UiT The Arctic University of Norway

Faculty of Health Sciences

Characterization of competition between commensal A2 and clinical A1 strains of *Enterococcus faecium*

An *in vitro* competition study

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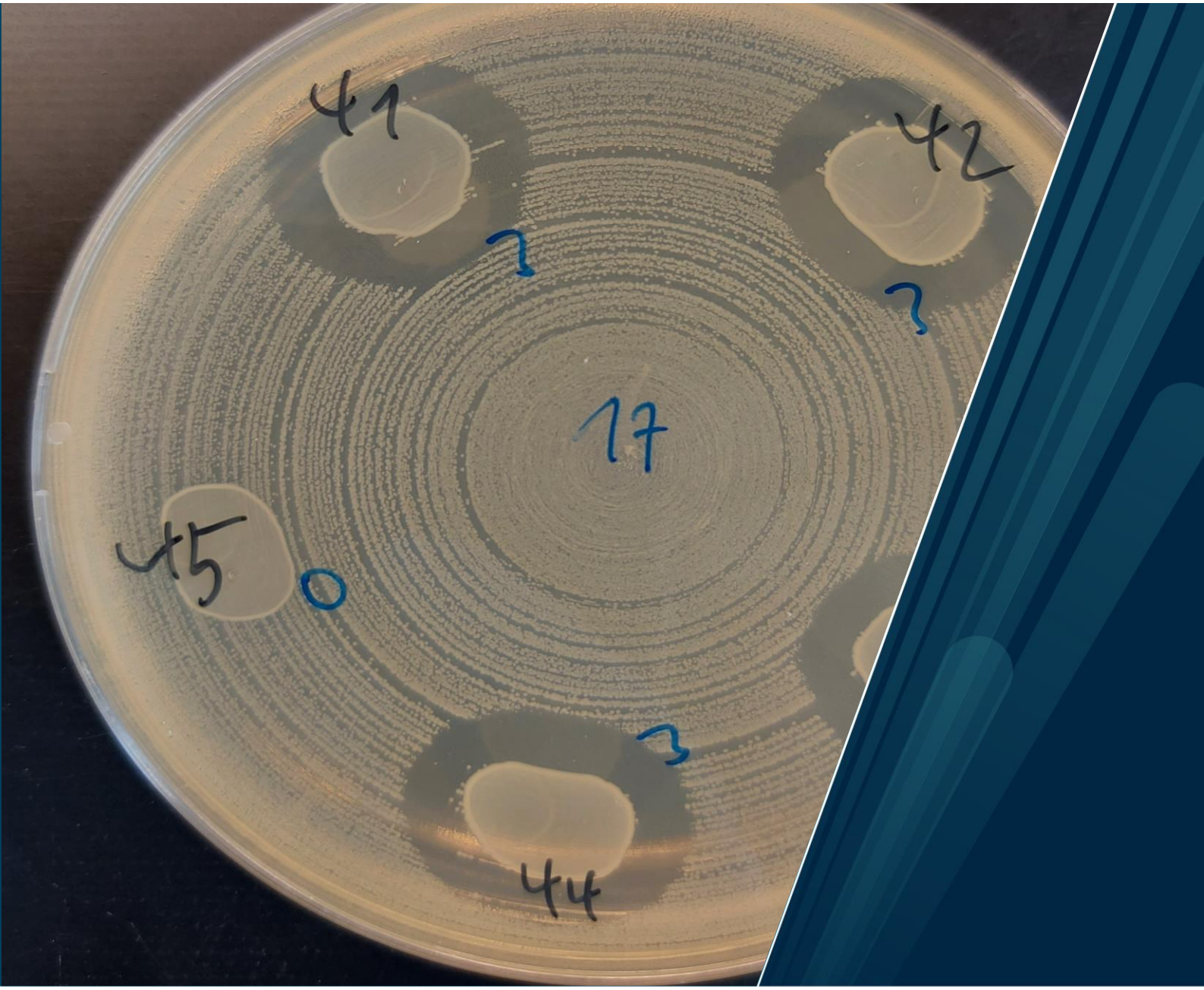
Master's thesis in Biomedicine, MBI-3911, May 2023



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Abstract

Enterococcus faecium (E. faecium) emerged from a gut commensal to one of the leading nosocomial multidrug-resistant pathogens. The species of *E. faecium*, has a deep phylogenetic split into commensal and clinical strains. The dominance of clinical strains during nosocomial infections is only insufficiently understood. Previous studies revealed competitive characteristics between the commensal and clinical clade.

This thesis aimed to study interaction and competition dynamics *in vitro* between a diverse range of different strains of commensal and clinical *E. faecium* clades. For the commensals, the focus was set on clade A2. Competitive growth on agar plates was carried out to determine inhibition grades between competing strains. Furthermore, the degree of inhibition mediated by bacterial supernatant combined with different stress treatments was investigated. Previous studies suggested proteinaceous and heat-stable secreted compounds, such as bacteriocins, which might be involved in mediating inhibition. On genome level, bacteriocins were predicted using the bacteriocin prediction database BAGEL4.

It was shown that diverse clinical clade A1 and commensal clade A2 *E. faecium* strains generally could outcompete each other in some cases. Clinical strains showed a higher inhibition frequency and most of them could be associated with hospital-leading lineages. Some commensal strains showed high inhibition and at the same time resistance of being inhibited towards a whole range of clinical strains. Most of these inhibitions could be associated with secreted proteinaceous, heat-stable compounds, most likely bacteriocins. However, a lot of bacteriocins or other secreted compounds which might mediate *E. faecium* inhibition remain unknown and further studies are needed.

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Abbreviations

AMR	Antimicrobial Resistance
BHI	Brain Heart Infusion
CC17	Clonal Complex 17
CFU	Colony-forming Unit
cgMLST	Core Genome Multilocus Sequence Typing
ECDC	European Centre for Disease Prevention and Control
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal Gene Transfer
K-res	Norwegian National Advisory Unit on Detection of Antimicrobial Resistance
LAB	Lactic Acid Bacteria
LPSN	List of Prokaryotic names with Standing in Nomenclature
MDR	Multidrug-resistant
MGEs	Mobile Genetic Elements
MLST	Multilocus Sequence Typing
MWCO	Molecular Weight Cut-Off
NORM	Norwegian Surveillance System for Antibiotic Resistance in Microbes
PBP5	Penicillin-Binding Protein 5
SNP	Single Nucleotide Polymorphism
ST	Sequence Type
VRE	Vancomycin-resistant enterococci
VR*Efm*	Vancomycin-resistant *Enterococcus faecium*
WGS	Whole Genome Sequencing
WHO	World Health Organization

1 Introduction

1.1 *Enterococcus* as a commensal

In 1899 the first isolate of the genus *Enterococcus* was described as a commensal inhabitant of the intestine which can cause pathogenicity [1-3]. The same year an isolate from an acute and lethal case of endocarditis was mentioned as well [4]. A micrococcus which grew mostly in diplococci and appeared white-greyish *in vitro* was described [4]. However, a century later *Enterococcus* became its own genus, after being classified with *Streptococcus* due to morphological similarities [5, 6]. Nowadays, it is known that *Enterococcus* is genetically separated from *Streptococcus* [7].

The genus of *Enterococcus* displays facultative anaerobic microorganisms living in various niches including the gastrointestinal tract (GI-tract) of terrestrial animals and humans, soil, plants, marine and fresh water, all kinds of food- like fermented or dairy products or meat and even hospital environments [8]. To inhabit numerous different environments, they need to be able to survive and grow in extreme conditions [8]. Enterococci are Gram-positive, ovoid, non-spore-forming, homo-fermentative bacteria producing lactic acid during carbohydrate fermentation [3]. Besides growing most often in pairs (diplococci), they can appear in single colonies or chains [3, 4].

In healthy adult gut environments, enterococci are usually <0.1% of commensal colonizers within the microbiome [9]. The GI-tract represents a dynamic system of continuously changing conditions like nutrient supply, pH, oxygen tension and host molecules like digestive enzymes, the reason why enterococci had to evolve mechanisms to withstand this challenging niche [10].

Within the genus of *Enterococcus* over 60 child taxa are known according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) [8]. *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*) are the most frequent species known for clinical relevance in hospitals [8].

1.2 Enterococcus faecium as an emergent clinical pathogen

Enterococcus coevolved with different hosts over hundreds of millions of years [8, 9]. With the terrestrialization of animals, the species evolved and pathogenic mechanisms were developed to adapt successfully to novel harsh environments [8, 9]. Especially the successful adaption to hospital environments makes *Enterococcus* an emergent nosocomial pathogen [11].

Antimicrobial resistance (AMR) within *E. faecium* is increasingly spreading in hospitals [8]. Multidrug-resistance (MDR) of *E. faecium* towards a wide spectrum of antibiotics particularly represent an emergent health concern worldwide [8].

In the past, *E. faecalis* was found worldwide in 80 to 90% of clinical enterococcal isolates, whereas only 5 to 10% were *E. faecium* [11]. In the United States, enterococci-associated outbreaks in hospitals occurred in two waves, in line with the introduction of different antibiotic types and resistance development [12]. From 1970 *E. faecalis* represented 90 to 95% of clinical enterococcal isolates, when third-generation cephalosporins were introduced [12]. Since 1990 vancomycin- and ampicillin-resistant *E. faecium* are responsible for the second wave causing most of the infections in hospitals not only in the United States, but also worldwide [12].

In Europe from 1994 to 2005 ampicillin-resistant *E. faecium* related hospital infections increased from 2 to 32% [13]. According to the European Centre for Disease Prevention and Control (ECDC), AMR percentages of *E. faecium* continued to increase in Europe until 2020 and are likely to increase in the future [14]. Within vancomycin-resistant enterococci (VRE) causing infections, vancomycin-resistant *E. faecium* (VR*Efm*) represent the major species [15]. In 2020 percentages of VR*Efm* being invasive were above 25% in 13 countries (Figure 1) [14].

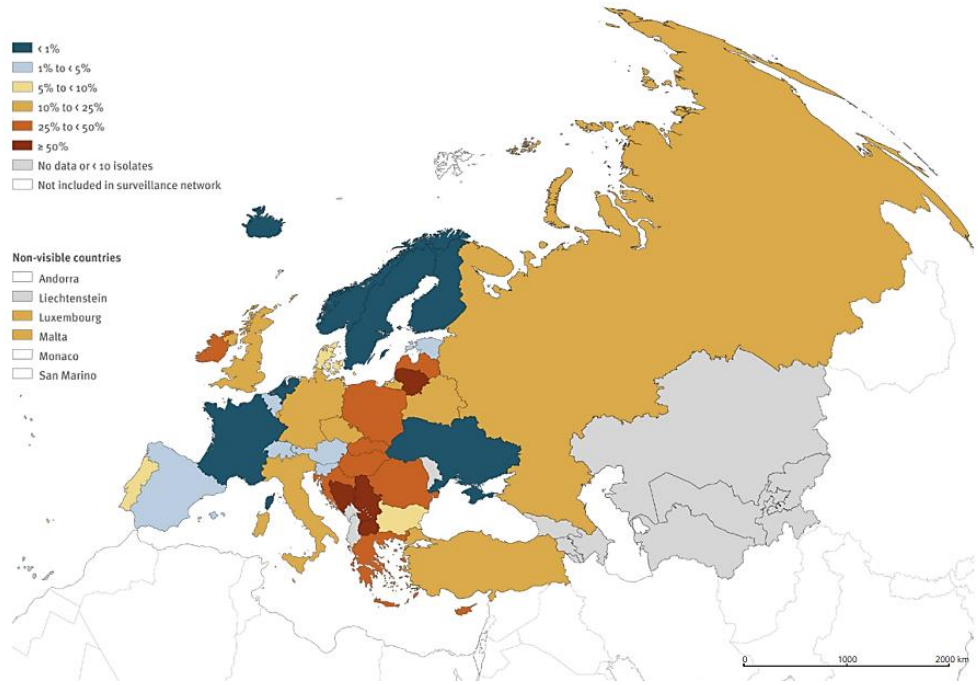

Figure 1: Percentage of invasive isolates of *E. faecium* resistant to vancomycin present in WHO European Region (EU/EEA), 2020. The level of vancomycin resistance per country is indicated according to the colour code to the left. Data sources: 2021 data from the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR, ©WHO 2021. All rights reserved) and 2021 data from the European Antimicrobial Resistance Surveillance Network (EARS−Net, ©ECDC 2021). Map production: ©WHO. Figure taken from yearly report of antimicrobial resistance surveillance in Europe by ECDC, WHO Regional Office for Europe/European Centre for Disease Prevention and Control (last access: 02.04.2023) [14]. Antimicrobial resistance surveillance in Europe 2022 – 2020 data with reprint permission under Creative Commons Attribution CC BY-NC-SA 3.0 IGO licence (https://creativecommons.org/licenses/by-nc-sa/3.0/igo/deed.en).

