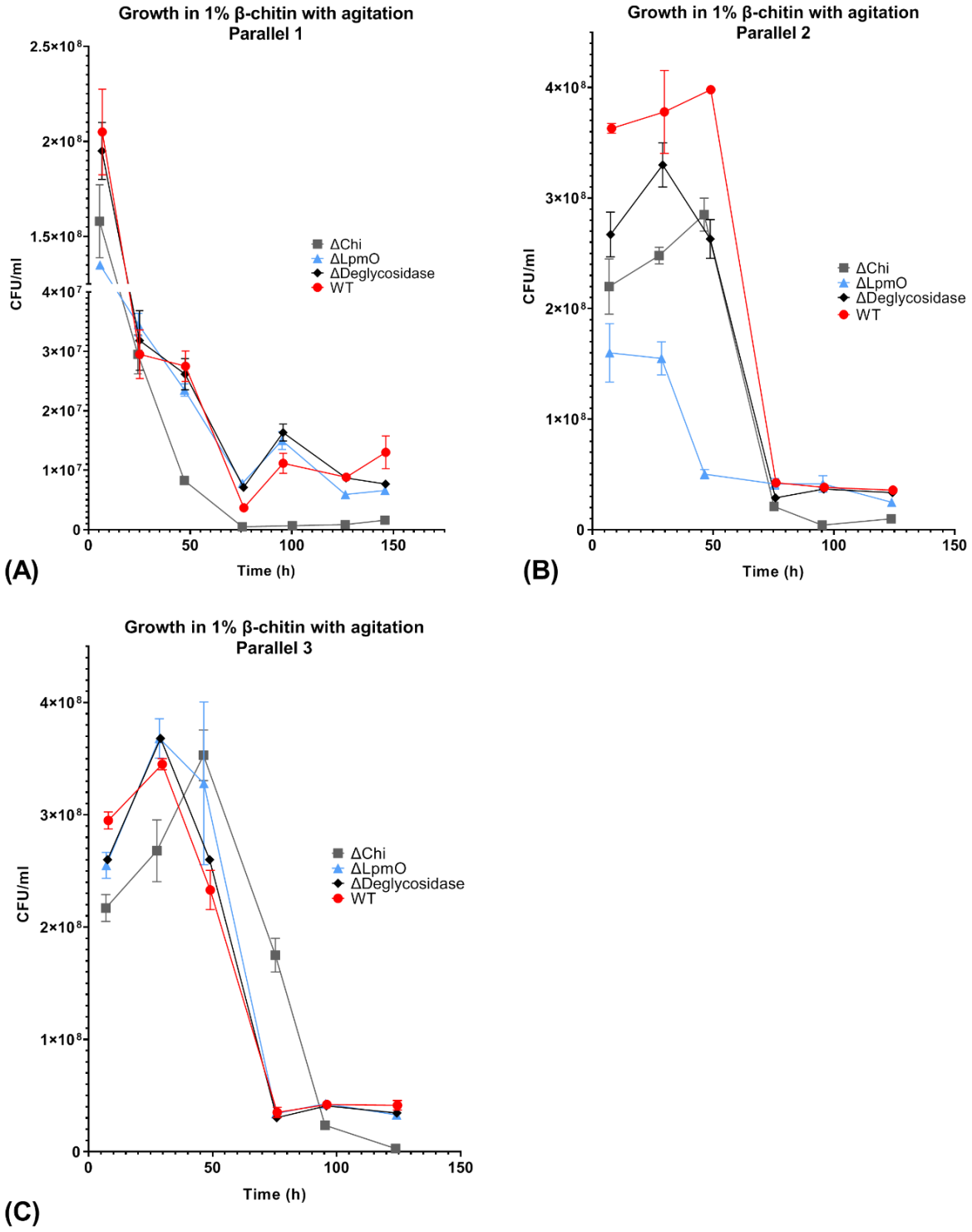

Figure 4.2.2.2.2 Growth of *E. faecalis* strains in 1% β-chitin with agitation. The growth was followed by plating and calculations of CFU/ml over a period of up to 146 hours. The growth assay was performed in three parallels; parallel 1 (A), parallel 2 (B) and parallel 3 (C). Thus, the assay was performed in three biological replicates, with two technical replicates. Standard errors of the mean are included as error bars in the figure.

Notably, the wild type seems to reach the highest bacterial numbers when grown in β-chitin, whereas ΔChi demonstrates the fastest decline in bacterial number in all the parallels (**figure 4.2.2.2.2**).

Following the growth assays performed with agitation, new assays were set up with static incubation of the strains in the insoluble substrate β-chitin. When the bacterial strains were grown statically (**figure 4.2.2.2.3)**, they demonstrated increasing numbers of CFU/ml over the first 70 hours. Compared to the results from growth with agitation, static growth conditions seem to better allow for cultivation of the bacterial strains. Towards the end of the growth assay, the bacterial numbers eventually declined, most likely due to oversaturation of bacteria and lack of nutrients in the flasks.

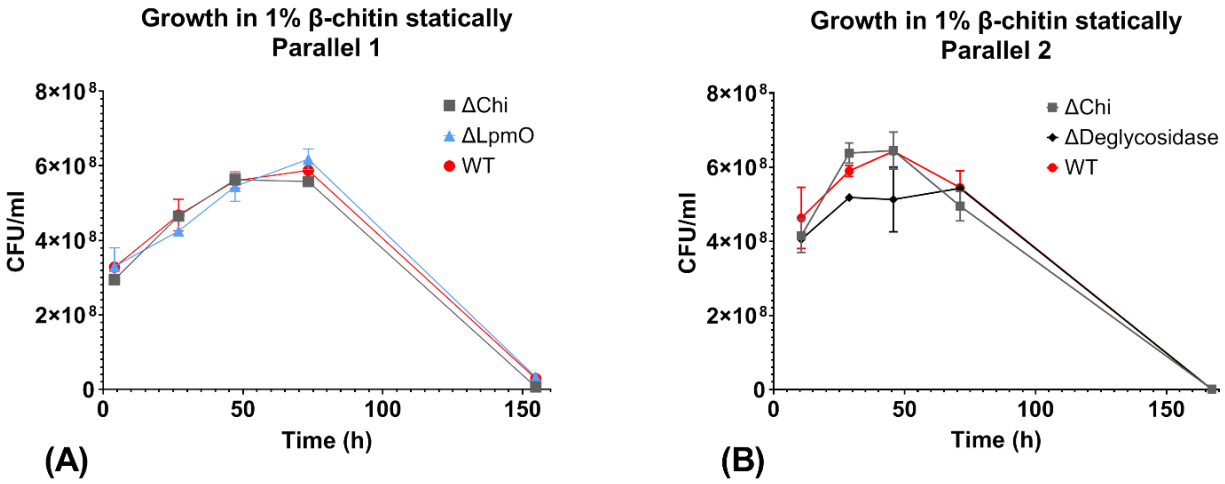

Figure 4.2.2.2.3 Growth curves of ΔChi, ΔLpmO, ΔDeglycosidase and the wild type, when grown statically on 1% β-chitin over a period of up to 170 hours. The growth was followed by plating and calculations of CFU/ml. The growth assay was performed in two parallels; parallel 1 (A) and parallel 2 (B).

4.2.3 Enzyme activity

Chitinase- and N-acetylhexosaminidase activity was assessed in culture supernatants of all the bacterial strains, using the $GlcNAc_3$ analogue 4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside (4-MU-$GLcNAc_2$) and the $GlcNAc_2$ analogue 4-methylumbelliferyl-β-D-N-acetylglucosamine (4-MU-GLcNAc) as substrates. The bacteria were cultivated in minimal media supplemented with 1% β-chitin over a period of seven days and samples were collected every day for analysis.

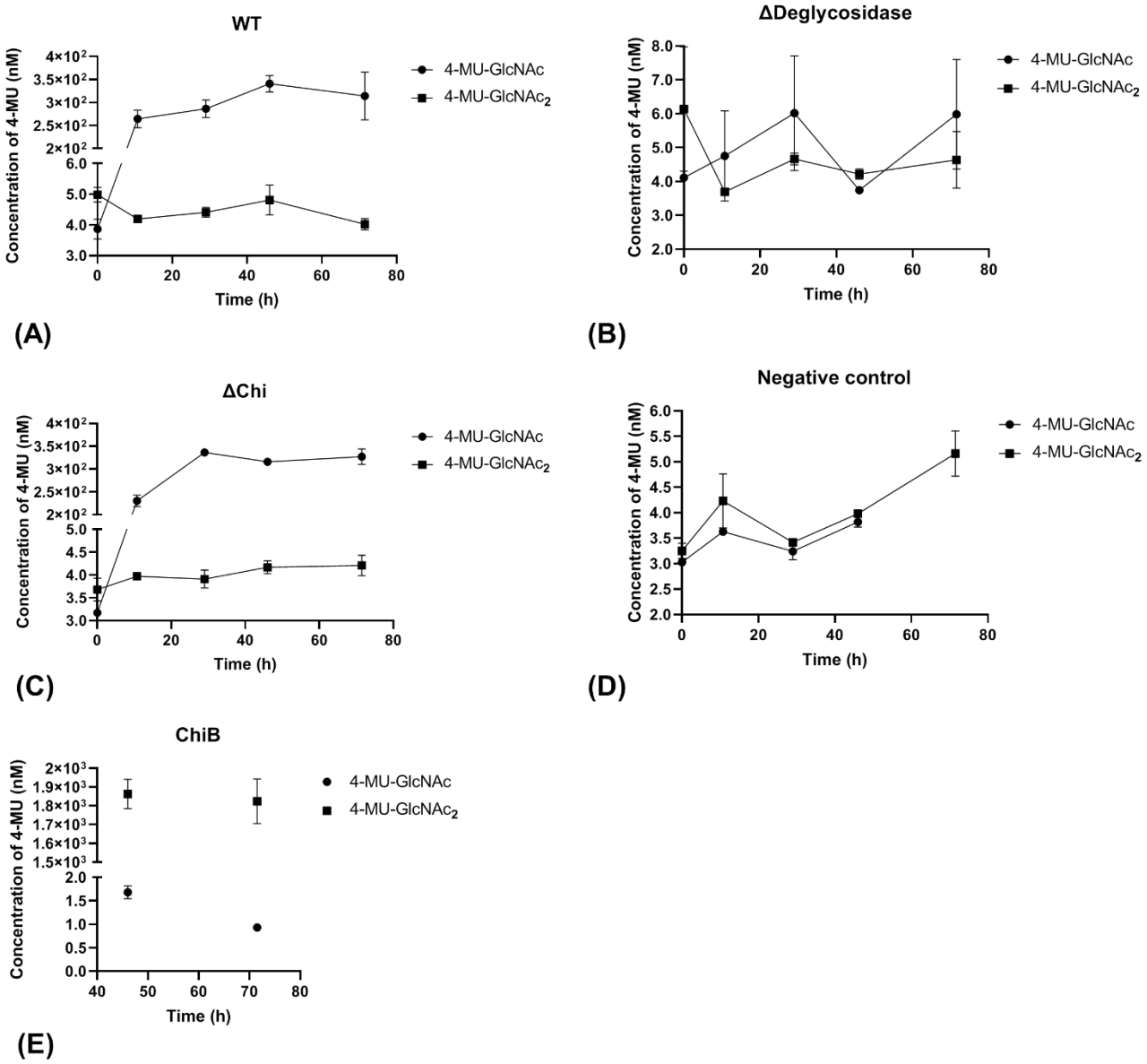

Figure 4.2.3.1 4-MU enzyme activity assay for analysis of chitinase- and N-acetylhexosaminidase activity in culture supernatants of the wild type (A), ΔDeglycosidase (B) and ΔChi (C), as well as in the negative control with media only (D) and the positive/negative control ChiB (E). Chitinase activity is represented by 4-MU-$GlcNAc_2$ and N-acetylhexosaminidase activity by 4-MU-GlcNAc. Enzymatic activity will result in cleavage of these substrates and subsequent release of the fluorophore 4-MU, hence the concentration of 4-MU serves as a quantitation of the enzymatic activity. The assay was performed at 37°C and pH 6, with substrate concentrations of 50 µM. The data presented are based on three technical replicates.

The results presented in **figure 4.2.3.1** are based on mean values calculated from three technical replicates of the assays, with the standard error of the mean presented as error bars. One value was removed from the data, as it deviated from the two other technical parallels and was considered an outlier. The data excluded was the measurement of 4-MU-GlcNAc activity 5.83 at the time point 71.5 h for ChiB.

The chitinase activity of all the bacterial strains, as well as the negative control, are approximately equal to zero (**figure 4.2.3.1**)**.** Statistical analysis (t-test with $p < 0.05$) reveals that there is no statistical significance in chitinase activity between any of the bacterial strains at the two last time points (46 h and 71.5 h). Thus, there is no statistically significant difference between the wild type and the ΔChi (46 h, p=0.2758; 71.5 h, p=0.5482), suggesting that *Ef*Chi18A is indeed not actively secreted in the wild type. Furthermore, the chitinase activity of the positive control, ChiB, is statistically significantly higher than of the wild type at both time points.

N-acetylhexosaminidase activity, the ability to hydrolyse $GlcNAc_2$ to 2xGlcNAc, is observed in all the bacterial strains, except for the ΔDeglycosidase. Likewise, the negative control with media only, as well as ChiB, here serving as another negative control, both show no activity on 4-MU-GlcNAc. The differences in N-acetylhexosaminidase activity between the ΔDeglycosidase and the two other strains are statistically significant, with p-values less than 0.05 at the time points 29 h and 46 h. There is no significant difference between the ΔDeglycosidase and the negative control at either time points. These results suggest that *Ef*EndoE and/or *Ef*Endo18A could be active on β-chitin.

In order to display the results in a figure that is easier to interpret, the bacterial strains are combined into two separate graphs with respect to chitinase- and N-acetylhexosaminidase activity (**figure 4.2.3.2**).