In 2017 the World Health Organization (WHO) listed VR*Efm* as a high priority within pathogens where research and development of new antibiotics for drug-resistant bacterial infections are needed [16]. WHO stated the length of treatment significantly increased during infections with VR*Efm* and limited treatment options are available [16]. A mortality rate higher than 40% for VR*Efm* is the consequence [16].

VRE in Norway In Norway, enterococci are the fifth most frequent genus isolated from bloodstream infections [17]. The Norwegian Surveillance System for Antibiotic Resistance in Microbes (NORM) represents a surveillance program for antimicrobial resistance in human pathogens in Norway [17]. Studies show VRE infections altered significantly over the last 10 years (Figure 2) [17]. From 2019 to 2020 VRE decreased within Norway (63%) as well as from 2020 to 2021 (55%) in contrast to many other European countries [17]. VR*Efm* represent the majority of Norwegian VRE isolates in hospitals [6, 17, 18].

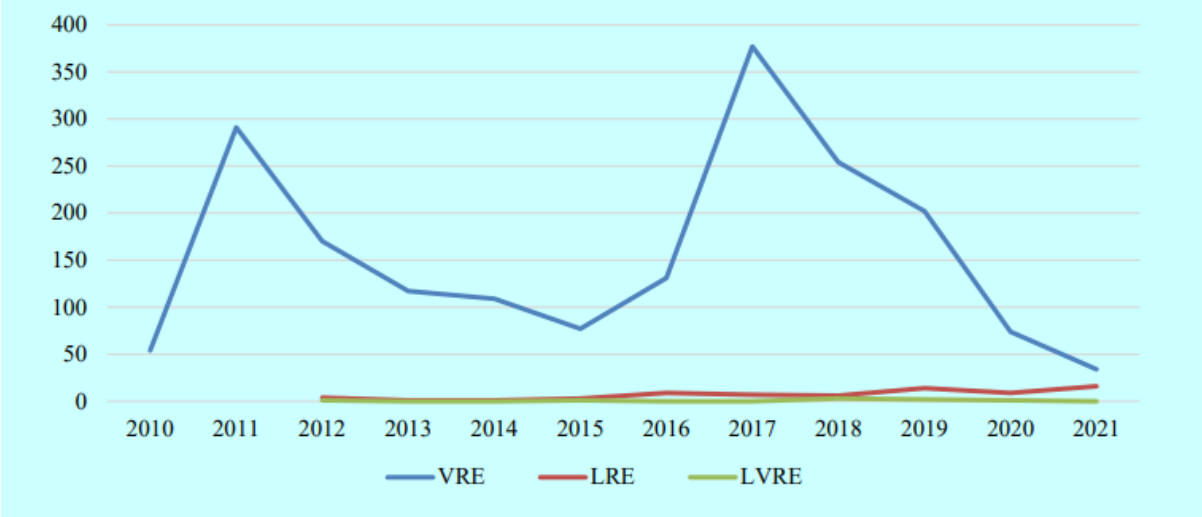

Figure 2: Number of isolates of vancomycin resistant enterococci (VRE), linezolid resistant enterococci (LRE) and both vancomycin and linezolid resistant (LVRE) enterococci in Norway 2010-2021. The y-axis shows the number of isolates and the x-axis the year. Figure taken from NORM/NORM-VET 2021 annual report [17] and reprinted with permission.

1.2.1 Evolution and phylogeny of *E. faecium*

Phylogenetic trees suggest that *E. faecium* arose later than *E. faecalis* and they locate to different branches (Figure 3) [9]. Enterococci evolved 425 to 500 million years ago when animals evolved and life on land was introduced [9]. Terrestrialization selected for mechanisms like hardening the cell wall or stress resistance of enterococci to survive in changing carbohydrate conditions in the host gut, which nowadays remarkably benefits *E. faecium* in hospitals to withstand challenging conditions like antibiotic use or disinfections on surfaces [9]. New enterococcal species evolved around 200 million years ago and colonization of a more diverse range of niches like different hosts drove this speciation [9].

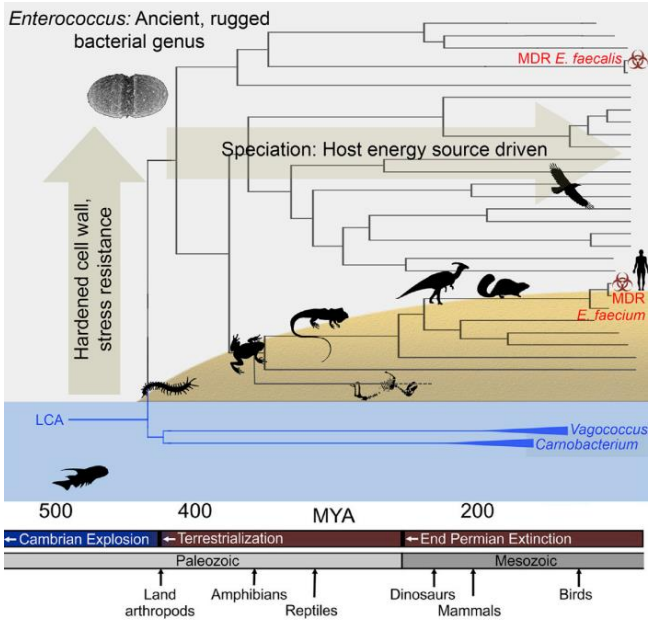

Figure 3: Evolution of *E. faecium* during the past 500 million years. Phylogeny of the genus enterococci is shown referring to different stages in earth history (Cambrian explosion, terrestrialization and end Permian extinction). *E. faecalis* and *E. faecium* evolved separate from each other and speciation drove enterococci to colonize more different hosts. Figure taken from [9] and reprinted with permission.

Around 3000 years ago, with the increase of agriculture and animal domestication, a deep phylogenetic split arose within the phylogenetic structure of *E. faecium*, which is until today one of the major characteristics of the species [7]. When sequences of *E. faecium* strains were investigated via whole-genome-sequencing two key subgroups (clades) were identified [7]. Clade B represents community-derived commensals living in healthy humans, whereas clade A includes both human- and animal-derived strains (Figure 4A) [7, 19]. About 75 years ago clinical strains separated from human commensal and animal-derived strains within clade A resulting in a new branching into subclades A1 and A2 (Figure 4A) [7]. Clade A1 perhaps evolved as a clone from clade A2 in hospital environments due to high recombination rates

[20]. *E. faecium* clade A1 strains are primarily associated with nosocomial hospital infections, visible in Figure 4A, since they are mostly isolated from infections [21]. Clade A2 mostly includes strains isolated from healthy humans and animals and can represent a reservoir for antibiotic-resistance and other genes [7, 19, 20]. The introduction of modern antibiotics coincides with the subclade separation [22].

In 2021 Daza *et al.* reinvestigated the *E. faecium* phylogeny [23]. The taxonomic relationship between isolates of different origin of *E. faecium* and *Enterococcus lactis* (*E. lactis*) from dairy origin were investigated via genomic-based approach [23]. Interestingly, this study revealed that *E. faecium* strains clade B grouped together with *E. lactis* strains in the phylogenetic tree (Figure 4B) [23]. Thus, clade B *E. faecium* strains should be considered *E. lactis* [23]. Considering the diverse characteristics of *E. faecium* clades is essential for future studies, as previous classifications have often neglected this aspect and lack representative isolates. [23].

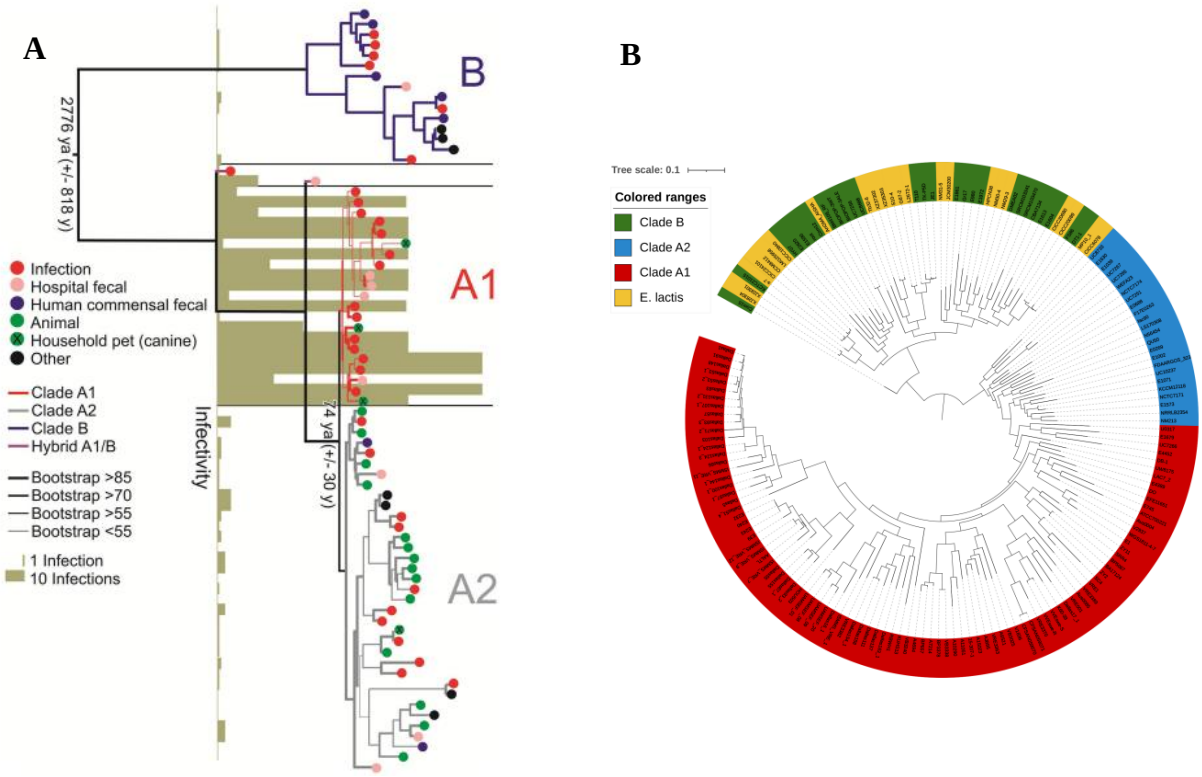

Figure 4: A: Phylogenetic tree of *E. faecium* displaying clade structure of the species. Commensal associated clade B (purple) and A2 (grey) is shown as well as clinical associated clade A1 (red). Color-coding depending on their isolation site (origin). Time is stated at the origins of the clades, when the split between the clades appeared in years ago (ya). Infectivity score is shown in grey-greenish indicating how many strains of a specific ST type were isolated from infection. Phylogenetic tree is based on alignments of DNA sequences of 1344 single-copy core genes in 73 *E. faecium* genomes. Figure taken from © 2013 Lebreton *et al.* [21] and reprinted with permission under Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported license (https://creativecommons.org/licenses/by-nc-sa/3.0/). **B: Phylogenetic tree of *E. faecium* after Daza *et al.* with *E. lactis* and *E. faecium* clade B strains clustered together.** *E. faecium* clade A1 strains are illustrated in red, clade A2 strains in blue, clade B strains in green and *E. lactis* in yellow. Maximum-likelihood tree is based on core genome alignment of 181 selected genomes. Figure taken from Daza *et al.* [23] and reprinted with permission under 1354458-1 licence ID. .