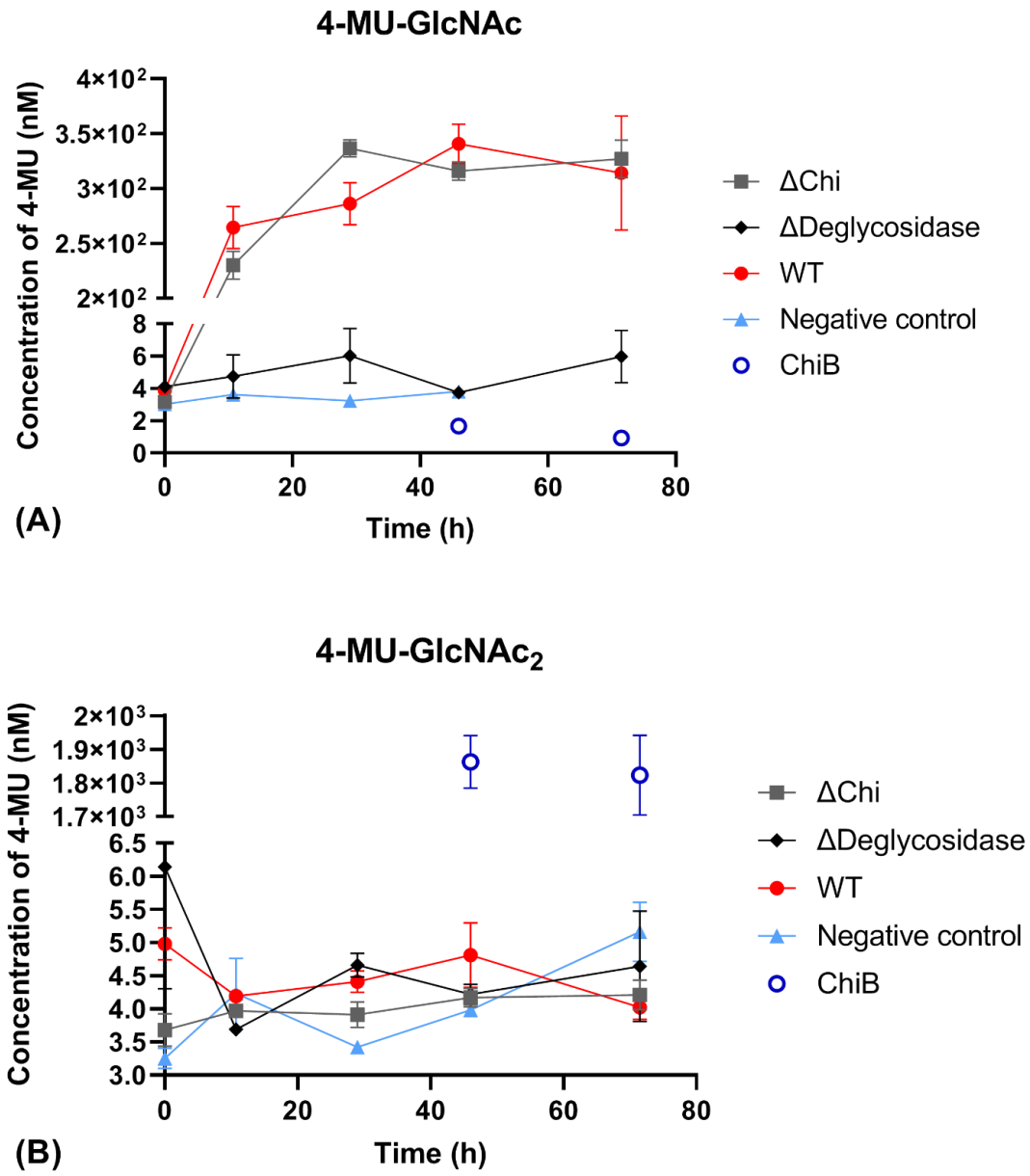

Figure 4.2.3.2 4-MU enzyme activity assay performed to analyse N-acetylhexosaminidase activity (A) and chitinase activity (B) in culture supernatants of the wild type, ΔDeglycosidase and ΔChi, as well as in the negative control with media only and the positive/negative control ChiB. Chitinase activity is represented by 4-MU-GlcNAc$_2$ and N-acetylhexosaminidase activity by 4-MU-GlcNAc.

Figure 4.2.3.2 thereby highlights these differences between the strains with regards to N-acetylhexosaminidase activity (panel A) and chitinase activity (panel B). The gap on the y-axis signifies the difference in activity between the separate strains and controls.

The enzyme activity assay was also conducted in a second parallel, this time including the ΔLpmO as well. These results are presented in a separate graph in **figure 4.2.3.3**, due to different time readings compared to the rest of the bacterial strains in the first parallel.

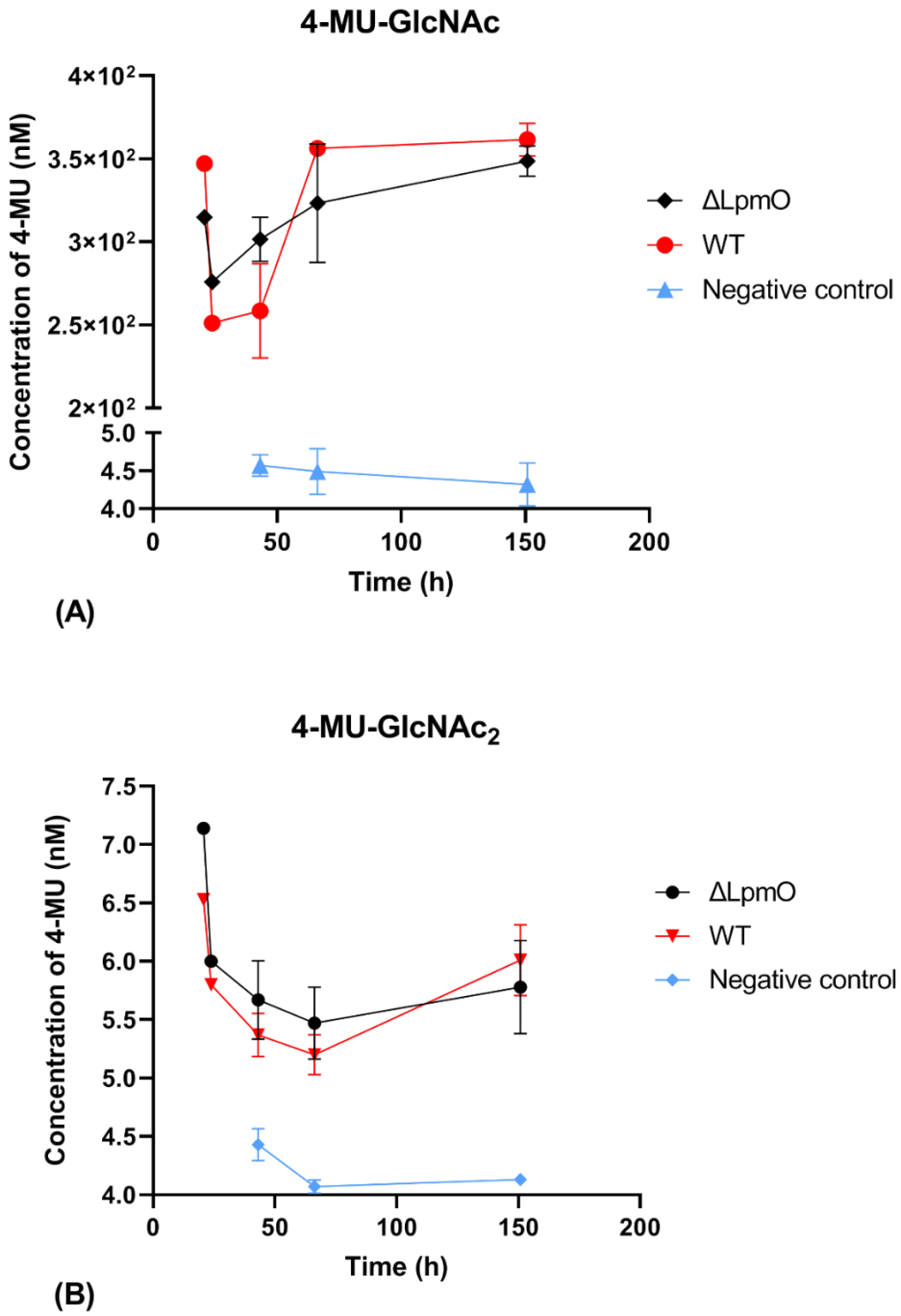

Figure 4.2.3.3 4-MU enzyme activity assay performed to analyse N-acetylhexosaminidase activity (A) and chitinase activity (B) in culture supernatants of the ΔLpmO and wild type, as well as in the negative control with media only. Chitinase activity is represented by 4-MU-GlcNAc$_2$ and N-acetylhexosaminidase activity by 4-MU-GlcNAc.

The first two time points of the assay plotted in **figure 4.2.3.3** only consist of one single measurement, differing from the rest of the time points with three technical replicates for each measurement. Therefore, the graph does not include error bars for these time points and the measurements involve greater uncertainty. One value (11.89) was removed from the data of 4-MU-GlcNAc$_2$ activity at time point 150.83 h for the wild type, as it was considered an outlier.

Nonetheless, **figure 4.2.3.3** show the same results for the wild type and the negative control as the first assay, presented in **figure 4.2.3.2.** The ΔLpmO occupy the same characteristics as of the wild type, with regards to both N-acetylhexosaminidase- and chitinase activity, and thereby only shows activity on the 4-MU-GlcNAc substrate.

4.3 Transcriptional analysis of *ef0361, ef0362, ef0114* and *ef2863*

Transcriptional analyses were performed on the wildtype to evaluate expression of the genes ef*0361, ef0362, ef0114* and *ef2863* under bacteriologic (different phases of bacterial growth) and under host mimicking conditions (serum), using ddPCR.

The cDNA generated from reverse transcription of the harvested RNA-samples were amplified through PCR using gene specific primers, to evaluate the presence of reverse-transcribed mRNA (cDNA) from the respective genes through gel electrophoresis. The expected sizes of the PCR-products were 194 bp for *ef0361* (*Ef*Chi18A), 165 bp for *ef0362* (*Ef*AA10A), 155 bp for *ef0114* (*Ef*EndoE*)* and 173 bp for *ef2863* (*Ef*Endo18A). The resulting gel images indicated expression of the genes of interest (**figure 4.3.1**). The sizes of the obtained bands were verified to the correct size by comparison to a Quick-Load® 100 bp DNA ladder.

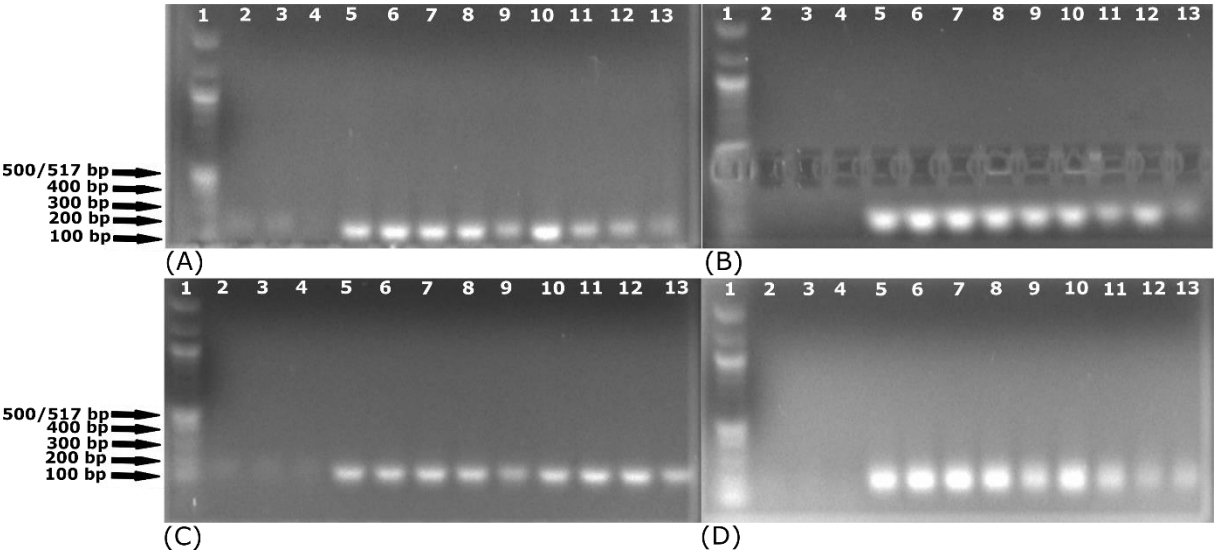

Figure 4.3.1 Gene-expression evaluation of the genes *ef0361* (A), *ef0362* (B), *ef0114* (C) and *ef2863* (D), following cultivation under bacteriologic conditions with bacteria in different phases of bacterial growth and under host mimicking conditions (serum). The wells are numbered, where well 1 contains a 100 bp ladder, well 2-4 contain cDNA from bacteria grown to exponential growth phase in BHI, well 5-7 contain cDNA from bacteria exposed to 10% serum, well 8-10 contain cDNA from bacteria grown in minimal medium (LM17ent), and finally well 11-13 contain cDNA from bacteria grown to stationary growth phase in BHI.

The PCRs were set up with control samples without reverse transcription (-RT), resulting in an additional row of -RT controls on the agarose gel. No PCR products were obtained for all control samples (results not shown).

PCR products generated with all four gene-specific primer sets were obtained for the samples harvested post-induction in serum, as well as for bacteria cultivated in minimal medium and in bacteriologic medium grown to stationary phase (**figure 4.3.1**). Slightly weaker bands were obtained for the latter, suggesting lower levels of expression compared to when the bacteria are exposed to serum or are grown in minimal medium. Bacteria grown until exponential phase in bacteriologic medium, however, only showed PCR products with very weak intensity barely seen on the gel. These results suggest minimal transcription of the genes when the wild type is grown to exponential phase of growth in BHI.

To further assess the expression of the *LpmO* (*ef0362*), which is of particular interest in this study due to its putative role in virulence, ddPCR was performed with the samples using specific primers for this gene to quantify the expression (**figure 4.3.2**).

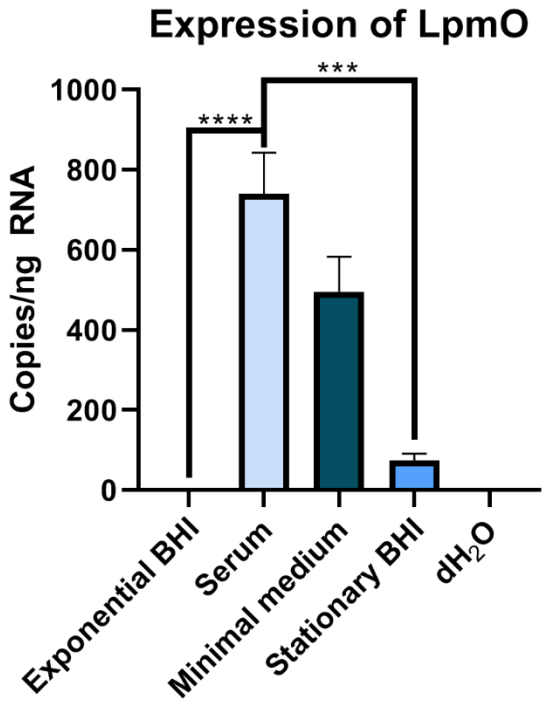

Figure 4.3.2 Transcriptional levels of *LpmO* in the wild type, under different phases of growth in bacteriologic medium and under host mimicking conditions with exposure to serum. Samples were harvested from bacteria grown to exponential and stationary phase of growth in BHI, from bacteria grown in the minimal medium LM17ent, as well as from bacteria exposed to LM17ent supplemented with 10% serum for 30 minutes. dH_2O was included as a negative control. Each column represents three biological replicates and three technical replicates.

One of the technical replicates from the first biological replicate of the samples exposed to serum was excluded, as the value was noticeably lower (16.05 copies/ng RNA) than the rest and was therefore considered an outlier.