Multilocus sequence typing (MLST) For identification of specific *E. faecium* strains during clinical outbreaks or molecular long-term epidemiology studies of nosocomial strains MLST is used, a method based on DNA sequences from different strains and the comparison of their allelic profiles of internal fragments of seven housekeeping genes [3, 24]. Evolutionary MLST studies of clinical *E. faecium* strains revealed strains clustered together in a distinct genetic lineage termed clonal complex 17 (CC17) which were later by genome sequencing classified as hospital associated subclade A1 [21]. Different sequence types (STs) were clustered within this lineage based on how many alleles were shared [25]. CC17 *E. faecium* strains are responsible in hospitals for increasing infections and mortality rates [26]. However, MLST has limited resolution, since it only includes a few genes [27]. Whole-genome sequencing (WGS) represents higher resolution, even though standards for comparability are not available yet [27]. Core genome MLST (cgMLST) signifies a more diverse standard typing scheme for *E. faecium* since genome-wide single nucleotide polymorphism (SNP) are used and 1423 cgMLST genes are included [27].

Genome characteristics *E. faecium* are particularly good at uptake and exchange of large amounts of new DNA [8]. Around 38% of the *E. faecium* genome is accessory, in contrast to *E. faecalis* with only up to 25% [8]. Furthermore, within the phylogenetic structure of *E. faecium* significant genomic differences between human commensal and clinical strains have been described [7]. Clinical associated clade A1 strains have a larger accessory genome, in particular mobile genetic elements (MGEs), such as plasmids, bacteriophages and transposable elements contributing to AMR [8, 28]. Clinical lineage CC17 shows a high genome plasticity which comes with a high recombination rate and allows fast adaption to environmental stressors, such as antimicrobials [26]. The absence of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas defense mechanisms also contributes to high-adaption since no protection against foreign DNA is given and thus genomes of multidrug-resistant clinical strains are larger [7, 26]. However, it is mostly believed, restriction modification (RM) systems, another type of defense mechanisms, are involved in *E. faecium* subspeciation with different plasmidome content [19].

1.2.2 *E. faecium* as an infectious agent

E. faecium frequently can cause, mostly hospital-acquired, bloodstream, urinary tract and intra-abdominal, pelvic or soft tissue infections in immunosuppressed or microbiota disturbed patients [7, 29]. Enterococcal bacteremia often occurs together with endocarditis, which represents the most common life-threatening infection regarding *E. faecium* in hospitals [29]. Within the gut microbiota, *E. faecium* is only present in a small amount, though enterococcal infections often are favored by an increase in colonization density of *E. faecium* strains in the GI-tract [12]. Through the intestinal lining and the liver, *E. faecium* can then migrate in the bloodstream towards the heart to cause diseases like endocarditis [12]. Contamination of the hospital environment and the spread of infections is caused via fecal transmission or the skin through bloodstream catheters and urinal catheters and transmission from healthcare workers [12].

E. faecium being resistant to specific types of antibiotics (e.g. cephalosporins, streptogramins, vancomycin, penicillins, linezolid) additionally challenges hospital infection treatment [6]. Cephalosporins often were used for enterococcal nosocomial infections [30]. However, studies revealed VRE colonization increased in the GI-tract when especially cephalosporins or antibiotics against anaerobes were used for infection treatment [30]. These types of antibiotics target various gram-negative and gram-positive bacteria except VRE favoring an immense change in the gut microbiota and therefore causing overgrowth of VRE within the patient [12]. Gram-negative organisms are essential for a functioning microbiota balance and mechanisms against invasive VRE [12]. Mouse model studies revealed REGIIIγ, a C-type lectin, which gets produced by Paneth cells upon stimulation by gram-negative bacteria and owns antimicrobial activity against gram-positive bacteria like VRE (Figure 5a) [12]. However, an influx of antibiotics decreases Gram-negative organisms within the gut as well as the production of REGIIIγ resulting in an overgrowth of Gram-positive organisms like VRE causing then threatening hospital infections (Figure 5b-c) [12]. VRE can then predominate the gut environment up to two months after stopping the antibiotic treatment, however healthy commensal *E. faecium* strains can overtake again the microbiome environment then as well and ensure a healthy microbiota balance [12].

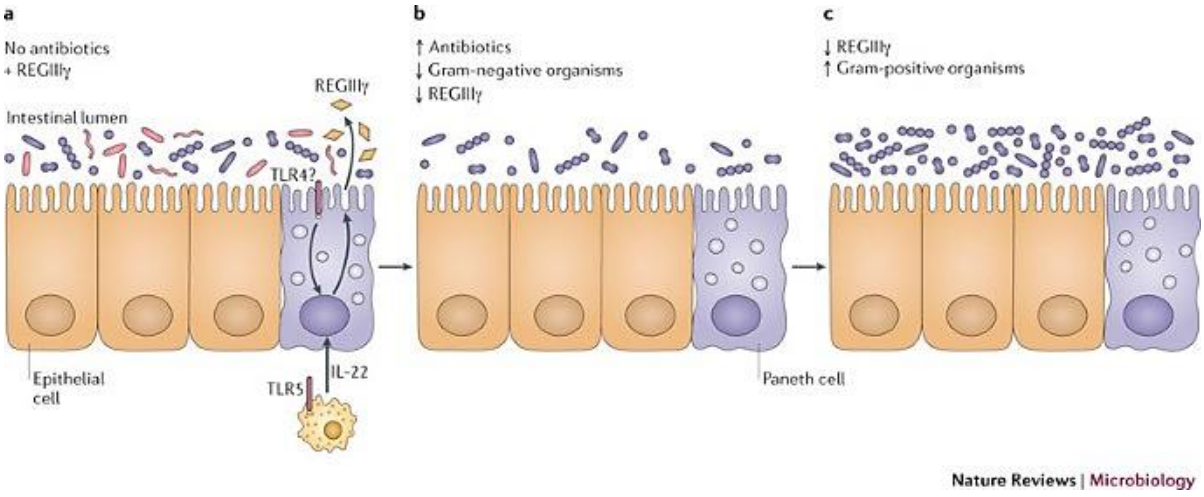

Figure 5: Effect of antibiotic treatment during hospitalization on gastrointestinal microbiota and the resulting predominance of VRE shown on intestinal epithelial cells and Paneth cells of a mouse model. a: No antibiotic treatment: mouse intestinal epithelial cells and Paneth cells produce C-type lectin REGIIIγ which has antimicrobial activity against Gram-positive bacteria (purple). Gram-negative bacteria (pink) trigger REGIIIγ production mediated via surface patterns and innate immunity cascades (TLR5 (toll-like receptor 5), IL-22 (interleukin-22). b: Antibiotic treatment present and reduction of Gram-negative bacteria and REGIIIγ production of the two cell types. c: Gram-positive bacteria like VRE overgrow since REGIIIγ is decreased. Figure is taken from Arias, C.A. and B.E. Murray [12] (reprinted with permission, licence number 5546451472101) but was already modified from the author, with permission, from Zaph, C., [31] © (2010) American Society for Clinical Investigation.

1.2.3 Adaption of nosocomial *E. faecium*

E. faecium's successful invasion of the hospital niche comes from its ability to survive outside of the GI-tract and its extraordinary adaption to extreme conditions within the healthcare sector [7]. Studies show that *E. faecium* can survive for up to 58 days on surfaces of countertops in the hospital [32]. Other risk areas are door handles, medical devices or bed rails as well as all kinds of fabrics used for patients [7, 12]. Healthcare workers can contaminate one out of ten sterile surfaces with VRE during their daily work [7]. Even disinfection with hand-wash alcohol solutions does not represent a safe prevention anymore, since studies show that MDR *E. faecium* can resist alcohols within hand-wash solutions, which could explain the emergent increase in survival of clinical *E. faecium* strains in hospitals [33]. Within the resistant strains towards hand-wash alcohols, genetic mutations were found in genes that are involved in carbohydrate uptake and metabolism [33].

Furthermore, *E. faecium* shows resistance to heat (up to 60°C for 30 minutes), chlorine and can persist in a wide range of pH (4.8 to 9.6) [6]. Enrichment of virulence factors within clade A1 enhancing colonization and infection properties in comparison to clade A2 strains was described as well [6, 28]

Antibiotic resistance The most challenging aspect when treating enterococcal infections is MDR and resistance to last resort antibiotics like vancomycin [34]. Most clinical strains belonging to CC17 lineage/clade A1 show high ampicillin resistance and within the last decades, VR*Efm* CC17 spread rapidly too [26]. Various *E. faecium* strains evolved antibiotic resistance independently since they each had to introduce mechanisms for survival within the hospital [7]. Therefore, the clinical CC17 lineage does not stem from a single ancestor strain [7]. Studies have shown that most of the nosocomial isolates clustered into the two subgroups 2-1 and 3-3 [25]. Three important hospital lineages originating from their STs could be confirmed as well belonging to these two subgroups, 2-1 including ST78 and 3-3 including ST17 and ST18 lineages [25].

Several mechanisms are known regarding antibiotic resistance development in *E. faecium* [12]. A distinction can be made between intrinsic resistance (antibiotic resistance is already encoded within the genome of Enterococci) and acquired resistance (resistance genes are transferred on MGEs between isolates via horizontal gene transfer) [7]. Ampicillin resistance is achieved via modification and resulting in inactivation of penicillin-binding protein 5 (PBP5) [7]. PBPs are involved in cell-wall synthesis of bacteria and therefore ampicillin inhibits bacterial growth [7]. PBP5 is intrinsically encoded in the genome of *E. faecium,* and its modification can be amplified resulting in higher resistance and reduced affinity to ampicillin [7]. Vancomycin is a glycopeptide, and it blocks enzymatic binding sites during the synthesis of the peptidoglycan cell wall by building complexes with a D-ala-D-ala peptide terminus [7]. However, acquired resistance to vancomycin is achieved by modifying the peptide terminus to D-ala-D-lactate or D-ala-D-serine ensuring a lower binding affinity for vancomycin [7]. These different modifications are encoded in different gene clusters on MGE [7]. *vanA* and *vanB* are the most common genes operating peptide modifications [7]. Non-enterococcal gut commensal anaerobes were described to carry vancomycin-resistance *vanD* gene clusters and therefore could act like a reservoir of antibiotic-resistant genes carried by enterococci [35].

1.3 Bacterial competition

Natural habitats including the GI-tract of animals and humans represent a species rich niche with different microbes [36]. Bacteria are known to communicate with each other via sending and receiving extracellular signals (quorum sensing) and also compete with other species for niche space and nutrients constantly [36]. Both interspecies and intraspecies competition is defined, as the ability of intraspecies interactions to influence interspecies interactions [36]. *Staphylococcus aureus* (*S. aureus*) colonies can impact *Pseudomonas aeruginosa* (*P. aeruginosa*) when competing for iron sources with a resulting increase in selection pressure [36]. Furthermore, both *S. aureus* and *P. aeruginosa* can have pathogenic synergy and can cause and even worsen disease [37].

Competition models have been described based on *Escherichia coli* (*E. coli*) demonstrating bacterial behavior between related strains [36]. Strains which produce antimicrobial toxins can outcompete a sensitive strain, however, the sensitive strain does not live on cost of encoding resistance towards antimicrobial toxins [36]. Thus, the sensitive strain can outcompete the resistant strain which can outcompete the toxin producing strain because the resistant strain does not carry the costs for toxin production [36]. Commensal *E. coli* strains are known to outcompete other interspecies pathogenic *E. coli* strains during biofilm growth phase in the gut [37].

1.3.1 Intraspecies growth competition between *E. faecium* clades

Colonization advantages and competitional growth are driven by diverse factors including nutritional acquisition, antimicrobial production and resistance, biofilm formation and bacterial niche competition [38]. The enterococcal genome acquired most of these strategies [38]. Differences in enterococcal genome content between the phylogenetic clades result in different competitive fitness levels which have an impact on GI-tract colonization [38].

The predominance of clinical *E. faecium* strains to commensal strains within the GI-tract during hospitalization remains elusive [28]. Overgrowth of gram-positive bacteria during antibiotic treatment due to lack of REGIIIγ as explained in 1.2.2 could be a contributing factor [12]. Furthermore, the interaction between different *E. faecium* clades and their inhibition towards each other is described as well in terms of hospital infections [28]. It is confirmed that ampicillin-resistant *E. faecium* strains replace ampicillin-susceptible, commensal *E. faecium* strains in the GI-tract in hospital environments [28].

Therefore, a replacement of commensal clade B strains by clinical clade A1 strains in the patient's gut in hospitals could be hypothesized and would be the first step to cause disease due to increased density in growth [28]. Also, clinical clade A1 strains might have higher fitness and acquired virulence towards the host to overtake commensal clade B strains during gut colonization [28]. Singh *et al.* confirmed this by growing different strains of clade A1 and B (2-3 strains of each clade) in a competitive *in vivo* GI-tract mouse model in the presence of β-lactam antibiotics [39]. Strains of clade A1 would outcompete strains of clade B as seen in patients during hospitalization and antibiotic treatment [39].

However, commensal clade B strains can overtake after time once the patient leaves the hospital. Thus, clade B implements a better colonization strategy in the human community outside of healthcare institutions and the absence of antibiotic treatment [28]. Montealegre *et al.* verified this within a study in a murine GI-tract mouse model where *E. faecium* strains of clade B, A2 and A1 (3-5 strains of each clade) were competitively grown [28]. Clade B showed predominant patterns towards clade A strains within the competitive *in vivo* model [28].

1.4 Bacteriocins

Microbes possess a wide range of different microbial defense systems e.g. classical antibiotics, metabolic by-products or lytic agents [40]. Bacteria can specifically inhibit similar bacterial strains, closely related strains, or other species by producing antimicrobial peptides called bacteriocins [41, 42]. They represent a diverse cluster of mostly proteinaceous, heat-stable compounds that are secreted by bacteria [41, 43]. Four genes are involved in generating bacteriocins, including a structural gene (prepeptide), an immunity gene and an ABC-immunity gene for the bacterium being protected against its own bacteriocin and a gene being responsible for the protein production for extracellular export of the bacteriocin [43] (Figure 6). The prepeptide is synthesized in the bacterial cell and matures then to its final form, the bacteriocin, when it is released outside of the cell [43]. Bacteriocins can act as colonizing peptides, killing peptides or signaling peptides [44]. Thus, they can enable the colonization of a specific niche, directly eliminate pathogens of mostly related species or send signaling pathways towards other bacteria or the immune system [44].

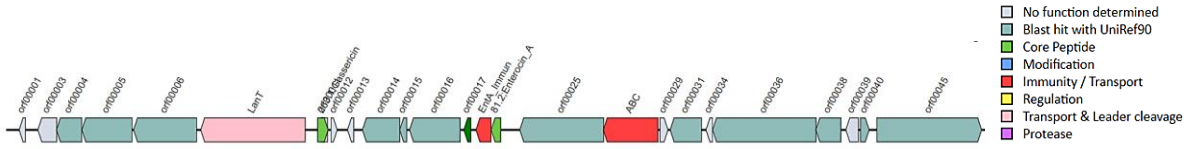

Figure 6: Genetic structure of bacteriocins shown in the example of Enterocin A which is often found in *E. faecium* strains. The color code on the right indicates different functionalities. The four main core genes are the core peptide (green), two immunity genes (red) EntA_Immun and ABC-immunity gene and a LanT gene responsible for encoding the protein for bacteriocin export (light pink). Figure taken from BAGEL4 database website [45] when predicting *E. faecium* clade A2 strain T7EF-50964995 from this study (Table 1 Appendix).

1.4.1 Use of bacteriocins and their role in enterococci

Between 30 to 99% of all bacteria and archaea produce at least one bacteriocin [43]. Interestingly, lactic acid bacteria (LAB) like enterococci produce a wide spectrum of bacteriocins, which are taken advantage of by the food industry for preservation and anti-contamination purposes [43]. Already 8000 years ago humans made use of bacteriocins unintendedly for cheese and fermented food production since bacteriocins could influence the microflora of food products [43]. 80 years ago, the first report of bacteriocins mediating inhibition between *E. coli* strains was described and later in enterococci enterocins as a group of bacteriocins were found targeting *Clostridum* species and *S. aureus* [43].

Enterococci are commercially available as probiotic supplements and used as treatment against diarrhea [44]. Therefore, different bacteriocins are described within the species and a wide range of enterocins are encoded within enterococci being active against foodborne pathogens including *Listeria* spp. [44]. Most enterocins are classified within class II bacteriocins which are characterized as non-modified and heat-stable substances [44, 46]. Class III describes cyclic heat-labile bacteriocins with a higher molecular weight [46, 47]. Presumably, enterococci evolved the ability to produce bacteriocins to ensure beneficial highly competitive patterns towards other microbes in the GI-tract environment of the host [44, 48].

Fewer resistance mechanisms are suggested to develop against bacteriocins compared to common antibiotics since a single bacterium produces its own bacteriocins and their mode of action is more specific [41, 43]. In contrast to antibiotics, bacteriocins are genetically encoded and ribosomally synthesized which makes them an interesting target and an eventual alternative to regular antibiotics in the future [49]. Interestingly, bacteriocins have shown antibacterial activities against hospital pathogens including multidrug-resistant pathogens [50]. Previous unpublished studies by Wagner and Engi *et al.* hypothesized bacteriocins being involved in competition of different *E. faecium* strains between clinical and commensal clades [51].

2 Objectives

The overall aim of this master thesis project was to study interaction and competition dynamics between different strains of commensal and clinical *E. faecium* clades. This included the determination of the amount of growth inhibition during competitive growth *in vitro* between commensal and clinical strains as well as bacterial supernatant characterization to characterize secreted antimicrobial compounds which might carry out inhibition between strains.

The specific aims of this study included:

(i) To screen via competitional growth 50 clinical *E. faecium* clade A1 strains and 25 commensal *E. faecium* clade A2 strains against each other and investigate whether they may inhibit strains of the opposing clade or whether they show resistance towards the competing strain

(ii) To investigate the bacterial supernatant of top-inhibitors from clade A1 and clade A2 strains (including clade B top-inhibitor strains) whether secreted proteinaceous, heat-stable compounds may be present and may carry out visible inhibition towards strains of the opposing clade

(iii) To detect genetically encoded bacteriocins within *E. faecium* clade A2 strains with the BAGEL4 bacteriocin database which may be associated with *in vitro* inhibition results of (ii)

3 Materials and methods

3.1 *Enterococcus faecium* strains

For this study, *E. faecium* strains from three different clades of the phylogenetic tree were used to cover a wide spectrum of different phylogenetic origins. 50 clinical strains belonging to clade A1 were selected from the Norwegian Surveillance System for Antibiotic Resistance in Microbes (NORM) 2008 and 2014 collections and Vancomycin-resistant *E. faecium* in Norway (VRE) collection from 2010 to 2015 (K-res/Al-Rubaye *et al.* [6]). The strains were isolated from blood and some from urine or feces from hospitalized patients in Norway (see Table 1 Appendix for more specific information).

25 commensal strains of clade A2 were selected from the Tromsø 7 collection (Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res)/Hegstad *et al.*, unpublished) and isolated from feces from non-hospitalized volunteers in Tromsø, Norway in 2014/15 (see Table 1 Appendix for more specific information). 19 commensal strains of clade B were selected as well from Tromsø 7 collection (K-res/Hegstad *et al.*, unpublished) in 2014/15 (see Table 1 Appendix for more specific information). The strains were isolated from feces. Strains belonging to clade B were only used for the supernatant assay, since a previous study by Wagner and Engi (Host Microbe Interaction research group, UiT) already described bacterial interactions between clade B and A1 [51].

All strains are phylogenetically different isolates of *E. faecium* and represent different ST types. They were selected to represent genetic diversity within the different subclades. Via pubMLST, different ST types were annotated and determined.

For long term storage of the bacterial strains freezing broth (Section for Infection Control, Education, Method Development and Production (SUMP), Department of Microbiology and Infection Control, University Hospital of Northern Norway, Tromsø, Norway) containing Brain Heart Infusion (BHI) (Oxoid CM1135 916), glycerol solution 85% (Honeywell 49783-1L 916) and filtered and deionized water, was used. About 20-30 µl of bacterial culture from a blood agar plate was added with an inoculation loop to 1.0 ml freezing broth and stored at -70°C. Glycerol protects the bacteria from cell death by reducing ice crystals.

3.2 Workflow overview of all experimental methods

In general, three different workflows were carried out when preparing the competitors, the indicator lawns and the supernatants of a bacterial strain. Competitors and indicators differ in their preparation and competitors were generally seen as strains that carry out inhibition towards the indicator (Figure 7). An overview is shown in Figure 7 and a more detailed description of the methods and why they were used will follow in the next section. It was important to plan at least two days in advance since the bacteria had to grow first on blood agar overnight to be able to inoculate a liquid BHI culture the next day and grow an overnight culture. It was distinguished between bacterial competition assay and supernatant assay. During a competition assay, different bacterial strains are competitively grown against each other on one plate. A supernatant assay represents the same, however, only the sterile supernatant of the bacterial culture is used and not the bacterial culture itself. The indicator lawn preparation was applied for both assays.