The transcription level of the *LpmO* is interestingly much higher in bacteria exposed to serum, than in bacteria grown to exponential phase and stationary phase of growth in BHI (**figure 4.3.2**)**.** For exponential-phase-of-growth-bacteria cultivated under bacteriologic conditions, the expression levels of *LpmO* are approximately equal to zero. This supports the findings of the PCR where no products were obtained with primers targeting the *LpmO*-gene (**figure 4.3.1,** panel B)**.** The difference in copies/ng RNA for bacteria exposed to serum and bacteria under bacteriologic conditions grown to exponential phase is statistically significant ($p<0.0001$). Furthermore, expression of *LpmO* is statistically significantly higher ($p=0.0003$) in bacteria exposed to serum than in bacteria grown to stationary phase in BHI. Both findings affirm the upregulation of *LpmO* in presence of serum. The difference between bacteria grown in minimal media supplemented with 10% serum and bacteria grown in minimal media alone, which served as a control, is however not statistically significant ($p=0.0897$), although the number of copies/ng RNA is higher in the serum-samples.

The ddPCR included one single -RT control from one biological replicate of the bacteria grown to exponential phase of growth in BHI, which contained 1.185 copies/ng RNA. This value was approximately equal to zero, suggesting minimal gDNA contamination in the sample.

4.4 Survival assays

4.4.1 Estimation of bacterial number at a certain OD

In order to know the approximate number of the added bacteria in each assay, the number of colony-forming units (CFU) per millilitre at $OD_{600nm} \approx 0.4$ was determined. Thus, the number of bacteria at stationary phase of growth was estimated using overnight culture, which was diluted to $OD_{600nm} \approx 0.4$. The overnight cultures were prepared at three different time points (8-9 am, 12 pm and 15-16 pm) to evaluate any differences in growth based on the time of inoculation. However, no statistically significant differences in the bacterial numbers were detected between the time points. The assay was performed using four biological and three technical replicates (n=12), and the bacterial number at this OD was 2.09E+08 [± 3.37E+07] CFU/ml.

4.4.2 Serum Assay

As transcriptional analysis revealed high induction of the *LpmO* in presence of serum, viability of the wild type and ΔLpmO were examined in an *ex vivo* normal human serum analysis. The wild type and ΔLpmO were grown to stationary phase of growth to ensure expression of *LpmO* (**figure 4.3.2**), washed twice with PBS and adjusted to the OD_{600nm} 0.4 in RPMI/HSA. The bacterial number was adjusted by diluting the bacterial stocks 2x (approximately 1E+09 CFU/ml). Twenty µl of the bacteria were incubated in LM17ent supplemented with 10% pooled normal human serum (v/v) for one hour and three hours.

The viability of the wild type (107.4%) was slightly lower compared to the ΔLpmO (116.4%) one-hour post infection (**figure 4.4.2.1**, panel A), however no significant difference was detected statistically ($p>0.05$). Both strains demonstrated resistance towards killing in serum as a slight replication was observed. When the incubation period was prolonged to three hours (**figure 4.4.2.1,** panel B), the percentages of wild type and ΔLpmO viability increased extensively to 387.0% and 462.0%, respectively. Interestingly, replication of ΔLpmO in serum was significantly higher compared to the wild type ($p=0.0065$), and this was not attributable to differences in bacterial growth rates between wild type and its isogenic mutant (**figure 4.2.2.1.1**).

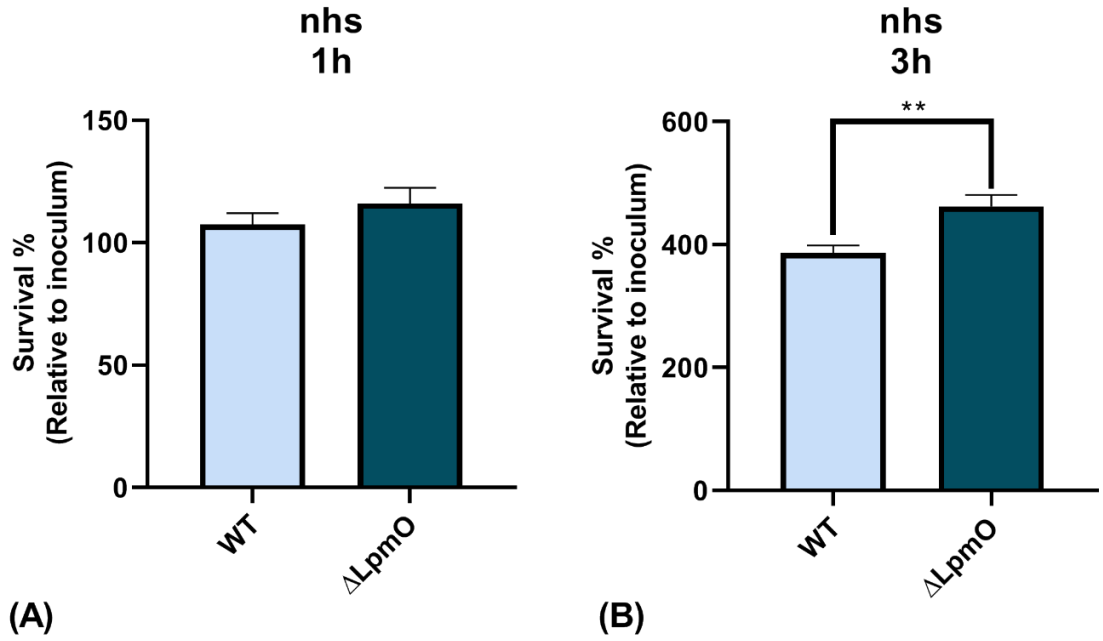

Figure 4.4.2.1 Serum assay of 2x diluted bacteria from OD_{600nm} 0.4 in stationary phase of growth. (A) Wild type and ΔLpmO incubated for one hour in nhs. (B) Wild type and ΔLpmO incubated for three hours in nhs. (Number of donors: >5; number of biological replicates: three; number of technical replicates: two).

The wild type and ΔLpmO was also incubated in heat-inactivated (HI) serum, which functioned as controls. Heat inactivation of serum is performed in order to inactivate the complement system, thus allowing for analysis of the contribution of complement-mediated killing of the bacteria.

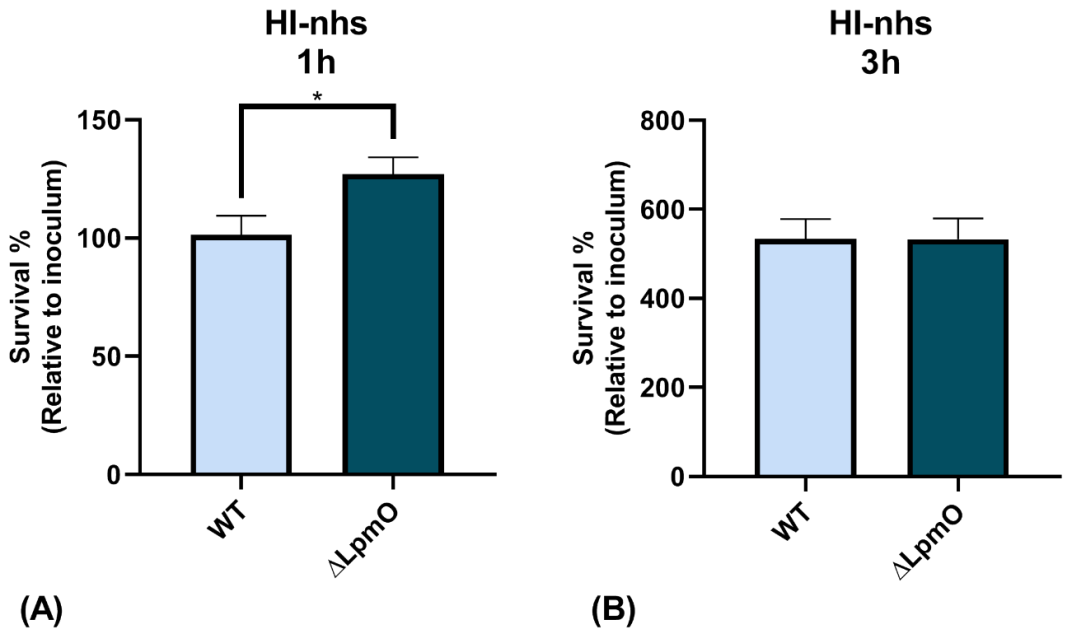

Figure 4.4.2.2 HI-serum assay of 2x diluted bacteria from OD_{600nm} 0.4 in stationary phase of growth. (A) Wild type and ΔLpmO incubated for one hour in HI-nhs. (B) Wild type and ΔLpmO incubated for three hours in HI-nhs. (Number of donors: >5; number of biological replicates: three; number of technical replicates: two).

Deletion of *LpmO* resulted in significantly higher viability of the mutant (127.0%) compared to the wild type strain (101.4%, p=0.0403) after a one-hour incubation in HI-serum (**figure 4.4.2.2**, panel A). Comparison of the percentages of viability of both strains when incubated in nhs and HI-nhs (**figure 4.4.2.1** and **figure 4.4.2.2**), reveals the same levels for the wild type, which indicate that the complement-mediated killing generally is not important in killing *E. faecalis* regardless of the mutation.

When the incubation period was extended to three hours post infection, no significant difference was observed between the viability of the wild type and ΔLpmO (p=0.9914; **figure 4.4.2.2**, panel B**).** The percentages of viability increased for both the wild type (533.6%) and ΔLpmO (532.8%) when incubated in HI-nhs compared to nhs. The highest increase was however observed for the wild type.

Following incubation in serum, viable bacteria were plated onto agar plates and enumerated in order to calculate the percentages of survival presented in the figures of this section. Interestingly, the colonies on several of the agar plates showed a distinctive phenotype called

small colony variants (SCVs) upon incubation in serum for a longer period (three hours) regardless of the mutation**.** Both the normal colonies and the SCVs can be seen in **figure 4.4.2.3**, which shows a selection of the plate parallels from the serum assays.

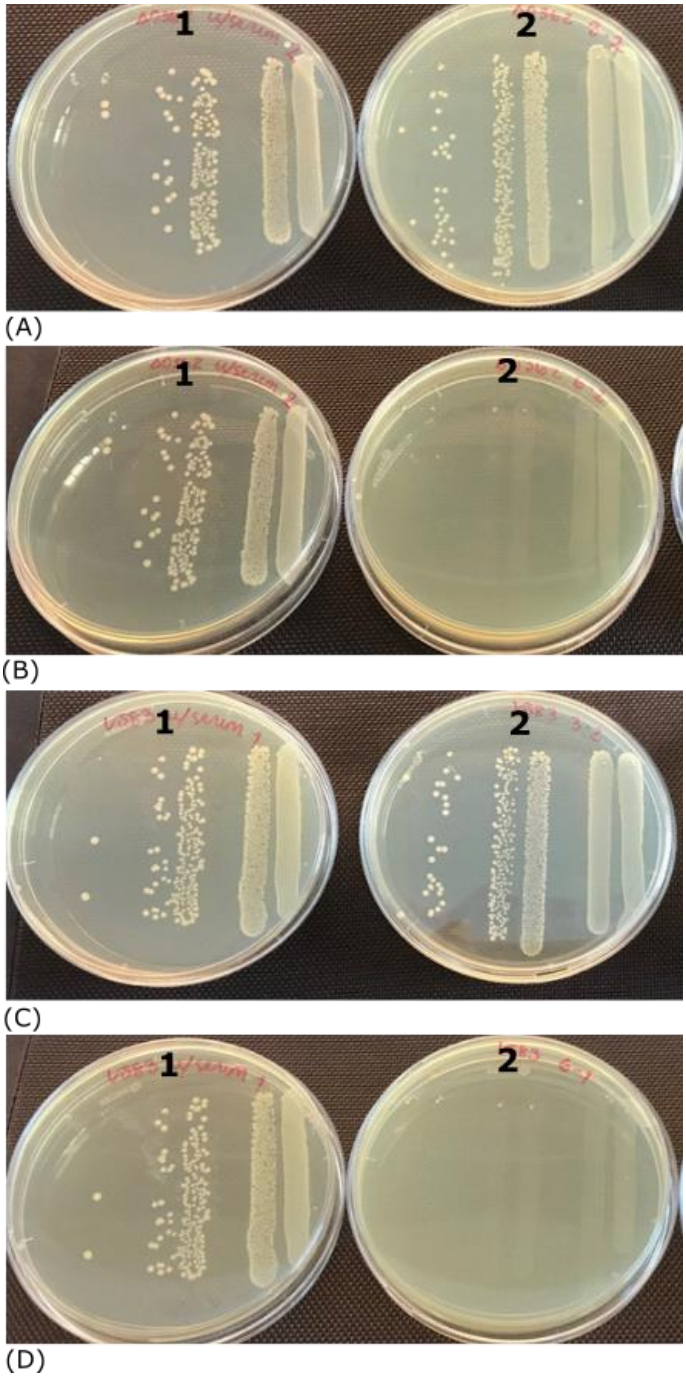

Figure 4.4.2.3 Plating of surviving ΔLpmO and wild type bacteria following one- and three-hour incubations in nhs. The agar plates are numbered, where plate 1 corresponds to the ΔLpmO (panel A and B) and wild type (panel C and D) controls without serum, while plate 2 contains the surviving ΔLpmO and wild type incubated in nhs. (A) ΔLpmO incubated for one hour. (B) ΔLpmO incubated for three hours. (C) Wild type incubated for one hour. (D) Wild type incubated for three hours.

The results from the serum assay demonstrated resistance of both the wild type and ΔLpmO to be killed by serum. Furthermore, the observed SCVs might be a general mechanism in which the bacteria use to protect themselves from complement-mediated killing.

4.4.3 Whole Blood Survival Assay

Viability of *E. faecalis* was further assessed in a more complex whole human blood system involving not only the complement proteins of the serum, but all immune components of the blood. The assays required optimizations in order to achieve a realistic infection model, where the genes of interest were expressed and the effect of the immune cells on the bacteria were detectable. This involved optimizations of the growth phase of the inoculated bacteria, the bacterial number of the inoculum, as well as the incubation times post inoculation. Therefore, results from several assays performed with different conditions are presented in this section.

Wild type and knockout strains were grown to stationary phase of growth to ensure expression of *LpmO,* washed twice with PBS and adjusted to the OD_{600nm} 0.4 in RPMI/HSA. Twenty µl of the bacteria were incubated in RPMI supplemented with 80% freshly drawn blood (v/v) for one hour and three hours.

Although the percentage of viability of the ΔDeglycosidase (44.38%) was slightly lower compared to the wild type (60.14%) one-hour post infection, no significant difference was statistically detected (**figure 4.4.3.1**, panel A; $p>0.05$). When the incubation period extended to three hours post infection, an increase in viability of both the wild type and the ΔDeglycosidase was detected (**figure 4.4.3.1**, panel B). The percentages of wild type and ΔDeglycosidase viability were increased to 170.6% and 230.0%, respectively. The ΔDeglycosidase showed resistance towards killing by whole human blood components and replication was observed, although the difference in viability was not significant in comparison to the wild type ($p=0.0837$).

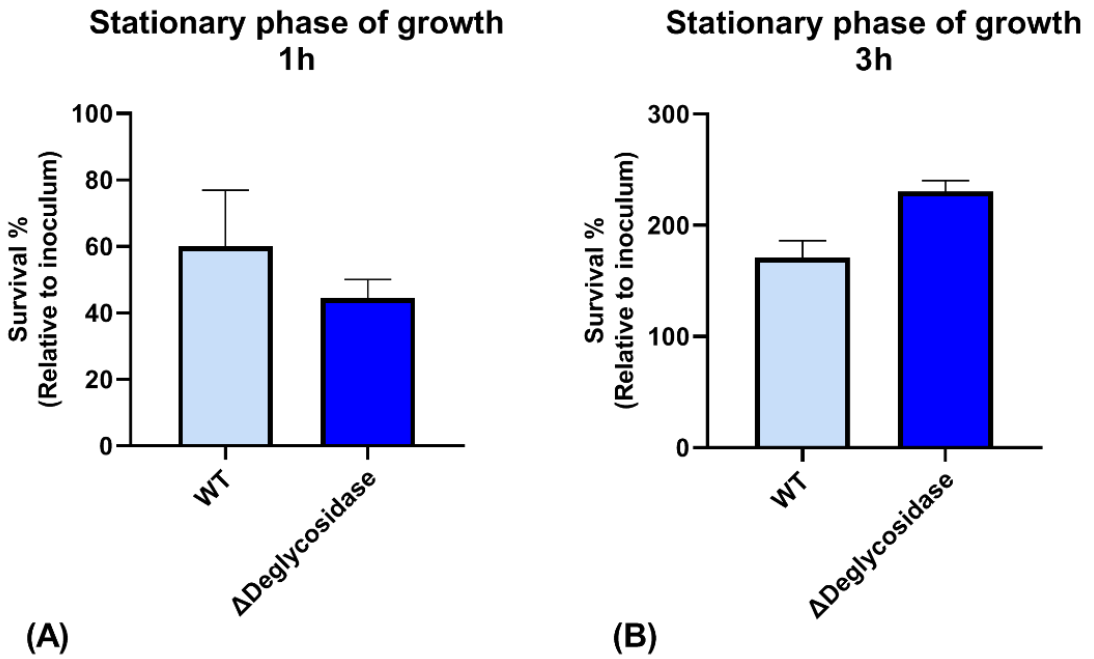

Figure 4.4.3.1. Whole blood survival assay of undiluted (OD_{600nm} 0.4) bacteria in stationary phase of growth. (A) WT and ΔDeglycosidase incubated in blood for one hour. (B) WT and ΔDeglycosidase incubated in blood for three hours. (Number of donors: one; number of biological replicates: one; number of technical replicates: two).

Fine tuning between the bacterial number and the amount of blood is crucial in the whole human blood assay. Overloading the system with high bacterial number might mask the effect of a protein in promoting/reducing bacterial killing in this complex system. Therefore, another killing assay was performed using lower number of bacteria (10x (approximately 2E+07 CFU/ml) and 100x (aproximately 2E+06 CFU/ml) diluted compared to the above assay) grown to stationary phase.

The results of the blood assay using 10x dilution (approximately 2E+07 CFU/ml) of the inoculum are presented as **figure 4.4.3.2**.

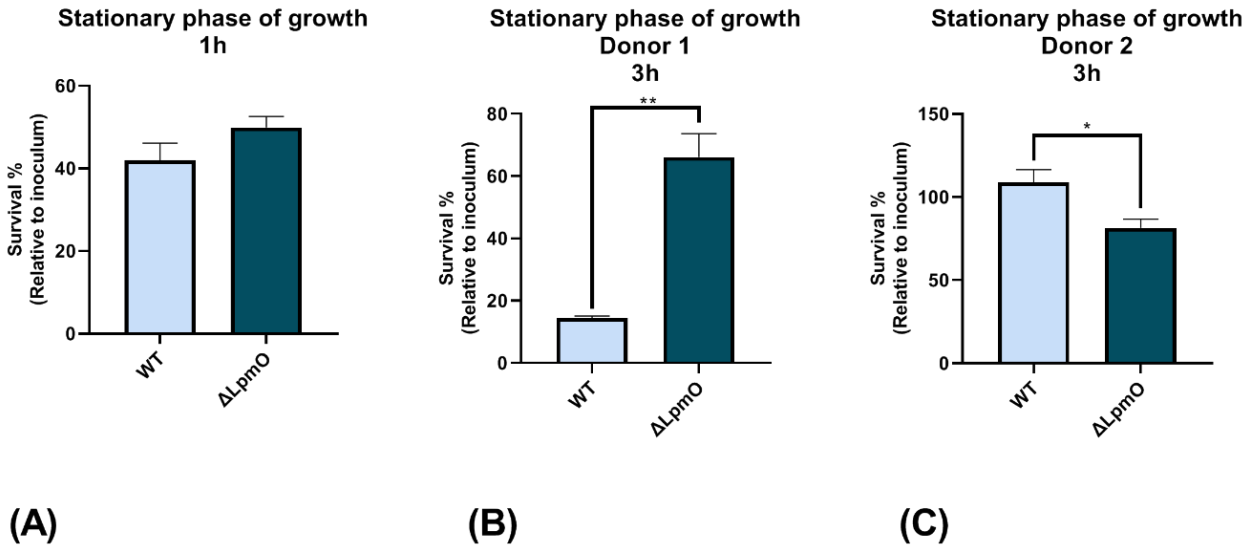

Figure 4.4.3.2. Whole blood survival assay of 10x diluted bacteria in stationary phase of growth. (A) Pooled results of the WT and ΔLpmO incubated in blood for one hour. (B) Three hour incubation of WT and ΔLpmO in donor blood 1. (C) Three hour incubation of WT and ΔLpmO in donor blood 2. (Number of donors: two; number of biological replicates: two; number of technical replicates: two).

Panel A of **figure 4.4.3.2** shows the pooled results of both blood donors, after a one hour incubation of the the wild type and ΔLpmO in the blood. The results revealed that deletion of *LpmO* resulted in increased viability of *E. faecalis* (49.95%) compared to the wild type strain (41.97%), although the effect is not significant (**figure 4.4.3.2**, panel A; p=0.1257). However, prolonging the incubation period to three hours significantly promoted ΔLpmO viability in blood (**figure 4.4.3.2**, panel B; p=0.0060). To ensure reproducibility of the results obtained from ΔLpmO, the blood assay was repeated. However, deletion of the gene resulted in significant reduction of ΔLpmO (81.28%) viability compared to the wild type (**figure 4.4.3.2**, panel C; 108.9%, p=0.0249).

The results of the blood assay using 100x dilution (approximately 2E+06 CFU/ml) of the inoculum are presented as **figure 4.4.3.3**.

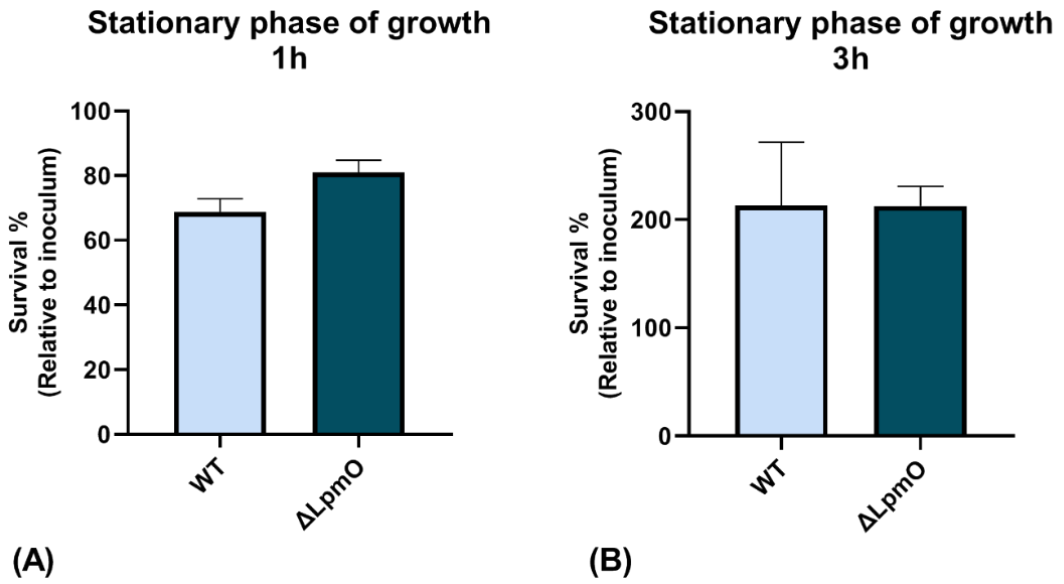

Figure 4.4.3.3. Whole blood survival assay of 100x diluted bacteria in stationary phase of growth. (A) WT and ΔLpmO incubated in blood for one hour. (B) WT and ΔLpmO incubated in blood for three hours. (Number of donors: one; number of biological replicates: two; number of technical replicates: two).

The percentage of the wild type and ΔLpmO viability one hour post infection were 68.78% and 81.06%, respectively (**figure 4.4.3.3,** panel A). Although the percentage of viability was slightly lower for the wild type, no significant difference was statistically detected (p=0.0696). When the incubation period was extended to three hours post infection, both the wild type and ΔLpmO resisted killing by whole human blood components (**figure 4.4.3.3**, panel B). The percentages of the wild type and ΔLpmO viability were increased to 213.5% and 212.7%, respectively. Thus, no significant difference was observed between the strains (p=0.9904).

The blood assay was also performed using bacteria in exponential phase of growth. Overnight cultures were diluted and re-grown until reaching to mid exponential phase. The bacteria were washed twice in PBS and adjusted to OD_{600nm} 0.4 in RPMI/HSA. Blood assays were performed with bacteria diluted 10x and 100x, and twenty µl of the bacteria were incubated in RPMI supplemented with 80% freshly drawn blood (v/v) for one hour and three hours accordingly.

The results of the blood assay using 10x dilution of the inoculum are presented as **figure 4.4.3.4**.

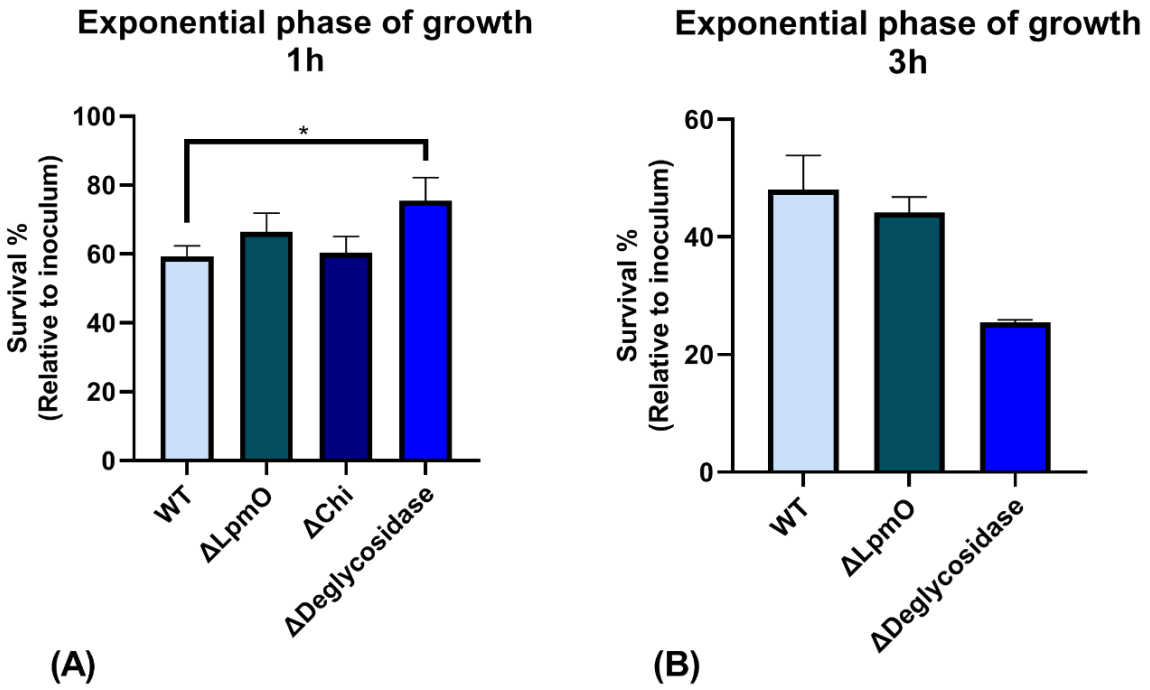

Figure 4.4.3.4. Whole blood survival assay of 10x diluted bacteria in exponential phase of growth. (A) WT, ΔLpmO, ΔChi and ΔDeglycosidase incubated in blood for one hour. (B) WT, ΔLpmO and ΔDeglycosidase incubated in blood for three hours. (Number of donors: three, one of which donated blood twice; number of biological replicates: two or three; number of technical replicates: two or three).

Panel A of **figure 4.4.3.4** shows the pooled results of three blood donors, after a one hour incubation of the the wild type, ΔLpmO, ΔChi and ΔDeglycosidase in the blood. Deletion of *ef0114* and *ef2863* resulted in significantly increased viability of ΔDeglycosidase (75.45%) compared to the wild type strain (59.32%, p=0.0470), suggesting a potential role of these genes in promoting *E. faecalis* survival in blood. However, prolonging the incubation period to three hours abolish this effect (**figure 4.4.3.4,** panel B; p=0.1252). The percentages of wild type, ΔLpmO and ΔDeglycosidase viability were reduced to 48.07%, 44.18% and 25.44%, respectively. No significant differences in viability were detected between the strains three hours post infection, although ΔDeglycosidase demonstrated reduction in viability compared to the other strains.

This assay was also performed on a fifth donor, but due to deviating results compared to the other donors, this particular assay was not pooled with the others but is instead presented in a separate graph in **Appendix D.**

The results of the blood assay using 100x dilution of the inoculum are presented as **figure 4.4.3.5**.