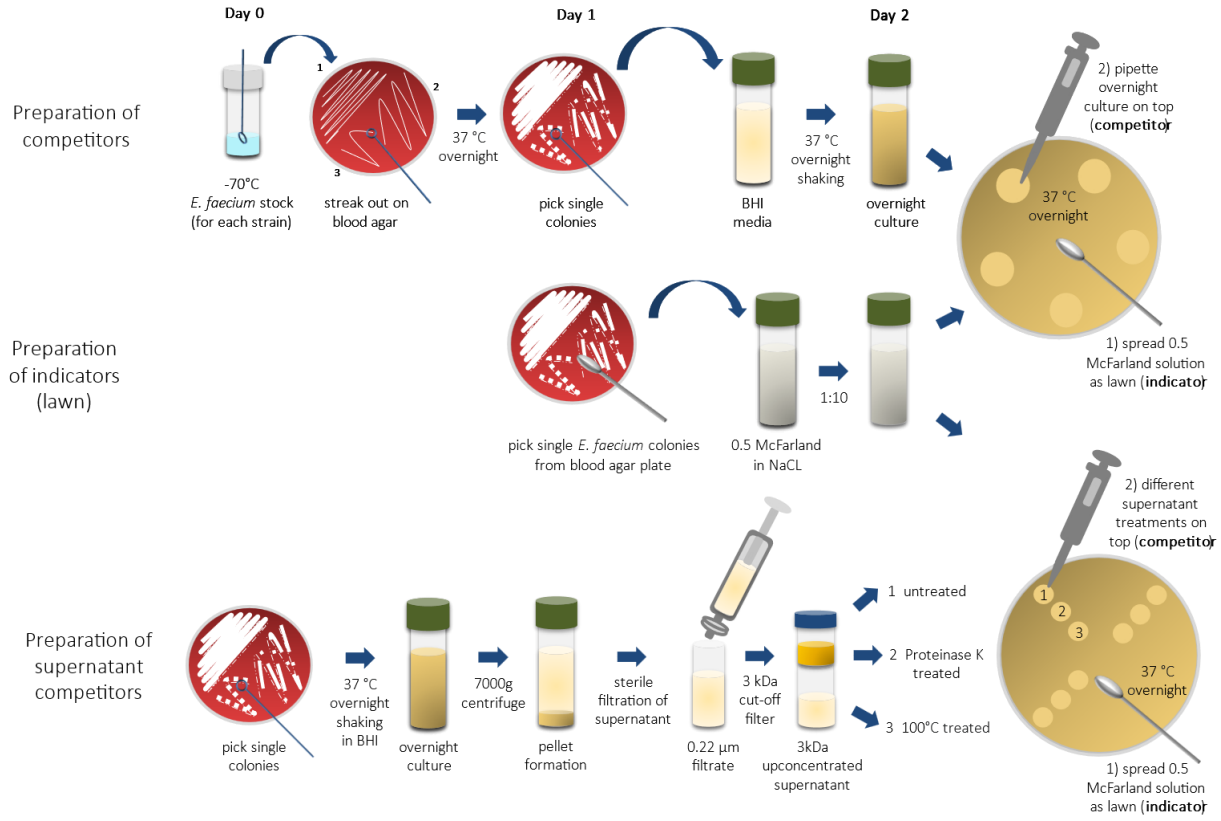

Figure 7: General overview of the workflow and methods used during investigation of interaction between different strains of *E. faecium* in this project. Preparation of competitor strains, indicator lawns and supernatants are shown. The general workflow for competitor strains includes growing of *E. faecium* strains on blood agar plates overnight and starting overnight cultures the next day by inoculating liquid BHI media with single grown colonies. Supernatant assays include extra steps, where sterile filtration and concentration of the sample was carried out to apply different treatments (Heat, Proteinase K) in the end. Indicator lawns were prepared as 0.5 McFarland in natrium chloride (NaCl) and 1:10 dilution in NaCl.

3.3 Growing routines of *E. faecium*

All kinds of media and diluents like blood agar plates, BHI agar and liquid media and 0.85% NaCl in water were ordered and provided by the SUMP. Storage of sterile media and diluents was at 4°C.

3.3.1 Growing *E. faecium* on blood agar plates

E. faecium strains were plated out from -70°C freeze stocks and grown overnight on blood agar plates (5-10% blood) since blood agar provides optimal growing conditions for *E. faecium* and functions as a nutrition source. Therefore, optimal harvesting conditions of the bacteria were ensured. Storage of grown bacteria on blood agar was possible as well for about two weeks at 4°C. Furthermore, the differentiation of hemolytic properties of bacteria with blood agar plates is visible. *E. faecium* bacteria appear white/silver when they are grown and good visibility of single colonies on red blood agar plates is ensured.

Ten frozen bacterial strains at a time were put on ice from the freezer (-70°C) to keep the samples frozen and avoid cell death. With an inoculation loop (10 µl), in sterile conditions under a flame, frozen bacterial stocks were plated out in blood agar plates. Streak plate method was carried out to ensure the growth of different densities of bacteria and decrease bacterial load to be able to pick single colonies after incubation (Figure 8). Plates were incubated for about 16 hours overnight at 37°C to grow *E. faecium* strains at optimal temperature. Observation for optimal growth was essential e.g. checking for contaminations where colonies differ in color or size.

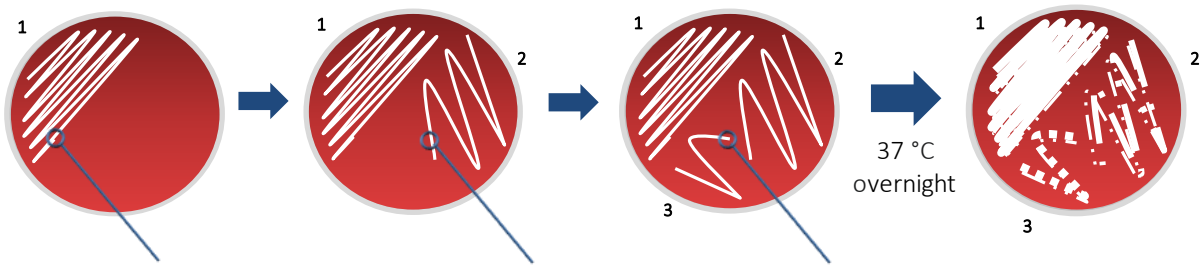

Figure 8: Streak plate method. Applied when plating out 10 µl of frozen *E. faecium* stock on blood agar plates starting with narrow lines (1) and continue with less narrow plating lines (2 and 3). Result will be single grown colonies at position 3 or already at position 2.

3.3.2 Growing *E. faecium* overnight cultures in liquid BHI

For preparing competitor strains for bacterial competition assays, overnight cultures in liquid BHI media were inoculated with bacteria from blood agar plates, which were grown in advance (see 3.3.1). From an area with visible single colonies, 3-5 single colonies were picked with an inoculation loop (10 µl) from the blood agar plate with the strain of interest and inoculated in a 5 ml glass tube with liquid BHI. Sterile conditions were ensured by working under a flame. With BHI medium bacteria can be cultured in a high-nutritious environment. Incubation was carried out overnight at 37°C in a shaking rack at 250 rpm. Shaking ensures the flow and distribution of oxygen and nutrients within the bacterial culture and avoids bacterial aggregations. Overnight cultures were always incubated around 16 hours to ensure that the competitor strains were in the same growth phase and are comparable to each other between different experiments. After they were taken out of the incubator they were put on ice until the experiment was carried out.

3.4 Preparation of bacterial assay and supernatant assay

3.4.1 Bacterial competition assay

To assess the competitive growth between *E. faecium* clade A1 and A2 strains, bacterial competition assays were carried out on BHI agar plates. The competition assay contained an indicator lawn and competitors which were put on top of the lawn (Figure 7). The indicator lawn was spread over the whole agar. After incubation, different intensities of inhibition zones around the competitor might be visible and can indicate a possible competitive interaction between two specific strains from different clades.

The preparation of the competitors started one day before the competition assay was carried out (Figure 7). The strains of clade A1 and A2 were already grown on blood agar plates (see 3.3.1). The competitors were grown as overnight cultures for about 16 hours (see 3.3.2) and kept on ice the next day. The indicator lawn was made on the same day the competition assay was carried out. A 0.5 McFarland dilution in 5 ml 0.85% NaCl (8.5g NaCl in 1L water, autoclaved, SUMP) was made by adding single colonies with a cotton swab from a blood agar plate and measuring the solution in a McFarland densitometer. A McFarland standard of 0.5 is equivalent to a bacterial solution containing 1–2 x 10^8 colony-forming units/mL (CFU/mL) of *E. coli* (ATCC 25922). In antimicrobial disk susceptibility testing concentration standardization is

used as well. Experiments in this study were based on disk diffusion, an approach for antimicrobial susceptibility testing (EUCAST - European Committee on Antimicrobial Susceptibility Testing) [52]. The 0.5 McFarland dilution of the strain was diluted 1:10 with 0.85% NaCl according to previous experience by Wagner and Engi in this project [51]. Using a swirl, the indicator lawn was spread evenly with a cotton swab on the BHI agar plate. After drying the lawn for about 10 minutes 10 µl of the competitor strains were pipetted on top of the lawn. It was important to distribute the competitors across the agar plate to avoid the competitors contaminating each other (see experimental layout in Figure 7). Incubation was carried out overnight at 37°C for about 17-20 hours.

3.4.2 Supernatant assay and stress treatments

In the second part of this study, supernatant assays were carried out. Besides studying growth inhibition mediated by bacterial cells, investigation of cell-free solutions which might contain bacterial compounds carrying out inhibition was essential. Via supernatant assays a sterile filtrate of the bacterial culture was examined and used as a competitor on top of a bacterial indicator lawn in order to investigate inhibition. *E. faecium* clade A2 and B strains (commensals) and clade A1 strains (clinicals) were tested against each other. Indicator lawns strains were chosen to represent different positions within the clade in the phylogenetic tree while competitor specific strains which showed inhibition were selected from previous bacterial competition assays. The selection was based on competitor strains that showed at least inhibition grade 2 with one strain (see results).

Supernatant competitors were made by starting overnight cultures in liquid BHI medium (see 3.3.2 and Figure 7). Three to five single colonies from the blood agar plate were inoculated in 20 ml of liquid BHI medium in a falcon tube and incubated overnight in a shaking rack. The next day overnight cultures of different strains were pelleted at 7000g for 10 minutes to separate solid particles from liquid ones. A bacterial pellet formed at the bottom, but only the supernatant was used for further steps. Sterile filtration of the supernatant was carried out twice using first 0.45 µm filter (VWR® Sterile Syringe Filter PES 25mm 0.45 µm, Cat. No. 514-1261, avantor delivered by VWR® International) and 0.2 µm filter (VWR® Sterile Syringe Filter w/ 0.2 µm Polyethersulfone Membrane, Cat. No. 514-0073, VWR® International). Two step filtration was done since the non-sterile supernatant would block the 0.2 µm filter (only one filtration step shown in Figure 7). A 5 ml syringe was used to press the supernatants through the filter into a falcon tube.

Previous experiments by Wagner and Engi revealed no visible inhibition of sterile filtrated supernatants [51]. Therefore, the solution was concentrated five times. 3 kDa molecular weight cut-off (MWCO) filters (VIVASPIN® 20 centrifugal concentrator cut-off filter, membrane 3000 MWCO PES, Cat. No. Z629464-48EA, Sartorius Stedim Lab Ltd. Stonehouse, UK) were used for concentration of the samples. MWCO is the smallest molecular size where compounds are not able to pass the filter. The size of 3 kDa was chosen based on previous research of known bacteriocins by Wagner and Engi for clade A1 and B strains (size 4.63–37.3 kDa) [51]. In this study bacteriocins for clade A2 were predicted via BAGEL4 database and their size was annotated (see 3.5.1).

Each 3 kDa MWCO filter tube was filled with 14 ml of sterile filtrated supernatant and centrifuged at 7000g for approximately 3 hours until only 2,5-3 ml of liquid was left in the upper part whereas the flow-through was collected in the bottom part of the tube (Figure 7). Since each concentrated supernatant needed different amounts of time for the process, the volume was not accurate in each tube. However, the volume was determined by using a weight and a 5x concentration of every supernatant was achieved. Storage of the finished supernatant competitor was done in Eppendorf tubes at -20°C. The preparation of the indicator lawn was done according to 3.4.1.