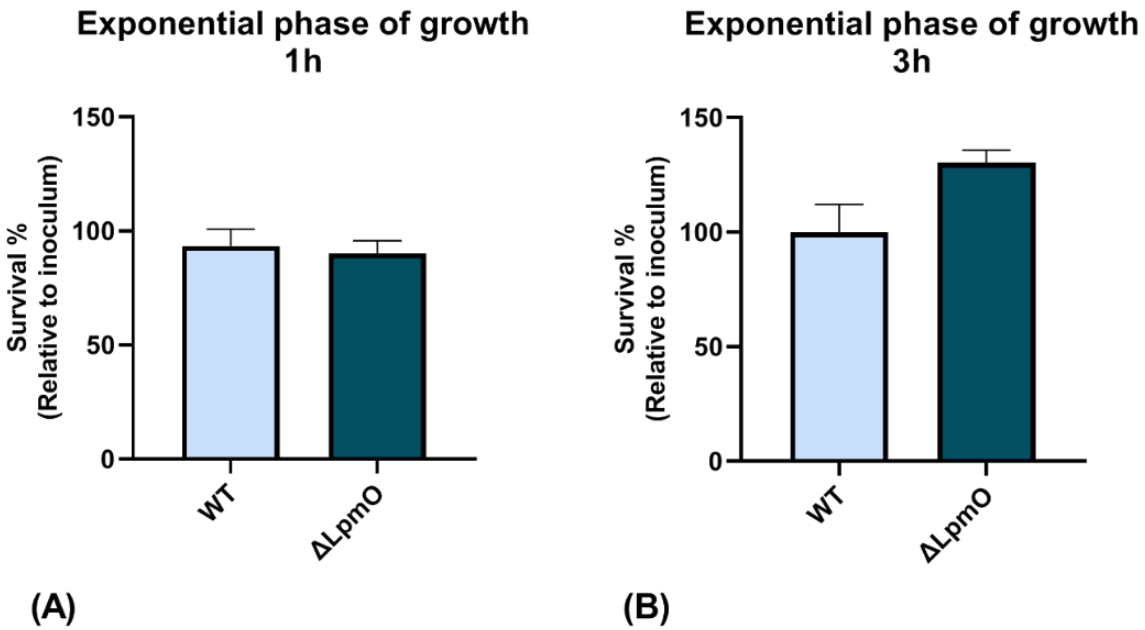

Figure 4.4.3.5. Whole blood survival assay of 100x diluted bacteria in exponential phase of growth. (A) WT and ΔLpmO, incubated in blood for one hour. (B) WT and ΔLpmO incubated in blood for three hours. (Number of donors: one; number of biological replicates: two; number of technical replicates: two).

Less than a ten percent reduction of viability was observed for the wild type (93.39%) and ΔLpmO (90.19%) one hour post-infection (**figure 4.4.3.5**, panel A), suggesting resistance towards killing by whole human blood components. Extending the incubation period to three hours post infection (**figure 4.4.3.5,** panel B), promoted both wild type (100.0%) and ΔLpmO (130.4%) viability in blood. The increased viability of ΔLpmO in blood three hours post infection was however not signifcant (p=0.0600) in comparison to the wild type.

Analysis of viability of the wild type and deletion strains in whole human blood resulted in tendencies of improved survival of the ΔLpmO compared to the wild type, with bacteria in stationary phase as well as in exponential phase of growth. The differences were however not statistically signficant, and in the cases with significance, donor variations gave contradicting results. The ΔDeglycosidase showed increased viability compared to the wild type three hours post infection when grown to stationary phase of growth, but demonstrated lower viability when grown to exponential phase.

5 Discussion

The proteins associated with the chitinolytic system of *Enterococcus faecalis V583* are proposed to have virulence properties. In order to analyse their proposed role in virulence, gene knockout strains of *E. faecalis V583* were utilized. Firstly, characterization of the wild type and gene deletion strains was conducted with regards to morphology, growth on soluble and insoluble substrates, and enzymatic activity. Secondly, virulence properties of the genes of interest were analysed through *ex vivo* whole human blood and serum assays, where viability of the wild type and knockout strains were determined.

5.1 Verification of gene-knockout strains

Confirmation of genomic deletion was successful for the knockout strains ΔChi, ΔLpmO and ΔDeglycosidase as verified through PCR (**figure 4.1.1** and **figure 4.1.3**) and sequencing of the respective regions. The double knockout strain ΔChi-ΔLpmO was however not verified, as DNA-bands resembling the wild type were obtained by PCR using several sets of gene-specific primers (**figure 4.1.1** and **figure 4.2.2**). These results were unexpected, as DNA sequencing of the knockout regions had previously been conducted revealing correct deletion of the genes. Since all the primers gave the same PCR products of sizes corresponding to the wild type, mistakes in the primer design were not likely to be the cause. The presence of both wild type- and ΔChi-ΔLpmO-products in lane 2 and 3 of **figure 4.1.1** highly indicate contamination between the neighbouring wells, potentially caused by a rift between the wells. This would mean that the gene deletions in actuality were correct, supported by the intensity of the obtained DNA bands. However, the following PCRs gave clear indications of the ΔChi-ΔLpmO as an unsuccessful gene deletion strain. Contamination between neighbouring lanes in the gel is not plausible in these cases, as fragments of the wild type occurred for the knockout strain with all the inspected primer sets, regardless of the person carrying out the experiment. A final explanation could therefore be contamination of the glycerol stock containing the ΔChi-ΔLpmO strain by wild type bacteria.

A final attempt of verification was planned through whole genome sequencing of the strain. Unfortunately, the results of the whole genome sequencing have not been analysed at the current time being, thus the strain was decided to be excluded from this study.

5.2 Characterization of the wild type and knockout strains

Light microscopy revealed no morphological differences between the wild type and knockout strains (**figure 4.2.1.1**) under the given conditions, suggesting that deletion of the genes do not

affect the normal cell function of the bacteria. Growth on soluble substrates was characterized (**figure 4.2.2.1.1**), where the wild type in general demonstrated tendencies of higher growth rates than the gene deletion strains. This was seen both in LM17ent supplemented with 0.4% GlcNAc and LM17ent supplemented with 0.4% $GlcNAc_2$, where both substrates represent products that may arise from chitin degradation. ΔChi showed the lowest growth rate in these substrates, which could indicate that *Ef*Chi18A is a functional chitinase involved in degradation of chitin. However, GlcNAc and $GlcNAc_2$ are not preferred substrates for chitinases as these are the end products generated from chitinase activity. The reduced growth rate observed for ΔChi might therefore not be caused by a growth defect at all, as the differences between the strains are small and likely to be insignificant. Another explanation could be that the deletion of *ef0361* affects the bacteria in additional ways than the loss of chitinase activity, thereby resulting in a growth defect due to change of the cellular integrity of the bacteria.

The preferred substrate for family GH20 proteins, such as *Ef*EndoE of *E. faecalis V583*, is the dimer $GlcNAc_2$ that is cleaved to two GlcNAc-monomers by the enzyme. The wild type was therefore expected to show higher growth rates compared to the ΔDeglycosidase when cultivated in $GlcNAc_2$, since the gene encoding the GH20 N-acetylhexosaminidase is knocked out in the latter. Surprisingly, the wild type and ΔDeglycosidase demonstrated very similar growth in $GlcNAc_2$ (**figure 4.2.2.1.1**, panel D), suggesting a possible involvement of a different family GH20 protein instead. However, CAZy reports no other family GH20 proteins secreted by *E. faecalis V583*. Several of the members of the GH3 family have nevertheless been shown to exhibit N-acetylhexosamindase activity (Li et al., 2002), and *E. faecalis V583* encodes a family GH3 protein (*ef1238*) which could exhibit activity towards chitin. The detection of substantial N-acetylhexosaminidase activity in the wild type compared to lack of such activity in ΔDeglycosidase however disproves this argument, as this observation clearly indicates chitobiase-activity of the *Ef*EndoE. Moreover, N-acetylhexosaminidase activity is generally induced by presence of chitin degradation products, such as GlcNAc and $GlcNAc_2$ (Beier & Bertilsson, 2013; Vaaje-Kolstad et al., 2013), thus the activity of *Ef*EndoE should be induced when grown on LM17ent supplemented with 0.4% $GlcNAc_2$. Perhaps separate studies of each of the two protein-coding genes of the ΔDeglycosidase would give insight in the exact role of *Ef*EndoE (*ef0114*) in chitin hydrolysis, given the ambiguous results obtained in the present study.

Growth on chitin was further assessed, more specifically on β-chitin as *E. faecalis V583* have been shown to utilize β-chitin better than α-chitin (Vaaje-Kolstad et al., 2012). Assessment of

bacterial growth on β-chitin was challenging, as the growth assay required extensive optimizations. Absorbance measurements (OD_{600nm}) were problematic due to the presence of particles in the solution which interfered with the passing light. Separate assays were performed to determine the time needed in order for the particles to sink to the bottom of the flasks and for the measurements to stabilize and become reproducible. Despite these efforts, the measurements were still unreliable, as shown in **figure 4.2.2.2.1.** Absorbance measurements therefore do not reflect the realistic growth of the different bacterial strains.

Another factor affecting the absorbance measurements, was the swelling of the chitin particles throughout the growth assay. Clear differences between freshly made medium and several days old medium were observed by visual perception, and these variabilities of the media may influence the absorbance measurements if not being accounted for. However, by setting the cell density meter to blank using medium that was following the same incubations as the bacterial cultures, this problem was solved to some extent.

Another aspect was optimization of the cultivation conditions. As Enterococci are versatile microorganisms and tolerate a wide variety of growth conditions, cultivation both with and without agitation were carried out. Initial assays were performed with agitation at 230 rpm, which resulted in declining numbers of bacteria immediately post inoculation (results not shown). Therefore, the number of revolutions per minute (rpm) were reduced to 140. The first parallel resulted in the same immediate decrease in CFU/ml, while the second and third parallel demonstrated growth during the first 40 hours of the assay (see **figure 4.2.2.2.2**). These results suggest that too high turbulence in the bacterial cultures do not allow for bacterial growth. One explanation to this might be that the bacteria are killed by mechanical lysis due to collisions between the bacteria and chitin-particles, thus no bacterial growth is observed at high levels of agitation. Another theory could be that the bacteria require longer contact time with the chitin particle to enable binding and degradation. Because of the constant movement of chitin particles and bacteria in the solution, the bacteria are not given the time to access the chitin provided in the medium. A third explanation might not have to do with the agitation at all, but with the sinking of the particles prior to sample harvesting. When the chitin particles sink to the bottom of the flasks, bound bacteria might sink with them. This could have a major impact on the results, as samples were harvested from the upper parts of the flasks, thereby excluding sunken particles with potentially bound bacteria. Consequently, calculations of CFU/ml might not reflect the actual growth in the flasks, as the bacterial numbers are underestimated. Analysis of binding abilities of the bacterial strains to chitin particles would be interesting and could

potentially shed light on the putative activity of the proteins related to chitin metabolism. The swelling trait of the chitin-particles is likely to increase the ability of the bacteria to bind the chitin, as swollen particles are more easily hydrolysed than native chitin (Doetsch, 2012). Biofilm formation could also be a factor increasing bacterial binding to the particles, as *E. faecalis* is capable of producing biofilms (Mohamed & Huang, 2007).

The growth assays were continued with static cultivation of the bacteria, with the aim of improving the bacterial accessibility to the chitin particles. Prior to sample harvesting, the bacterial cultures were vortexed quickly to ensure inclusion of the particles with potentially bound bacteria, thereby eliminating the chance of underestimating the bacterial numbers. These assays did indeed result in improved bacterial growth (**figure 4.2.2.2.3**).

Although bacterial growth was observed in LM17ent supplemented with 1% β-chitin, the increase in bacterial numbers were rather small, indicating a general poor growth on β-chitin. The wild type was expected to show higher growth rate compared to ΔChi and ΔLpmO, where the respective chitinase and LpmO are knocked out. However, the strains demonstrated very similar growth rates, suggesting that these proteins are not involved in chitin degradation after all. These findings are interesting, as Vaaje-Kolstad et al. (2012) found *E. faecalis V583* to grow well on β-chitin and showed both chitinase- and LpmO-activity on this substrate. It could be that the β-chitin batch utilized in the present study is less attainable for *E. faecalis*, and that the assay should have been performed using colloidal chitin instead, which is more amorphous and easier accessible. Nonetheless, Vaaje-Kolstad et al. reported the bacterial number of the wildtype 72 hours post inoculation as 1.5E+06 CFU/ml, which is consistent with the results of the present study (from the three biological replicates: 3.67E+06, 4.25E+07 and 3.50E+07 at ~76 hours post inoculation). Vaaje-Kolstad et al. stated that a final proof for the contribution of the chitinase and LpmO-proteins to the ability of *E. faecalis V583* to grow on chitin, would require functional testing of knockout strains. As the present study have demonstrated, there are however no clear differences in growth rates between the wild type and the gene knockout strains, suggesting a different function of these proteins.

This was further supported by enzymatic activity assays, where chitinase- and N-acetylhexosaminidase activities were analysed. The substrates utilized were analogues of $GlcNAc_2$ and $GlcNAc_3$, which are soluble sugars derived from enzymatic chitin hydrolysis. Neither the wild type nor any of the gene deletion strains showed chitinase activity in assays using the $GlcNAc_3$-analogue 4-MU-$GlcNAc_2$ (see **figure 4.2.3.2** and **figure 4.2.3.3**). Furthermore, there were no statistically significant difference between the wild type and ΔChi,

which highly indicates that *Ef*Chi18A is not involved in chitin degradation. This is surprising, as the wild type possesses a chitinase (*Ef*Chi18A) that is presumed to be active when the wild type is grown in β-chitin. N-acetylhexosaminidase activity was however induced in all the strains using the $GlcNAc_2$-analogue 4-MU-GlcNAc, apart from the ΔDeglycosidase. These results suggest that *Ef*EndoE and/or *Ef*Endo18A are involved in degradation of β-chitin, while the chitinase *Ef*Chi18A along with the LpmO *Ef*AA10A most likely have other functions than digestion of chitin in *E. faecalis V583*.

A weakness of the present study was the exclusion of controls without β-chitin, but with bacteria and minimal medium LM17ent, as part of the growth-assays on β-chitin as well as the enzyme activity assays. The negative controls included, with LM17ent supplemented with 1% β-chitin, without addition of bacteria, does not account for any enzymatic activity or growth of the bacteria on the minimal medium components alone. Although growth of the bacteria in LM17ent was analysed in the growth assays on soluble substrates, these assays were performed by cultivation in microtiter plates, thus are not comparable to the growth curves from cultivation on β-chitin in erlenmeyer flasks, given that the growth was measured differently in the two assays. Moreover, enzymatic activity was not analysed in the growth experiments in LM17ent. Inclusion of such controls would have allowed for determination of the contribution of the minimal components to the bacterial growth on β-chitin. It could indeed be expected that chitinases or other chitin-degradable proteins are secreted under nutrient limiting conditions, as a way for the bacteria to seek new food sources.

5.3 Role of *E. faecalis V583* chitinolytic machinery in virulence

Several studies have linked chitinases and chitin binding modules to virulence, where glycoproteins of host organisms might be potential targets during infection. These proteins contain the monomer GlcNAc that also is the building block of chitin. The chitinase *Ef*Chi18A and the lytic chitin monooxygenase *Ef*AA10A of *E. faecalis V583* have been suggested as putative virulence factors in several previous studies (Solheim et al., 2007; Vebo et al., 2009; Vebo et al., 2010).

E. faecalis are known to survive within macrophages (Gentry-Weeks et al., 1999; Zou & Shankar, 2016), a topic of interest for the present study. Originally, it was intended to analyse survival and persistence of *E. faecalis V583* in the macrophage-like human cell line THP-1, through internalization assays (see **Appendix A**). However, as the lab was granted a REK approval, it was decided to conduct more relevant *ex vivo* assays (serum and whole blood

killing) rather than *in vitro* assays in the THP-1 model system. Analysis of viability *ex vivo* in hirudin whole blood allows investigation of the effects of the entire immune system (complement and immune cells), rather than restricting it to the macrophages alone. Furthermore, *ex vivo* whole blood assays keep the natural conditions intact with minimal alterations, and thereby is likely to mimic a systemic *E. faecalis* infection more realistically than what the THP-1 model system would, for the purpose of this study.

The LpmO is of particular interest, as similar chitin-binding modules have been shown to contribute to virulence in other bacterial species such as *Listeria monocytogenes* (Chaudhuri et al., 2010), *Vibrio cholerae* (Bhowmick et al., 2008) and *Serratia marcescens* (Kawada et al., 2008).

5.3.1 Transcriptional analysis of LpmO in presence of serum using ddPCR

Analysis of *LpmO* transcription was performed using Droplet digital PCR (ddPCR). ddPCR is highly precise and provides absolute quantification of nucleic acids, in comparison to other methods such as real-time PCR (Hindson et al., 2013).

Transcriptional analysis of *LpmO* revealed induction of expression in presence of 10% pooled human serum and under nutrient limiting conditions (LM17ent), compared to low expression when the bacteria were incubated in bacteriologic medium (BHI) (**figure 4.3.2**). The differences between expression in the presence of serum and in minimal medium alone was not significant. Higher numbers of copies/ng RNA were indeed observed in the bacteria exposed to serum, but due to the lack of significance, the results rather imply an upregulation of LpmO-expression in response to stress caused by lack of nutrients as the main factor, although serum might be an additional stress-factor contributing to the upregulation. These findings agree with previous gene regulation studies which too have shown upregulation in response to bile stress, urine and blood (Solheim et al., 2007; Vebo et al., 2009; Vebo et al., 2010). The results suggest that the *LpmO*-gene is upregulated when conditions are critical for the bacteria, such as under nutrient limiting conditions (minimal medium) and under stress. Low levels of expression are also seen for bacteria in stationary phase of growth cultivated in BHI, where the nutrient levels start to become limiting/are depleted, the media is enriched with wastes, and the bacterial number evens out due to equal growth and death rates. However, when conditions are satisfying, such as for the bacteria grown to exponential phase in BHI, the *LpmO* is not transcribed. These observations therefore suggest that the regulation of *LpmO*-expression is mainly related to nutrient acquisition and stress factors.

Presence of gDNA can lead to an overestimation of copies/ng RNA reported. To ensure absence of residual genomic DNA (gDNA) from the RNase treatment, -RT (without reverse transcriptase) controls went through normal PCR together with the +RT (with reverse transcriptase) samples (**figure 4.3.1**; -RT controls not shown). The number of amplification cycles (30X) and the duration of each elongation step (30 seconds) were adjusted accordingly to reveal possible contaminations. While bands of the correct sizes were detected in the +RT samples, no fragments were visualized on the gel in -RT treated samples. In addition, some of the -RT samples (Exponential BHI 10x and 100x diluted) were included in the ddPCR, where the number of copies/ng RNA were approximately equal to zero. These results thereby indicate that the analysed cDNA samples were free from contamination.

5.3.2 Viability of *E. faecalis* in 10% serum

Response to serum was further assessed through an *ex vivo* normal human serum assay, which included the wild type and ΔLpmO. The strains were grown to stationary phase of growth to ensure expression of the *LpmO*-gene, as observed in the transcriptional analysis by ddPCR (**figure 4.3.2**). The serum assay was set up to get confirmation on how much complement mediated killing was involved and to evaluate the impact of LpmO on bacterial viability in human serum. However, both the wild type and ΔLpmO demonstrated resistance towards the lytic components of complement (**figure 4.4.2.1)**, which indicates that complement-mediated killing generally is not important in killing *E. faecalis* regardless of the mutation. Gram-positive organisms are in fact generally not directly killed by complement and are defined as serum resistant (Brown, 1985). While human serum effectively kills Gram-negative bacteria (Berends et al., 2015), the thick cell wall of Gram-positive bacteria, including Enterococci, generally acts as a barrier and prevents complement-mediated Membrane Attack Complex (MAC) lysis (Brown, 1985). Therefore, complement has a very different role in host defence against these bacteria. Here, the objective of complement activation is to lead to opsonization of the bacteria. Complement factor C3b binds to bacterial cell walls and acts as signals for the ingestion and destruction of the bacteria by host phagocytic cells (Brown, 1985). As there is no presence of phagocytes in serum, killing is not performed and the bacteria thus survive.

Although it is well-established that complement-mediated killing is not common for Gram positive bacteria, the chitinolytic enzymes of *E. faecalis* could potentially affect the cell wall through modification of peptidoglycan, thereby allowing for MAC-mediated lysis of the cells. However, the performed serum assay showed resistance and survival of *E. faecalis* in serum as expected for Gram positive bacteria (**figure 4.4.2.1**).

Heat-inactivated serum serves as important controls of this assay, as certain heat-labile complement components are inactivated and MAC-dependent lysis is blocked (Berends et al., 2015). Thus, the effect of complement activity on the bacteria can be determined by comparing viability in nhs and HI-nhs. The wild type and ΔLpmO demonstrated almost equally high viabilities in both nhs and HI-nhs, which confirms *E. faecalis'* resistance towards serum.

Following incubation of the bacteria in serum, plating was performed to determine bacterial viability. Several of the colonies on the plates showed a distinctive phenotype called small colony variants (**figure 4.4.2.3**). SCVs are a slow-growing subpopulation of bacteria with an atypical colony morphology and a size of almost one-tenth of the colonies associated with the wild type bacteria. Interestingly, SCVs are known to facilitate pathogenic behaviour of the bacteria, as they are specialized for intracellular persistence in mammalian cells and are less susceptible to antibiotics compared to the normal wild type (Proctor et al., 2006). SCVs have been shown to be associated with persistent and recurrent infections, such as osteomyelitis, arthritis, abscesses and respiratory infections in humans (Proctor et al., 2006). During intracellular infection, the bacteria may alter their phenotype into SCVs which increases resistance to intracellular immune responses (Tuchscherr et al., 2011). Limited research is conducted on SCVs in *E. faecalis* and the underlying mechanisms are not fully understood. However, *E. faecalis* SCVs in chicken have been shown to be more virulent in an *in vivo* infection model than the normal phenotype (Petersen et al., 2008). Furthermore, *E. faecalis* SCVs have been characterized in a human patient suffering from a clinical case of chronic aortic valve endocarditis (Wellinghausen et al., 2009). These findings can be linked to the bacteria in the present study, as SCVs might be a mechanism that increases the viability of the bacteria through resistance to immune responses. The SCVs appeared on the plates with both wild type and ΔLpmO incubated for three hours in nhs, and some cases were also observed on plates with bacteria incubated in HI-nhs. SCVs thus seem to be a reaction to stress upon exposure to serum and may contribute to resistance towards killing.

5.3.3 Viability of *E. faecalis* in 80% hirudin human whole blood

Since *E. faecalis* demonstrated resistance towards serum, viability of the bacterial strains was evaluated in human whole blood instead. This is a more complex system where all the immune components are present. Contrary to the serum assays, the professional phagocytes are present in the blood. Host phagocytic cells, such as macrophages, dendritic cells and neutrophils, will be recruited to the bacteria due to deposited C3b complement components on the bacterial

surfaces. The phagocytes will then initiate eradication of the bacteria through phagocytosis, resulting in killing of the bacteria.

Indeed, killing of *E. faecalis* by whole blood was observed in the assays performed (**section 4.4.3**). Regardless of bacterial gene deletion, blood donor and assay conditions, the bacterial numbers were reduced one-hour post inoculation with few exceptions. The main trend was therefore efficient killing of the bacteria in blood, which further confirms the resistance of *E. faecalis* towards the C5b-9 membrane attack complex, as no killing was observed in serum.

Following a three-hour incubation, the results were varying, showing both killing of the bacteria as well as resistance to killing. The results are not straight forward to discuss and interpret, as the assay might need further optimizations in order to give clear results. Firstly, reproducibility was a challenge, and the large variations observed were highly donor-dependent. **Figure 4.4.3.2** presents separate results from donor one and donor two following a three-hour incubation of bacteria grown to stationary phase of growth diluted 10x. These results show major variations, as viability in the blood of donor one is much lower in comparison to the second donor. In addition to this, the donors demonstrated contradicting results, as donor one resulted in significantly higher viability of the ΔLpmO than the wild type, whereas donor two showed significantly higher viability of the wild type. Furthermore, another donor (see **Appendix D**) performed highly efficient killing which deviated from the results obtained with other donors. These major variations may be due to upregulated immunity of the donor, for instance due to an approaching or ongoing disease. Healthy individuals were a requirement and the donors were obliged to report any outbreak of disease the following days post blood donation. However, none of the donors reported such health declines.

Another factor that could contribute to variations are the experimental conditions. The vertical rotator utilized for incubation of the bacteria in blood might not be optimal, as the movement of the rotator might result in clogging of the tubes, thereby hindering free movement of the components. Another aspect is the speed of the rotator, as too high speed might lead to mechanically stressed cells. Optimally, the rotator should mimic the movement of the blood within the human body to recreate a realistic, natural environment of an *E. faecalis* infection. Furthermore, optimizations with regards to growth phase and bacterial numbers are also important aspects of the assay. Too high bacterial numbers might mask the effect of the whole blood system, thus preventing the effect of the protein of interest in promoting or reducing bacterial killing from being observed. Moreover, as the bacteria might attach to each other during incubations in blood, sufficient vortexing of the bacteria prior to plating is crucial in

order to ensure colonies on the plate caused by single-growing bacteria. If not, the enumeration of CFU/ml will be underestimated, and the calculated viability will be too low.

Due to these variations, differences in viability of the wild type and gene deletion strains are hard to evaluate and the putative effects of the proteins cannot be confirmed. However, the growth phase of the bacteria seems to affect the viability as the highest viability is seen for bacteria grown to stationary phase of growth with a three-hour long incubation. The viability is much lower for bacteria in exponential phase of growth, following the same assay conditions, in comparison. The LpmO is only expressed in stationary phase of growth, which could link these results to the hypothesis of a stress-induced expression of LpmO. However, the results of the assay exhibit large variations and whether expression of LpmO increases the viability of the bacteria, cannot be determined based on the data provided. Neither can the effect of the two proteins *Ef*EndoE and *Ef*Endo18A. *Ef*EndoE contains both a GH20- and GH18-module, and both catalytic modules have been shown to possess endoglycosidase activity directed at cleaving off glycans from N-glycosylated proteins, such as immunoglobin G. This results in inhibition of immunoglobin-mediated opsonophagocytosis and has been shown to increase survival of *S. pyogenes* in blood (Collin et al., 2002; Collin & Fischetti, 2004). Unfortunately, this role of *Ef*EndoE cannot be confirmed based on the blood assays conducted in this study.

5.4 Concluding remarks and future prospects

The focus of this study was the chitinolytic machinery of *E. faecalis V583* and its role in virulence.

Characterization of the wild type and gene knockout strains was conducted based on morphology, growth rates on several substrates and enzymatic activity. Light microscopy revealed no apparent differences between the strains with regards to morphology. Characterization of growth on the soluble substrates GlcNAc and $GlcNAc_2$ demonstrated slower growth of ΔChi than the wild type, thereby indicating the involvement of the chitinase in degradation of chitin. However, when the strains were grown on β-chitin, the differences between the wild type and chitinase-knockout were abolished, suggesting a different functional role of the protein than chitin hydrolysis. Measurements of chitinase activity further corroborated this, as no activity was reported for the wild type or any of the other knockout-strains. The proteins *Ef*EndoE and/or *Ef*Endo18A are however most likely involved in chitin degradation, as N-acetylhexosaminidase activity was demonstrated for all strains except for the ΔDeglycosidase when cultivated in β-chitin. This activity is likely to be caused by *Ef*EndoE,

which possesses hexosaminidase-activity. Further studies should include single knockouts of the *ef0114* and *ef2863*-genes for individual characterizations of each protein, in order to determine their exact role in chitin degradation and/or virulence.

It would be very interesting to study the binding abilities of the bacteria to the chitin particles in more detail. Fluorescence microscopy could be utilized to check the individual strains' binding abilities to the particles. A strategy could be to stain the wild type and knockout strain with different dyes, before incubating both strains in 1% β-chitin, followed by analysis of their individual binding abilities by fluorescence microscopy. Differences between the wild type and the ΔChi-ΔLpmO double knockout strain would be of particular interest, but this would require verification of the ΔChi-ΔLpmO-mutant.

Transcriptional analysis of *E. faecalis V583* upon exposure to serum showed upregulation and induced expression of the LpmO. Bacterial viability of the wild type and LpmO-knockout was therefore assessed in pooled normal human serum, revealing high resistance towards serum, regardless of the gene deletion. Further assessment was therefore conducted in human whole blood. Although the whole human blood assay did not disclose the specific roles of the proteins in survival in blood, the general findings are that the complete set of immune components perform more efficient killing of *E. faecalis* than the complement proteins alone. Resistance towards killing was however observed following longer incubation periods (three hours), particularly for the bacteria in stationary phase of growth. Further optimizations of the assay are required in order to improve reproducibility and reduce the obtained variations. By including more donors and increasing the number of biological replicates, perhaps the proteins' proposed effect on survival in blood will be uncovered.

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7 Appendices

7.1 Appendix A

7.1.1 Intracellular survival of *Enterococcus faecalis*

Extensive studies of *Enterococcus faecalis* have been conducted under infection-relevant growth conditions such as urine, blood, bile and antibiotics. The bacterium's ability to internalize and survive in human host cells is of huge interest, as the bacteria may spread systemically upon internalization and survival within macrophages (Koch et al., 2004). Previous studies have shown that *E. faecalis* can survive within macrophages for extended periods of time (Gentry-Weeks et al., 1999; Zou et al., 2014; Zou & Shankar, 2014; Zou & Shankar, 2016).

Several bacterial effectors have been proposed as promoters of enterococcal survival and persistence within professional phagocytes, including macrophages. For instance, *E. faecium* possesses an operon encoding genes with homology to the TIR-domain of toll/interleukin-1 receptor (TIR)-domain genes, which promote bacterial survival within macrophages and blood (Wagner et al., 2018). Also in *E. faecalis*, a TIR-domain containing protein (tcpF) is more common in isolates from urinary tract infections than in human faecal flora and has been shown to promote intracellular survival, further affirming the role of TIR-like proteins in virulence (Kraemer et al., 2014). Toll like receptors together with interleukin-1 receptors form a receptor superfamily and all receptors of this family share a so-called toll/interleukin-1 receptor-domain (Rana et al., 2013). Bacterial TIR-domain-containing proteins structurally mimic host domains, and thereby negatively interfere with toll-like-receptor (TLR)-signalling through adaptor blockade or adaptor degradation (Wagner et al., 2018). This immunoevasive property allows the bacteria to persist at the site of infection and possibly spread systemically through the bloodstream.