3.4.2.1 Stress treatments for supernatant characterization

In previous studies by Wagner and Engi, it was indicated that competition might be mediated by secreted heat-stable proteinaceous compounds of bacteria (bacteriocins) [51]. By treating the supernatant with stressors like proteinase K or heat it could indicate whether proteinaceous, heat-stable compounds are present in the supernatant of the bacterial culture.

For the proteinase K treatment, proteinase K was added to a final concentration of 1 mg/ml (original protocol) or doubled concentration of 2 mg/ml (1.5 µl or 3 µl of 10 mg/ml working solution, diluted proteinase K (Thermo Fisher Scientific, Cat. No. EO0491) stock in deionized water) was added to 15 µl of the bacterial, sterile-filtrated supernatant and incubation was carried out at 37°C for one hour or extended to two hours. The proteinase K concentration and incubation time were varied to compare different protocol outcomes and to increase the chances of proteinase K being active and inhibiting the compounds of interest in the supernatant.

For heat treatment 30 µl of bacterial, sterile-filtrated supernatant was boiled at 100°C for 10 min and was then kept on ice. As a control the supernatant samples were treated with a heat-treated proteinase K as well (proteinase K for 10 minutes at 100°C) to inactivate its properties

to destroy proteinaceous compounds. 10 µl of each supernatant competitor, treated or untreated was then pipetted on top of the lawn (untreated, Proteinase K and heat treated - Figure 7).

Inhibition grade scale The inhibition grades of the competitors of the bacterial and supernatant assay were set according to Figure 9.

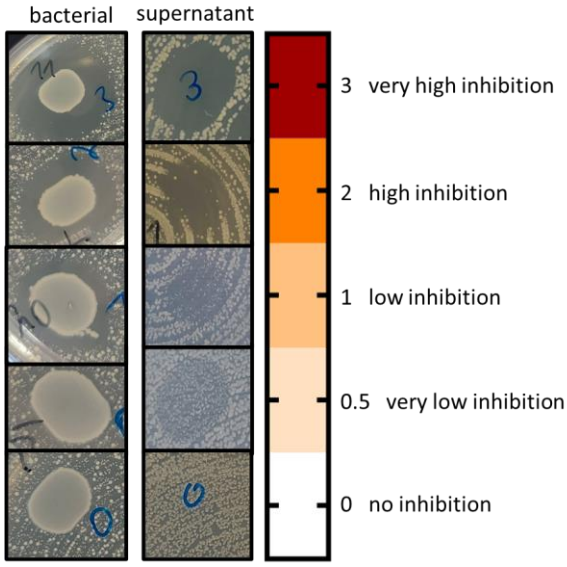

Figure 9: Inhibition scale of either bacterial or supernatant competitors with their visual related inhibition zone. Inhibition grade 0 = no inhibition to 3 = very high inhibition was set.

3.4.3 Co-culture assay

Supernatant assays only considered one strain being present when sterile filtrating the supernatant of the bacterial culture. However, the expression of compounds like bacteriocins might require the presence of competitors or other environmental cues [53]. To take this into account, co-culture assays were conducted. Indicator lawns of clade A1 (1-I-3, K59-20, K60-29) and competitors of clade B (6E1G, 4E1E, 6E7B, 2E7F) and clade A2 (6_T7EF-51025019) were selected. Initial experiments were conducted using these strains of clade B and were tested with clade A1 as indicators where no inhibition was seen in previous supernatant assays (see results). Strains were grown on blood agar plates according to 3.3.1. Overnight cultures in 5 ml BHI medium were carried out according to 3.3.2. The next day 20 ml of BHI media were inoculated with 100 µl of each strain from the overnight culture the day before. The two strains that will later compete as supernatant competitors on a BHI agar plate were grown overnight together in one tube. Incubation of the co-overnight culture was done according to 3.3.2. The next steps for preparing the competitors for supernatant assay were carried out according to 3.4.2 including sterile filtration and concentration.

3.5 Bioinformatic analyses

All figures (heatmaps) represented in this work which represent generated data from own experiments were created with Graph Pad Prism (Version 9.0.0, © 2020 GraphPad Software).

All strains used in this study of clade A1, A2 and B were whole genome sequenced (WGS) by Illumina and reads were processed according to AL-Rubaye *et al*. [6].

3.5.1 Using BAGEL4 database to predict bacteriocins

Searching for encoded bacteriocins within sequences of bacterial strains of *E. faecium* might help in cases where inhibition could not be explained using laboratory methods. Finding a significant pattern between visible growth inhibition on an agar plate and present bacteriocins within bacterial sequences would contribute to new knowledge regarding mediation of bacterial inhibition.

Sequences of bacterial clade A2 strains were searched in a database looking specifically for bacteriocins which might be encoded. BAGEL4 is an online database publicly accessible [45] Users can upload fasta formatted DNA sequences and search for genetic patterns and clusters which are encoding secondary metabolites like ribosomally synthesized and post-translationally modified peptides (RiPPs) and bacteriocins [45]. When scanning sequences for matches BAGEL4 is independent of looking for open reading frames (ORFs) [45]. Consequently, no areas of interest (AOIs) are missed [45]. Links to UniProt and NCBI are accessible as well [45]. Only already known compounds can be found through BAGEL4 [45].

Sequences of clade A2 were uploaded in BAGEL4 and were annotated with found bacteriocins. Information for the size of the bacteriocins was then added as well.

3.5.2 Phylogenetic trees

Understanding genetic relationships between *E. faecium* clades and their strains helps to reveal evolutionary patterns. Strains with a similar origin might show similar inhibition patterns and might have evolved similar techniques to prevent growth of competing strains.

All phylogenetic trees in this study were made by Anna Pöntinen, K-res, using the program parsnp based on WGS sequences (parameters: parsnp -x -c -o Parnp_com_cli_ncbi -d all_genomes/ -r !). The tree is rooted at the midpoint. For visualization, the applications figtree (for individual clades for dataset Figure 10-12) and Microreact (for visualizing all clades Figure 15 Appendix) were used.

4 Results

4.1 Clinical *E. faecium* clade A1 strains inhibit commensal clade A2 strains at a higher level

To investigate inhibition between commensal and clinical *E. faecium* strains competition assays between strains of different clade origin were carried out. During the competitive screening 50 clinical *E. faecium* strains, mostly of clade A1, and 25 commensal strains of clade A2 were tested against each other. The clinical strains will be termed clinical clade A1 strains to distinguish them from the commensal isolates in this thesis. Competitor strains are defined as strains which were put on top of an indicator lawn representing one specific strain of the opposing clade (either commensal or clinical).

In general, inhibition was exerted from both clade A1 and A2 competitors towards the opposing competitively grown strains (Figure 10). Commensal competitors of clade A2 inhibited 41% of the 50 clinical clade A1 indicator strains, whereas clinical competitors inhibited 59% of the 25 commensal clade A2 indicator strains. Within commensal competitors, 453 interactions were found to be either grade 1, 2 or 3. Of these 49% were grade 3 inhibitions. In contrast, clinical competitors showed 64% of grade 3 inhibitions. Therefore, a trend is visible of clade A1 strains inhibiting clade A2 strains more strongly and with a higher amount of inhibitions than the opposing way.

Of all commensal competitors, 20% did not show any inhibition towards clinical indicator strains, though there was no clinical competitor strain which carried out no inhibition towards commensal indicator strains (Figure 10). Some commensal competitors showed a trend of constant inhibition of all opposing strains (Figure 10A). Commensal competitors rather inhibited all the opposing strains (100% constant inhibition) or only a few or none of the opposing strains. Of all commensal competitor strains which showed inhibition, 80% of these strains showed constant or nearly constant inhibition towards all opposing strains. Clinical competitors showed no constant inhibition towards all strains, since commensal indicator 01_T7EF-51021120 did not get inhibited higher than 0.5 and commensal indicator 25_T7EF-51010478 did not get inhibited in any case (Figure 10B). Both commensal indicators don't share a common branch in the phylogenetic tree. However, 20% of all clinical competitors who showed inhibition indicated a nearly constant inhibition (92% of commensal indicators got inhibited) towards commensal indicators.

Though, commensal and clinical strains revealed top inhibitors which inhibited most of the opposing group. Top commensal inhibitors were 01_T7EF-51021120, 03_T7EF-51024681, 05_T7EF-51007961, 10_T7EF-51025019, 19_T7EF-50994001, 22_T7EF-50976613, 24_T7EF-50980395 and 25_T7EF-51010478. Top clinical inhibitors mainly belonged to ST117, ST80, ST203 and ST192 (strain names and STs in Table 1 Appendix). The top inhibitors are marked in Figure 10. Commensal clade A2 strains 01_T7EF-51021120 and 25_T7EF-51010478 could carry out constant high inhibition towards clinical indicator strains and towards clinical competitor strains of clade A1 they showed constant resistance of being inhibited by them. This trend of commensal strains inhibiting a full range of clinical strains and their resistance to being inhibited by clinical strains appears throughout the screening results from Figure 10. Commensal strains 3_T7EF-51024681, 10_T7EF-51025019, 16_T7EF-50997139, 19_T7EF-50994001 and 23_T7EF-51024665 showed less inhibition strength than commensal strains 01_T7EF-51021120 and 25_T7EF-51010478, however, they indicate visible constant inhibition towards all clinical strains. Clinical strains were able to inhibit them in a few cases, however, clearly less inhibition strength is seen. Furthermore, commensal strains which mostly did not carry out any inhibition towards clinical strains often get highly inhibited from most clinical strains and show no inhibition resistance (e.g. clade A2 strains 11-15). Though, this pattern is not seen the other way round: clinical strains (e.g. clade A1 strains 36-49) which highly inhibit commensal strains showed no obvious pattern when commensal competitors carried out inhibition towards clinical strains.

The phylogenetic relationship between *E. faecium* strains revealed that in general strains which share the same branches in the phylogenetic tree also often showed similar inhibition patterns (with some exemptions). Clade A2 strains 20-25 are more related to each other and show in general more frequent inhibition towards clinical strains, in contrast to related clade A2 strains 12-14 (located at a different branch) which showed mainly no inhibition. However, within the commensal clade, the inhibition pattern related to phylogeny remains vague, since strains 04_T7EF-51001128, 10_T7EF-51025019, 16_T7EF-50997139 and 23_T7EF-51024665 showed different patterns compared to their related strains. Closely related strains 15_T7EF50964995 and 16_T7EF-50997139 indicated different inhibition results. Though, the more related clinical clade A1 strains 36-41, 42-47 and 48-50 indicate high inhibition towards commensal strains. Furthermore, the more related clade A1 strains 1-6 and more related strains 27-31 illustrate almost no inhibition. Clade A2 strains showed inhibition all over the phylogenetic tree, in contrast to clade A1 strains where inhibition appeared more in groups.