7.1.2 THP-1 cells

Immune response can be studied in an *in vitro* model system using cell lines as tools. The THP-1 cell line is a model for human monocytes and are derived from the peripheral blood of a boy with acute monocytic leukaemia (Bosshart & Heinzelmann, 2016). The cells are immortalized and monocyte-like, in that they act as an *in vitro* model for the primary monocytes from peripheral blood. However, the THP-1 cells only represent the human peripheral blood monocytes, thus may behave differently when cultured than what monocytes would do ex vivo.

In this study, the THP-1 cells were utilized as a model system to study the survival of *E. faecalis* in human-like cells. The cells were grown in the MIDI 40 CO2 Incubator at 37°C. This incubator was designated for cell studies only, to reduce the risks of potential contaminations.

All work with the THP-1 cell line were done under sterile conditions and thus at a laminar flow workbench. Prior to start, the UV-light in the laminar flow workbench was left on for minimum 30 minutes to prevent any contaminations.

THP-1 medium

- 1 bottle RPMI 1640 (without phenol red), 500 ml
- 5 ml Penicillin-Streptomycin (50 units/mL penicillin, and 50 µg/mL streptomycin)
- 50 ml FBS
- 5 ml 1 M HEPES
- 5 ml 1 M Sodium pyruvate
- 2.8 ml Glucose

All components were mixed in the RPMI 1640-bottle, and Penicillin-Streptomycin is always added first. The medium was stored at 4°C.
Based on the experimental plan, antibiotic was omitted from the experimental conditions.

7.1.2.1 Counting THP-1 cells

The cell number is of crucial importance in cell studies. If the cell number becomes too high, the cells will die due to oversaturation and lack of nutrients. To ensure cell culture with good viability, counting the cells is therefore essential. For the THP-1 cell line, 10-15% dead cells are accepted. The method used to count the cells is trypan blue staining. This cell stain is impermeable and will only bind to dead cells, as they are permeable and are able to take up the dye. Viable cells do not take up impermeable dyes and will therefore not be stained.

Materials:

- Incubator, MIDI 40 CO_2 Incubator, 37°C
- Cellstar® Tubes, 50 ml
- Centrifuge, Heraeus™ Multifuge X1R
- Liquid Aspiration System, Vacusafe Comfort
- Vacuum Hand Operator, Vacuboy
- Sterile glass pipets
- THP-1 medium

- Eppendorf tube, 1.5 ml
- Trypan Blue Stain 0.4%
- Automated Cell Counter, Countess™ II
- Countess™ Cell Counting Chamber Slides

Method:

The THP-1 medium was preheated at 37°C. The THP-1 cells were taken out of the incubator and transferred to a 50 ml Cellstar® Tube. The cells were centrifuged at 1300 rpm for 6 minutes at 24°C to collect the cells. The supernatant was removed using the liquid aspiration system, and the cell pellet was resuspended in 10 ml THP-1 medium. 10 µl of the cell suspension was transferred to an Eppendorf tube, followed by 10 µl of Trypan blue which was added to the cells by pipetting up and down three times. 10 µl were then loaded onto the chamber of the Countess™ slide. The Countess™ slide was placed in the Countess™ II Automated Cell Counter Machine, the light and focus were adjusted, and the machine gave the total concentration of cells, the number of living cells and the number of dead cells as output.

7.1.2.2 Splitting THP-1 cells

Cell culture needs to be split to give the cells fresh media to grow on, and to prevent the cells from dying. The number of cells for the THP-1 cell line should not exceed one million cells/ml.

Materials:

- Incubator, MIDI 40 CO2 Incubator, 37°C
- THP-1 medium
- Cellstar® Tube, 50 ml
- Centrifuge, Heraeus™ Multifuge X1R
- Liquid Aspiration System, Vacusafe Comfort
- Vacuum Hand Operator, Vacuboy
- Sterile glass pipets
- Falcon™ Tissue Culture Treated Flask, 250 ml, 75 cm^2 culture area

Method:

The THP-1 medium was preheated at 37°C. The THP-1 cells were taken out of the incubator and transferred to a 50 ml Cellstar® Tube. The cells were centrifuged at 1300 rpm for 6 minutes at 24°C to collect the cells. The supernatant was removed using the liquid aspiration system, and the cell pellet was resuspended in 10 ml THP-1 medium. If needed, the cells were counted as explained in **section 7.1.2.1**. A new 75 cm^2 flask was marked with the name of the cells and

the passage number, and 20 ml THP-1 media was added to the flask. 2 ml of the THP-1 cells were then transferred from the Cellstar® tube to the new flask.

7.1.2.3 Freezing down THP-1 cells

Freezing of eukaryotic cells should be performed slowly, to prevent intracellular formation of ice crystals that will damage the cells. Slow cooling will allow sufficient efflux of water, so that the chance of intracellular ice formation is minimized. From one big cultivation flask (almost confluent) there will be enough cells for about 4-6 vials (cryogen tubes, 1 ml in each). The number of cells should be somewhere between 1 and 3 million cells/ml.

Materials:

- Fetal Bovine Serum, 500 ml
- Cellstar® Tube, 50 ml
- Centrifuge, Heraeus™ Multifuge X1R
- Liquid Aspiration System, Vacusafe Comfort
- Vacuum Hand Operator, Vacuboy
- Sterile glass pipets
- CryoPure Tube, 1.8 ml
- Dimethyl sulfoxide (DMSO)
- Box with ice

Method:

The FBS was transferred from the freezer to the refrigerator the day before seeding, so that it was defrosted and cold when used to freeze down the cells the next day.

The THP-1 cells were transferred to a 50 ml Cellstar® Tube and centrifuged at 1200xg for 6 minutes at 24°C. The supernatant was removed using the liquid aspiration system, and the cell pellet was resuspended in cold FBS. 900 µl FBS was added for each cryogen tube to be made. Cryogen tubes were marked with name of the cells, initials, date and which passage number they were from. The cell suspension was then transferred to the cryogen tubes, before 100 µl DMSO was added to a total volume of 1 ml in each tube. DMSO was added in the middle of the cell suspension by pipetting once, before the cryogen tubes were quickly put on ice. The tubes were then moved to the -20 °C freezer where they were kept for 1 hour, before they were moved to -80°C.

7.1.2.4 Thawing THP-1 cells

THP-1 cells should be thawed quickly, to ensure that a high proportion of the cells survive the procedure. The thawing should be done rapidly to prevent water from recrystallizing inside the cells. To increase the chance of cell survival, the media is added extra FBS as it's a rich supplement that will help the cells recover more quickly.

Materials:

- Cellstar® Tube, 15 ml
- THP-1 medium supplemented with 20% FBS (v/v)
- Centrifuge, Heraeus™ Multifuge X1R
- Falcon™ Tissue Culture Treated Flask
- Microscope
- THP-1 medium supplemented with 10% FBS (v/v)

Method:

1. The cells were gently transferred to a 15 ml tube containing THP-1 medium supplemented with 20% FBS to wash off DMSO.
2. The cells were washed by centrifugation for 5 minutes at 1000xg.
3. The cells were resuspended in THP-1 medium supplemented with 20% FBS and were transferred to a small flask.
4. The cells were checked the next day to ensure good growth and low levels of stress. If the cells were growing well, they were transferred to another flask using the normal THP-1 medium supplemented with 10% FBS (**Section 7.1.2**)

7.1.2.5 Internalization assay

Materials:

- Phorbol 12-myristate 13-acetate (PMA)
- Assay plates
- Bacteria
- Antibiotic
- 1X PBS
- Centrifuge, Heraeus™ Multifuge X1R
- Saponin
- Microtiter plates
- BHI-plates

Method:

1. THP-1 cells are differentiated into macrophages by addition of Phorbol 12-myristate 13-acetate (PMA). PMA is added to the THP-1 media to a final concentration of 25 nM, in which the THP-1 cells are resuspended after being counted.
2. The cells are then transferred to assay plates. Depending on the size of the plate, different amounts of cells are seeded to the individual wells. 1-5x10^5 cells are seeded in each well in a 24-well plate, 5x10^5-1x10^6 cells/well in a 12 well plate, 3x10^6 cells/well in a 6-well plate, and for a 10 cm plate normally 10-12x10^6 cells are seeded to each well. The cells will be differentiated in the wells for 48 hours.
3. The differentiated THP-1 cells are infected with the wild type and deletion strains at a certain multiplicity of infection (MOI). Bacteria are added accordingly and the assay plates are incubated for one hour at 37°C.
4. Following the infection step, the extracellular bacteria are killed by addition of antibiotic and the assay plates are incubated for one hour at 37°C.
5. Following antibiotic treatment, the cell suspensions are centrifuged and the supernatants are removed. Extracellular bacteria may be quantified by plating the bacteria from the supernatants.
6. The cells are washed once with 1X PBS. 1 ml Saponin is added to the cell pellets and the cells are incubated at 37°C for 5 minutes in order to lyse the cells.
7. The cell suspensions are pipetted vigorously to ensure efficient cell lysis. Serial dilutions are then prepared in microtiter plates in 1X PBS for plating on BHI-plates, in order to quantify the number of viable, uptaken bacteria.

7.1.3 Minimum Inhibitory Concentration (MIC)-assay

The original plan involved infection of differentiated THP-1 cells with the wild type and gene deletion strains at a certain multiplicity of infection (MOI), followed by antibiotic treatment in order to kill the extracellular bacteria, and finally evaluation of the number of viable, uptaken bacteria through enumeration. These planned internalization assays require that the extracellular, non-internalized bacteria are killed prior to cell lysis, in order to quantify the number of viable, internalized bacteria only. Several antibiotics were therefore tested to determine the MIC necessary to kill the bacteria.

Materials:

- Overnight culture of the wild type
- BHI-medium
- Sarstedt tube, 13 ml
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R
- RPMI supplemented with 5% BHI (v/v) (RPMI/BHI)
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes
- Antibiotic
- Microtiter plate
- Sealing tape
- Multiskan™ FC Microplate Photometer
- BHI-plates
- Incubator, Termaks, 37°C

Method:

Day 1

One single colony of the wild type was inoculated in 10 ml BHI at 37°C in ambient air with agitation (230 rpm).

Day 2

The overnight culture was washed and resuspended to OD_{600nm} = 0.4 in RPMI containing 5% bacteriological media (RPMI/BHI) in order to support the bacterial growth. The washing was performed by centrifugation at 4255xg for 15 minutes and the pellet was washed once in 10 ml RPMI/BHI, before being resuspended in 1 ml RPMI/BHI. The bacteria were gradually added to a new tube containing RPMI/BHI until reaching OD_{600nm} 0.4 (approximately 85 µl bacterial suspension to 6 ml RPMI/BHI). The bacterial concentration was then adjusted to 5 x 10^6 CFU/ml in RPMI/BHI and placed aside.

The concentration of antibiotic was adjusted 10x higher than the working concentration.

The assay was set up in triplicates, with three wells per antibiotic concentration to be tested. Two additional parallels were included for plating after two hours and twenty-four hours of incubation, resulting in a total of five rows of parallels on the plate. The assay also included

controls with no bacteria and with no bacteria or antibiotic for all the examined concentrations. The setup is shown in **figure 7.1.3.1**, with an example of concentrations of antibiotic.

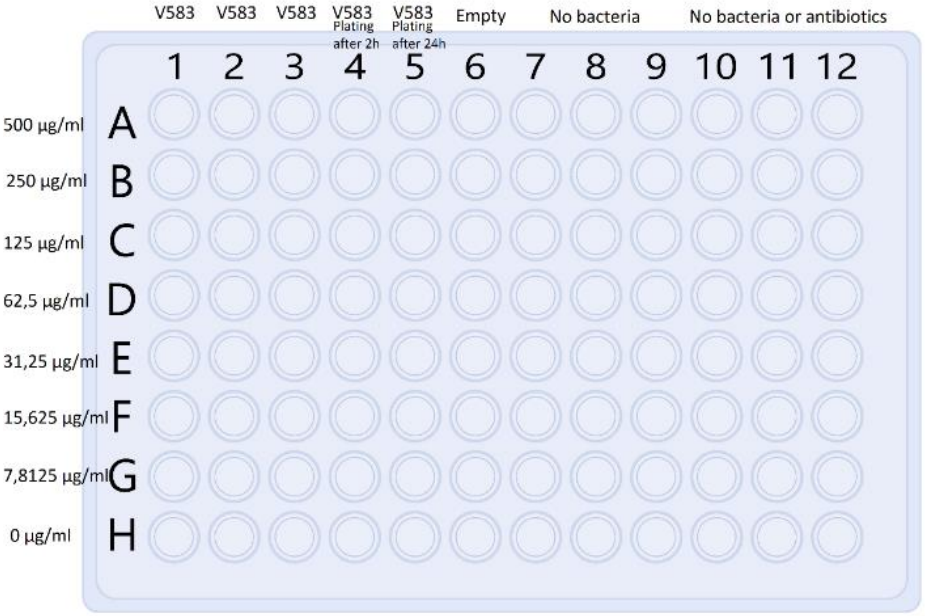


Figure 7.1.3.1 Example of the Minimum Inhibitory Concentration (MIC) assay setup. *Figure template adapted from Sigma Aldrich (2019).*

Each well contains 10 µl of 5 x 10^6 CFU/ml bacteria, 10 µl of antibiotic (10x) and 80 µl RPMI/BHI. This gave final concentrations of 5 x 10^5 CFU/ml bacteria and 1x of the antibiotic. The microtiter plate was covered, sealed with parafilm and placed in the incubator at 37°C with agitation (230 rpm). After two hours, the plate was taken out and one of the parallels was plated onto BHI plates to find minimum inhibitory concentration (MIC). The microtiter plate was then put back in the incubator overnight, and 24 hours after inoculation the assay was ended by plating another parallel on BHI plates and measuring the OD_{595nm} with the plate reader.

The plan of the designed MIC-assays is depicted in **table 7.1.3.1.** In the cases where to antibiotics were mixed, the concentration of Gentamicin was held constant (2500 µg/ml) while the other antibiotic was diluted twofold accordingly.

Table 7.1.3.1 The different antibiotics and the examined concentrations used in the MIC-assays.