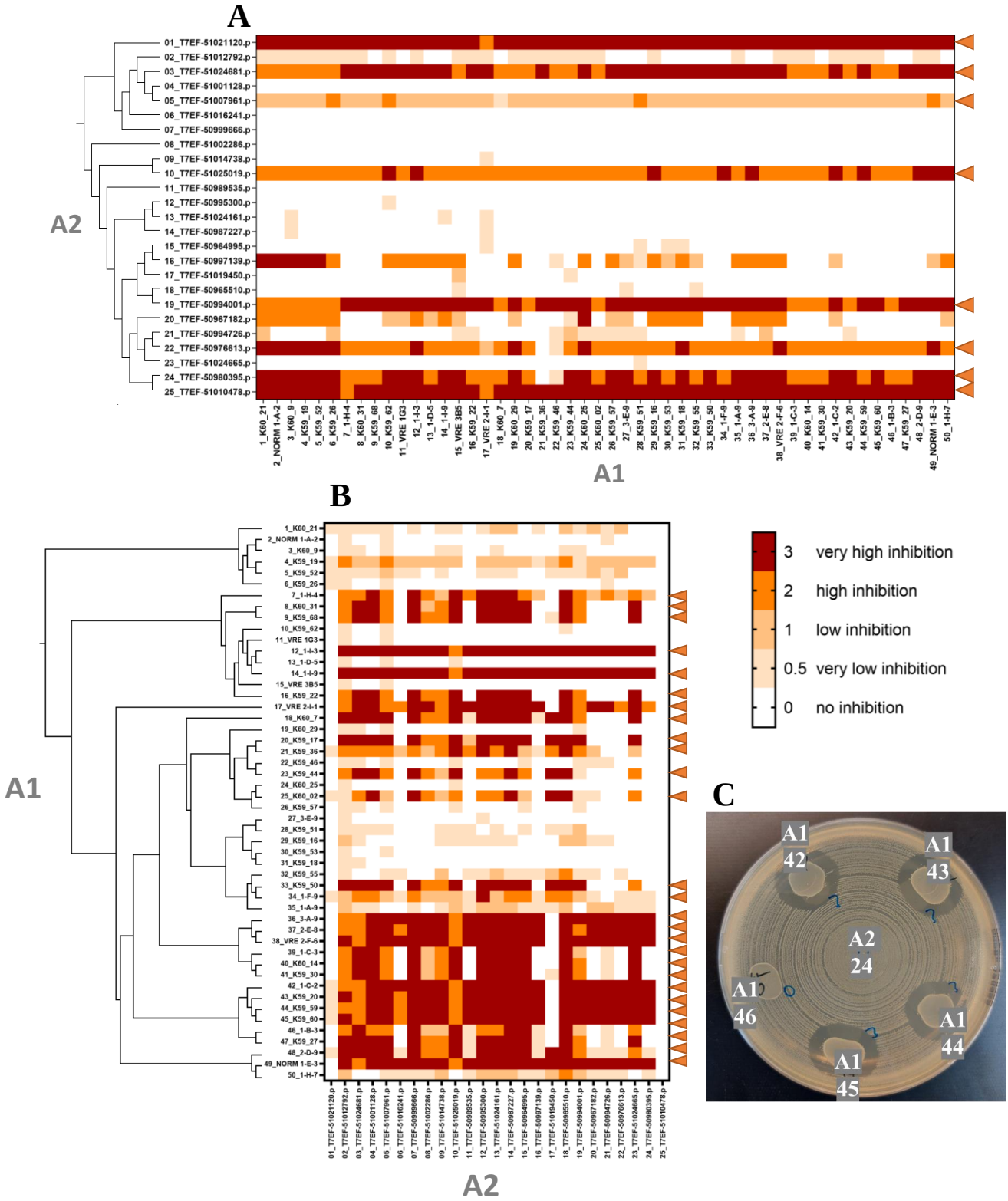

Figure 10: A. Heatmaps summarizing growth inhibition grades of bacterial competition assays between A: *E. faecium* clade A2 strains (commensal competitors) and *E. faecium* clade A1 strains (clinical indicators as lawn). Growth inhibition exerted by commensal competitors. Phylogenetic relations between clade A2 strains are indicated on the y-axis and clade A1 strains are shown on the x-axis. **B: *E. faecium* clade A1 strains (clinical competitors) and *E. faecium* clade A2 strains (commensal indicators as lawn).** Growth inhibition exerted by clinical competitors. Phylogenetic relations between clade A1 strains are indicated on the y-axis and clade A2 strains are shown on the x-axis. All bacterial strains are sorted by their order in the phylogenetic tree. Commensal competitors were grown as overnight cultures. 10 µl of each competitor was put on top of the indicator lawn and BHI agar plate was incubated overnight at 37°C. Top inhibitors marked with orange arrow. 0 (white) = no inhibition zone seen around the grown competitor strain, 3 (dark red) = high inhibition zone seen around the grown competitor strain. **C: Visual bacterial competition assay of clinical clade A1 competitors (strain 42-46) with commensal clade A2 indicator lawn 24_T7EF-50980395.p on a BHI agar plate**. Inhibition zones are visible.

4.2 Supernatants mediate inhibition of *E. faecium*

Studying supernatants of bacterial cultures, supernatant assays with characterization via different stress treatments were carried out to investigate whether secreted proteinaceous, heat-stable compounds may be present and may carry out visible inhibition towards strains of the opposing clade. The bacterial supernatant was sterile filtrated and concentrated five times with a 3 kDa cut-off filter. Top inhibitors and commensal and clinical bacterial strains which showed at least one strain with inhibition grade 2 in previous experiments were investigated for supernatant characterization. The supernatant was treated with proteinase K and heat since secreted bacterial compounds e.g. proteinaceous, heat-stable compounds were described. As indicator lawns three bacterial strains per clade (A1, A2 and B) were chosen which were located on different branches in the phylogenetic tree. For comparison bacterial assay results with clade B are indicated according to a previous study by Wagner and Engi [51].

Figure 11A displays the comparison between bacterial competition and supernatant competition of the clade competition with their different stress treatments. Clinical supernatant competitors of clade A1 showed a decrease in inhibition towards commensal indicators of clade A2 and B in comparison to bacterial competitors (Figure 11A). Only about 77% of inhibition interactions within 186 combinations were seen in the untreated supernatant whereas the bacterial competition had shown 95% inhibition interactions within these 186 combinations. Inhibition grade 3 was about 56% present in bacterial competition, though it decreased almost to half when using untreated supernatants (29% of interactions with inhibition grade 3). Still, visible inhibition was carried out by sterile-filtrated, concentrated clinical supernatant competitors towards commensal indicators. Interestingly, commensal A2 strain 15_ T7EF-50964995 did not get inhibited with an inhibition grade bigger than 0.5 by the clinical supernatants, whereas this bacterial strain was highly inhibited by the clinical bacterial strains in more than half of the interactions. Furthermore, within the bacterial assay commensal strains of clade A2 were inhibited more strongly from opposing clinical strains than indicator strains of clade B. Within 186 interactions between clinical competitors and commensal A2 indicators, 39% showed an inhibition grade of 3, whereas clade B indicators were only inhibited in 18% out of 186 interactions with inhibition grade 3. However, during the supernatant assay no clear difference between clade A2 and B was seen anymore.

Commensal supernatant competitors of clade A2 and B indicated a drastic decrease in inhibition interactions towards clinical indicators of clade A1 as well (Figure 11B). Only approximately

half of inhibition interactions were seen (bacterial assay: 94% within these 78 combinations, supernatant assay: 49% within these 78 combinations). Interactions with inhibition grade 3 decreased as well, but about 28% were still visible in the untreated supernatant assay (bacterial assay: 42% of inhibition grade 3 interactions). Strains which showed inhibition grade 3 in bacterial assays also showed the same pattern for the supernatant of these bacterial strains in most cases. Some interactions showed higher inhibition by supernatants than by bacterial suspension, though only with a difference of maximum 1 inhibition grade. Clade A2 supernatants inhibited more strains of clinical A1 indicators, which were more resistant towards clade B supernatant competitors.

The proteinase K treatment protocol got adjusted during the project, the data of the old protocol is attached in supplementary data (Figure 15, proteinase K (2) – 1 mg/ml) and the adjusted protocol data can be found in Figure 11 as proteinase K (2 mg/ml - protocol indicated in 3.4.2.1). In general, clinical supernatant competitors showed clearly less inhibition towards the three commensal A2 and three B indicators with proteinase K treatment of 2 mg/ml (Figure 11A). 77% of inhibited interactions were seen in the untreated supernatant assay in comparison to proteinase K treated clinical competitors which showed 55% of inhibited interactions. However, most of the inhibitions (78%) had only an inhibition grade of 0.5, while the rest mostly showed an inhibition of 1 or 2. Proteinase K (1 mg/ml) (Figure 15, supplementary data) showed a higher frequency of inhibited interactions (67%). 45% of the interactions had an inhibition grade of only 0.5, while the rest showed an inhibition of 1-3.

On the other hand, commensal supernatant competitors of clade A2 and B treated with proteinase K (both protocols) did not inhibit any of the clinical indicators during the supernatant assay (Figure 11B). During all interactions, the bacterial lawn of the clinical indicators grew 100% and no inhibitory characteristics were seen.

Heat treated supernatants of clinical strains revealed approximately the same amount of inhibited interaction as the untreated supernatant assay towards commensal strains (77%) (Figure 11A). However, the numbers of very highly inhibited commensal strains (inhibition grade 3) were approximately 15% higher than for the untreated supernatants. Furthermore, commensal A2 strain 15_ T7EF-50964995 showed the same pattern, as it showed high resistance towards inhibition from all the clinical A1 strains. Commensal supernatant competitors of clade A2 and B showed approximately similar inhibition percentages towards clinical A1 indicators (Figure 11B).

Co-culture supernatant assays In addition, co-culture assays were carried out to investigate if strains that did not show any inhibition in the supernatant assay but in the bacterial assay would show different results if the competitor strain was grown together with the indicator strain. The presence of an opposing competitive strain might be essential for the bacteria to secrete certain compounds. However, the assay did not show any inhibitory results (used strains indicated in 3.4.3, data not shown in this work).

Phylogeny of clade A1, A2 and B *E. faecium* strains used in this study got displayed in a phylogenetic tree using the program parsnp based on WGS sequences (Anna Pöntinen, K-res) and visualized in FigTree. With the web application Microreact the phylogeny between clade B, A2 and A1 was visualized (Figure 14 Appendix). Clade A2 and B indicate more diversity in contrast to clade A1. Furthermore, six out of 50 clinical A1 strains (1_K60-21, 2_NORM 1A2 and 3_K60-9, 4_K59-19, 5_K59-52 and 6_K59-26) clustered with the commensal clade B strains.