MIC-assay	Gentamicin (µg/ml)	Tetracycline (µg/ml)	Spectinomycin (µg/ml)	Rifampicin (µg/ml)
1	500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0			
2	3000, 2000, 1500, 1000, 500, 0			
3	3000, 1000, 0			
4	2500	6.25, 3.125, 1.5625, 0.78125, 0		
5	2500		500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0	
6	2500		3000, 1500, 750, 375, 187.5, 93.75, 46.875, 0	
7	2500			50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0

The results of the performed MIC-assays are presented in **figure 7.1.3.2.**

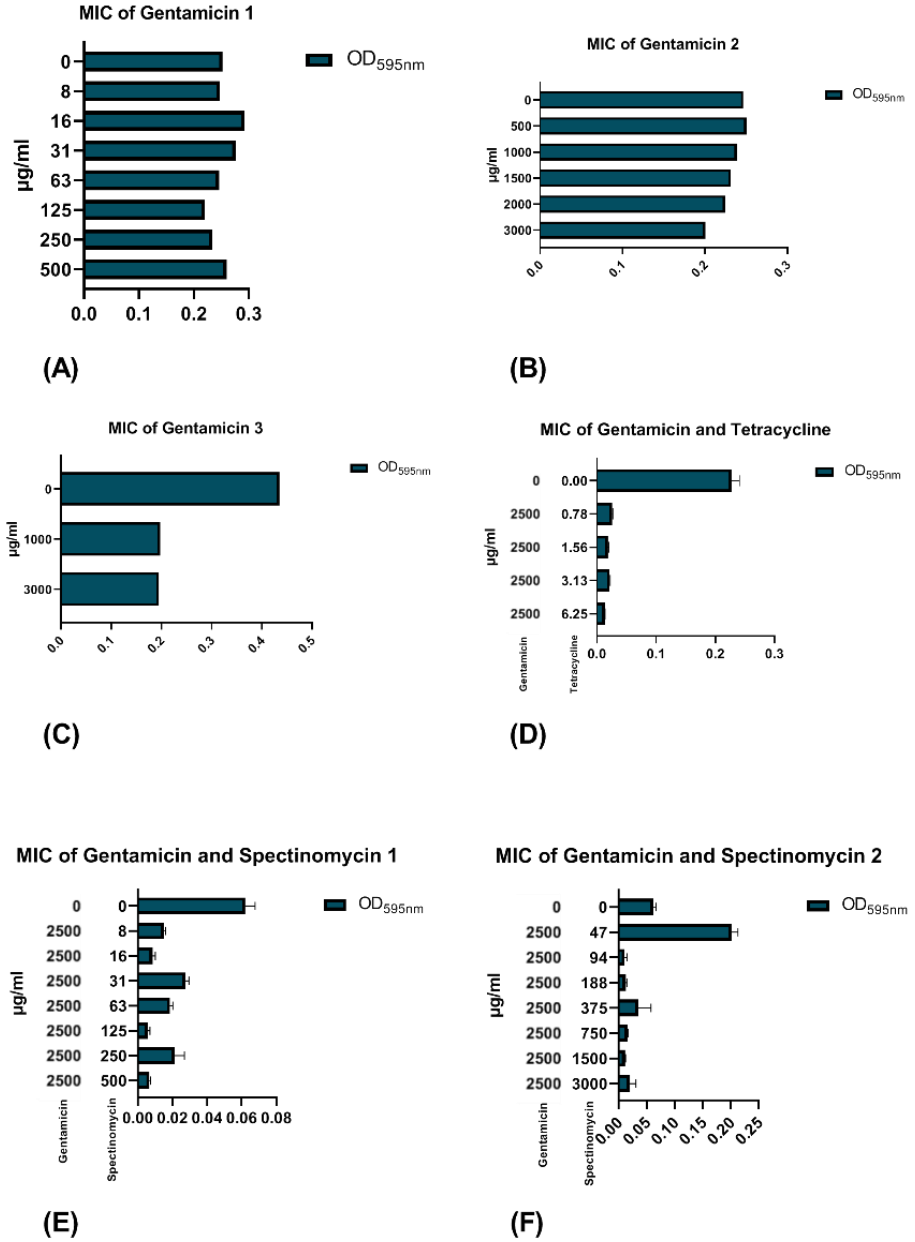


Figure 7.1.3.2 MIC-assays with various types of antibiotics conducted on the wild type strain. The x-axis shows the measured absorbance at 595 nm, and the y-axis presents the different concentrations of the antibiotics that were tested. (A) First MIC-assay for Gentamicin, with twofold dilutions starting at 500 µg/ml. (B) Second MIC-assay for gentamicin. (C) Third MIC-assay for gentamicin. (D) MIC-assay for Gentamicin and Tetracycline combined. (E) First MIC-assay of Gentamicin and Spectinomycin combined. (F) Second MIC-assay for Gentamicin and Spectinomycin combined.

Treatment with Gentamicin was not sufficient to kill *E. faecalis* (**figure 7.1.3.2**, panel A, B and C**).** Even at very high concentrations (3000 µg/ml), the bacteria resisted killing, although the treatment resulted in 50% reduction in growth. Thus, to obtain a more efficient killing Gentamicin was combined with a second antibiotic such as tetracycline (**figure 7.1.3.2**, panel D) and spectinomycin (**figure 7.1.3.2**, panel E and F), which resulted in improved killing.

However, plating of the bacteria showed bacterial viability, suggesting a bacteriostatic rather than bactericidal effect of the drugs.

In addition to the antibiotics mentioned so far, Rifampicin was also tested in combination with Gentamicin. This assay, however, only included plating of the bacteria at the different time points (2 hours and 24 hours) and did not include absorbance measurements, thus is not included in **figure 7.1.3.2.** Similarly to the other performed MIC-assays, bacterial growth occurred on all the plates with bacteria treated with Rifampicin and Gentamicin as well.

7.1.4 THP-1 internalization assay

Because of unsuccessful MIC-assays and none-sufficient killing with the suggested antibiotics, quantification of the internalized bacteria was not possible through the proposed internalization assays. A solution to this problem was treatment of the cells with Cytochalasin D (CytD); a cell permeable fungal toxin that polymerizes the actin of the cytoskeletal network. The internalization assay is then set up both with and without addition of CytD. In the cell suspension with CytD, bacteria will only be attached to the cell surface as they are unable to enter the cell. Without presence of CytD, the bacteria will be both attached and internalized. The number of internalized, viable bacteria can then be calculated by subtracting these numbers. However, this assay was not performed as it was decided to conduct more relevant *ex vivo* assays in whole blood rather than *in vitro* assays in the THP-1 model system (see **section 4.4.3** for the results).

7.2 Appendix B

Whole genome sequencing

Due to uncertainty to whether the *ef0361* and *ef0362* genes were correctly knocked out in the ΔChi-ΔLpmO strain, whole genome sequencing was planned to verify the presence of these mutations. The MagAttract® HMW DNA Kit was utilized for extraction and purification of high molecular weight DNA.

Materials:

- Overnight culture of ΔChi-ΔLpmO
- Sarstedt tube, 13 ml
- BHI-medium
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R

- The MagAttract® HMW DNA Kit
- Magnetic Rack
- Buffer P1 (50 mM Tris, 10 mM EDTA, pH 8.0)
- FastPrep®-24 Tissue and Cell Homogenizer
- FastPrep-tubes
- Glass beads
- Eppendorf Thermomixer

Method:

Preparation of overnight culture:

An overnight culture of ΔChi-ΔLpmO was prepared and grown overnight in 10 ml BHI at 37°C with agitation (230 rpm).

Harvest bacterial cells from overnight culture:

The number of bacterial cells to be harvested should be up to 2 x 10^9. The overnight culture was therefore diluted in BHI to $OD_{600nm} \approx 0.3$ and 0.4 in two new culture tubes. Subsequently, 4.5 ml of these diluted bacterial suspensions were centrifuged at 4255xg for 10 minutes at room temperature to harvest the bacterial cells. The supernatants were removed and discarded, and the bacterial pellets were resuspended in 900 µl Buffer P1.

Extraction and purification of DNA from harvested cells:

The extraction and purification of DNA from the harvested cells was performed following the manufacturer's protocol "Manual Purification of High-Molecular-Weight Genomic DNA from Gram-Positive Bacteria" from the MagAttract® HMW DNA Handbook, involving some changes to the procedure.

Since *Enterococcus faecalis* is resistant to lysozyme (Hébert et al., 2007), the bacterial cells were ruptured with lysing matrix particles in a FastPrep-24 instrument. The bacterial pellets resuspended in 900 µl Buffer P1 were transferred to FastPrep tubes, containing approximately 0.5 g glass beads. The tubes were run at 6.5 m/s for 30 seconds three times, with a one-minute interval between each run, to mechanically lyse the cells. Hereafter, the protocol was followed as written until the elution step. Since the purified DNA was to be used in enzymatic downstream applications, 100 µl of dH_2O was added to each tube to elute the DNA.

The concentrations of the eluted and purified DNA were measured with the Eppendorf D30 BioPhotometer®, as described in **section 3.6.2**.

The sample with the highest dsDNA concentration measured, out of the two samples harvested at $OD_{600nm} \approx 0.3$ and 0.4, was chosen for sequencing. 50 µl of the eluted DNA was transferred to a new Eppendorf tube, which in turn was to be sent to MicrobesNG (England) for whole genome sequencing.

7.3 Appendix C

Optimization of sinking time

Presence of chitin-particles will interfere with the absorbance-measurements as the light passes through the sample. Required sinking times were therefore established, which is the amount of time the chitin-particles are allowed to sink to the bottom of the flask for OD_{600nm} to stabilize and become reproducible.

Since growth on β-chitin was monitored over seven days, the chitin-particles might change over this period and behave differently due to swelling of the particles for instance. Therefore, sinking times were determined both in fresh 1% β-chitin medium and in medium that had been incubated with bacteria for five days.

Materials:

- Overnight culture of the wild type
- Sarstedt tube, 13 ml
- GLM17ent-medium
- Incubator, New Brunswick™ Scientific Innova® 44, 37°C
- Centrifuge, Allegra X-30R
- 1X PBS
- LM17ent-medium
- LM17ent supplemented with 1% β-chitin (w/v)
- Ultrospec 10 Cell Density Meter
- Disposable cuvettes

Method:

1. An overnight culture of the wild type was prepared and grown overnight in 10 ml GLM17ent at 37°C with agitation (230 rpm).
2. The next day, the overnight culture was centrifuged at 4255xg for 10-15 minutes and washed once in 1X PBS, before being resuspended in 1 ml LM17ent.

3. The bacterial suspension was diluted to $OD_{600nm} \approx 0.2$ in 50 ml 1% β-chitin medium. Given the difficulties in measuring a stable absorbance in the β-chitin medium, the bacterial suspension was instead of being added directly to the β-chitin-medium, first adjusted to the correct OD_{600nm} in a tube with 5 ml LM17ent-medium.
4. Samples were taken out in triplicates every minute to measure OD_{600nm} until the absorbance had stabilized.

The resulting absorbance measurements are presented in **table 7.3.1.**

Table 7.3.1 Optimization of sinking time prior to absorbance measurements (A_{600nm}) of bacterial culture grown on β-chitin. Absorbance measurements of five days old and freshly made 1% β-chitin media were performed in triplicates, both in media with and without wild type bacteria.

Time (min)	5 days old media		Fresh media	
	A_{600nm}		A_{600nm}	
	Only media	Media with bacteria	Only media	Media with bacteria
0	1,54	2,00	1,25	1,40
	1,35	1,73	1,21	1,26
	1,23	1,79	0,95	1,20
1	1,06	0,96	0,97	1,02
	1,08	1,00	0,82	0,86
	1,04	1,00	0,77	0,86
2	0,79	1,02	0,44	0,81
	0,95	1,11	0,67	0,71
	0,93	1,09	0,58	0,67
3	0,72	0,94	0,43	0,62
	0,75	1,01	0,36	0,59
	0,72	0,94	0,45	0,69
4	0,59	0,97	0,35	0,62
	0,63	0,99	0,26	0,52
	0,62	0,99	0,26	0,54
5	0,56	0,96	0,27	0,65
	0,60	0,95	0,23	0,51
	0,60	0,96	0,24	0,55
6	0,45	0,94	0,22	0,51
	0,54	0,92	0,29	0,51
	0,54	0,94	0,25	0,45
7	0,53	0,90	0,23	0,49
	0,53	0,90	0,11	0,48
	0,47	0,92	0,31	0,53
8	0,58	0,91	0,19	0,45
	0,57	1,12	0,21	0,43

	0,59	1,35	0,11	0,45
9	0,46	0,87	0,22	0,46
	0,58	0,90	0,08	0,45
	0,54	0,85	0,14	0,44
10		0,86	0,11	0,45
		0,86	0,13	0,46
		0,82	0,14	0,46
11			0,14	
			0,12	
			0,11	

The optimization assay was concluded with a required sinking time of minimum 10 minutes in order for the absorbance measurements to stabilize and become reproducible.

7.4 Appendix D

Whole blood survival assay

Incubation of bacteria in exponential phase of growth diluted 10x from OD_{600nm} 0.4. was also performed in blood from a fifth donor, but due to deviating results compared to all other donors, this particular assay was not pooled with the others but is instead presented in a separate graph in **figure 7.4.1.** This assay was only performed with an one-hour incubation.

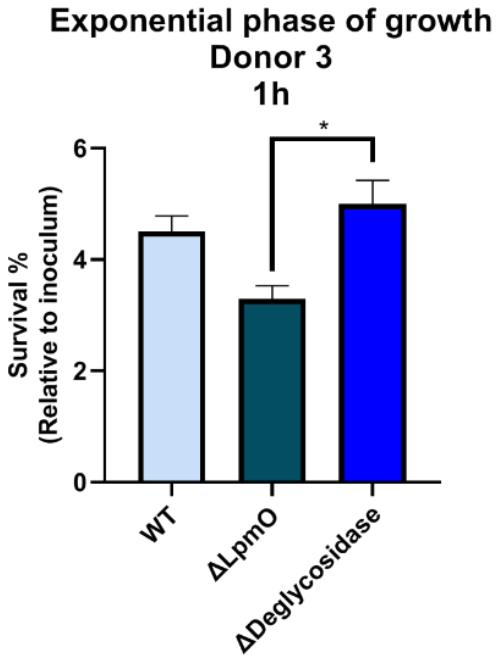
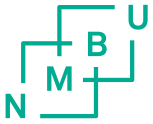


Figure 7.4.1 Whole blood survival assay of 10x diluted bacteria in exponential phase of growth for donor 3. WT, ΔLpmO and ΔDeglycosidase incubated in blood for one hour. (Number of donors: one; number of biological replicates: two; number of technical replicates: two).

The donor blood used in the assay of **figure 7.4.1** performed efficient killing of most of the bacteria, as the percentages of viability for all strains are remarkably low compared to all other assays conducted in this study. The percentages of the wild type, ΔLpmO and ΔDeglycosidase viability one hour post infection were 4.500%, 3.295% and 5.000%, respectively. There were no statistical significant differences between the wild type and ΔLpmO ($p=0.0629$), nor between the wild type and ΔDeglycosidase ($p=0.4971$). The viability of the ΔLpmO was significantly lower than the survival of the ΔDeglycosidase in the blood of donor 3 though, with a p-value of 0.0153. The surprisingly low viability detected in the blood of donor 3 and the high ability of the blood to clear the bacteria, might be caused by an upregulation of the immune system due to illness of the donor.



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