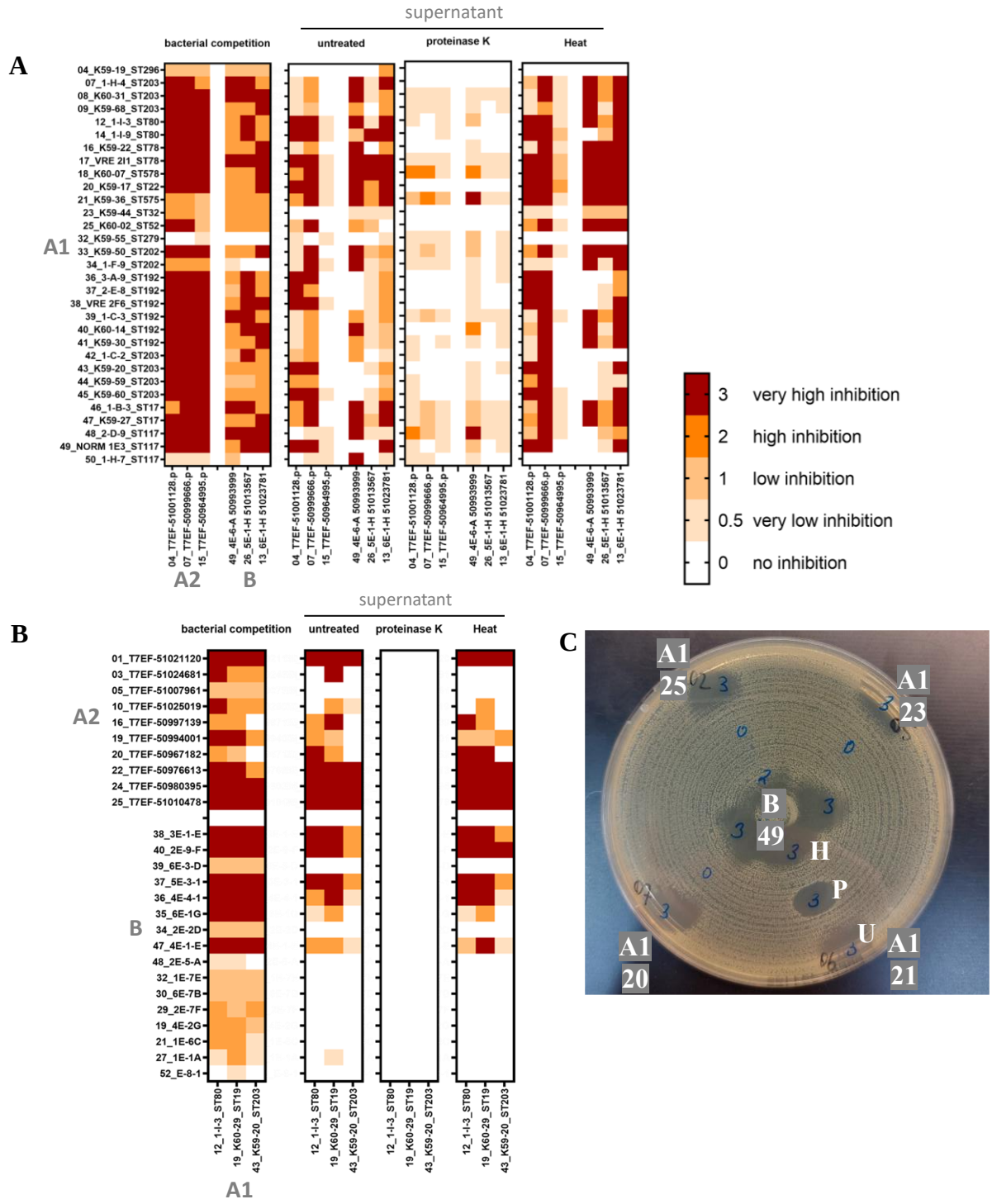

Figure 11: Heatmaps summarizing growth inhibition grades of bacterial competition assays versus supernatant assays with stress treatments (Proteinase K (2mg/ml), heat) between A: *E. faecium* clade A1 strains (clinical competitors) and *E. faecium* clade A2 and B strains (commensal indicators as lawn). Growth inhibition was exerted by clinical competitors (both bacterial and supernatant competitors) and **B: *E. faecium* clade B/A2 strains (commensal competitors) and *E. faecium* clade A1 strains (clinical indicators as lawn).** Growth inhibition was exerted by commensal competitors (either bacterial or supernatant competitors). All bacterial strains are sorted by their order in the phylogenetic tree. Indicator lawns are always indicated on the x-axis and competitor strains on the y-axis. **Bacterial assay:** clinical competitors were grown as overnight cultures (data shown in Figure 10 is taken from previous dataset from Figure 9 for comparison). *E. faecium* competitor strains were considered which showed at least one strain with inhibition grade 2 in the heatmap. **Supernatant assay:** for competitors, supernatant got sterile filtrated (0.2µm) and concentrated (3kDa MWCO filter). Supernatant characterization included treatment of Proteinase K (2 mg/ml for 1h at 37°C) and heat (10 min at 100°C). 10 µl of each competitor was put on top of the indicator lawn and BHI agar plate was incubated overnight at 37°C. 0 (white) = no inhibition zone seen around the grown competitor strain, 3 (dark red) = high inhibition zone seen around the grown competitor strain. **C: Visual BHI agar plate with supernatant assay of clade B indicator strain 49_4E-6-A with clade A1 competitors 25_K60-02, 23_K59-44, 21_K59-36 and 20_K59-17.** H = heat treated, P = proteinase K treated, U = untreated supernatant competitor.

4.3 Bacteriocins are present in clade A2 genomes

Bacterial sequences of clade A2 were uploaded into the bacteriocin database BAGEL4 [45] to predict bacteriocins. The goal was to find patterns between experimental results and bioinformatic approaches and detect unknown bacteriocins encoded in specific strains. Additionally, the bacteriocin size and classification were annotated by using BAGEL4.

Within clade A2 strains class II bacteriocins could be predicted via BAGEL4 including Enterocin A (6.89 kDa), Enterocin B (7.48 kDa), Enterocin P (7.21 kDa), Enterocin_SE-K4 (4.62 kDa), Bacteriocin_T8 (10.17 kDa), Enterocin_L50a (7.33 kDa), Bacteriocin_31 (7.97 kDa), Bac32 (10.03 kDa), UviB (7.76 kDa), Hiracin JM79 (5.09 kDa) and a class III bacteriocin Enterolysin A (42.71 kDa) (Figure 12).

Firstly, commensal clade A2 strains 01_T7EF-51021120, 03_T7EF-51024681, 05_T7EF-51007961, 10_T7EF-51025019, 19_T7EF-50994001, 22_T7EF-50976613, 24_T7EF-50980395 and 25_T7EF-51010478 showed constant inhibition, some stronger than others, towards clinical A1 strains (Figure 10A). Most of these strains encode Enterocin P, Enterocin A, Enterolysin A and one to three additional bacteriocins like Bac 32 and Bacteriocin 31 (strain 03_T7EF-51024681 and 10_T7EF-51025019 in Figure 12). Strain 22_T7EF-50976613 and 24_T7EF-50980395 do not encode Enterocin P, but Enterocin B. The most inhibiting strains and at the same time inhibition resistant strains 01_T7EF-51021120 and 25_T7EF-51010478 revealed different bacteriocins predictions. Strain 01_T7EF-51021120 only encoded Enterocin A, whereas strain 25_T7EF-51010478 encoded five more bacteriocins (Figure 12). Enterocin A was found in 84% of clade A2 strains and represents the most common bacteriocin within clade A2 strains. Bacterial strains which only encode one bacteriocin (12_T7EF-50995300, 13_T7EF-51024161, 14_T7EF-50987227) also showed mainly no inhibition towards clinical A1 strains (with exception of strain 01_T7EF-51021120) (see Figure 10A and 12).

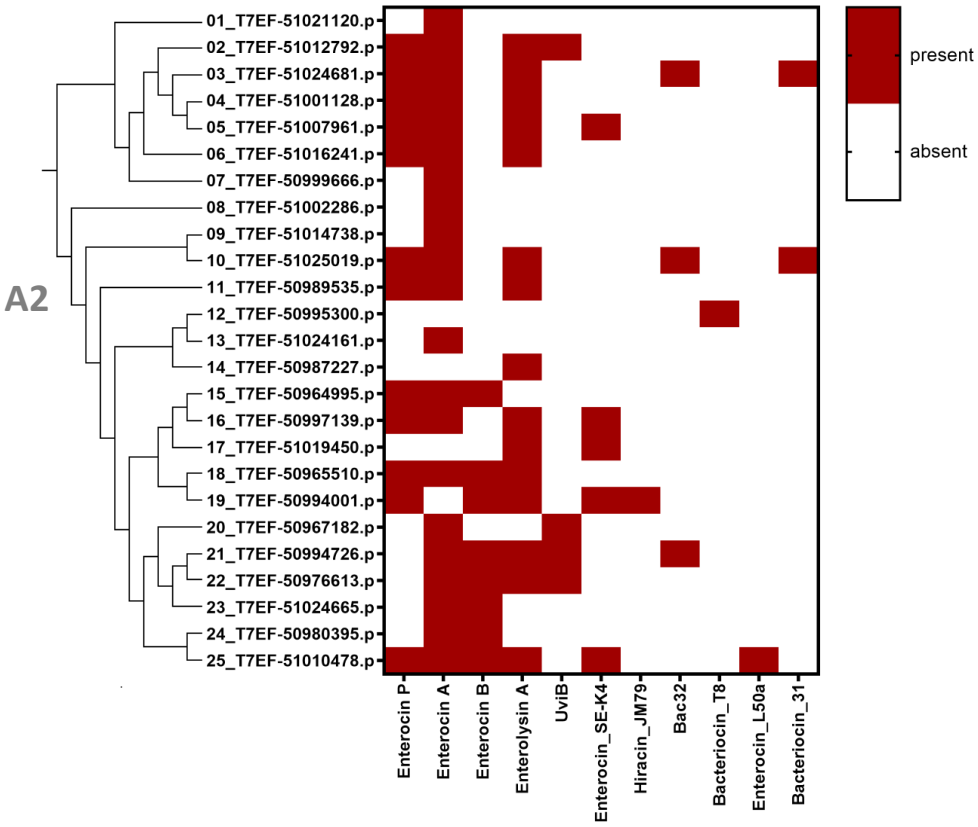

Figure 12: Heatmap summarizing predicted bacteriocins within the commensal clade A2 of *E. faecium*. Present bacteriocin types are indicated on the x-axis and strains of clade A2 on the y-axis. Clade A2 strains are sorted like in the phylogenetic tree and their phylogenetic relations are represented on the y-axis. FASTA format sequences of bacterial strains were uploaded in BAGEL4 database [45] (02.11.2022) to scan for present bacteriocins (red = present, white = absent).

5 Discussion

5.1 Clinical and commensal *E. faecium* can inhibit each other

Intra- and interspecies dynamic interactions of microbes and their outcomes are known to affect the host in a beneficial or harmful way e.g. causing disease [37]. *E. faecium* can inhibit the growth of a wide range of different Gram-positive bacteria, especially pathogens causing food poisoning [44, 54]. Only a few studies have been conducted on intraspecies interaction within *E. faecium*. Colonization and particularly overgrowth of resistant hospital *E. faecium* strains within the GI-tract can be the first step in causing serious infections in humans and therefore *E. faecium* clade interaction should be further investigated [28]. This could help to gain a deeper understanding of *E. faecium* clades and their role in healthcare.

5.1.1 Hospital outbreak associated clinical STs show high inhibition

Various studies revealed different *E. faecium* ST types being more invasive or prevalent than others in hospital outbreaks [6, 55]. Different STs have dominated in recent years in Norway [6]. ST117, ST80 and ST203 belong to the most prevalent ones in Norway [6] and in Germany as well [56] and were found to act highly inhibiting towards commensal A2 strains when growing them competitively in this study (Figure 10B). ST117 was named as being responsible for increasing hospital outbreaks in Germany from 2008 to 2018 [57] as well as in other European countries like Norway [58] and worldwide [55, 59]. In this study, ST117 clinical strain 49_NORM1E3_ST117 belonging to clade A1 showed a remarkably high inhibition pattern towards almost all commensal strains of clade A2 (Figure 10B). ST117 strains 48_2-D-9 and 50_1-H-7 showed less inhibition or only weak inhibition (Figure 10B). The success of ST117 remains not completely solved, however, high production of bacteriocins was described in ST117 strains already back in the 1990s hypothesizing clinical outcompeted commensal strains and then caused infection [55].

In Ireland, linezolid-resistant and vancomycin-resistant ST80 *E. faecium* was found to be increasingly involved in hospital outbreaks as well [60]. Clinical strains 14_1-I-9_ST80 and 12_1-I-3_ST80 from this study showed a very high inhibition pattern as well towards almost all commensal strains (Figure 10B) correlating with the literature. Conversely, ST80 strain 13_1-D-5_ST80 almost showed no inhibition towards commensals (Figure 10B). Some ST80 strains were found to carry a conjugative plasmid which encodes genes involved in antibiotic resistance [60]. Bacteriocins could be encoded on conjugative plasmids as well within ST80 strains being responsible for inhibition of commensal A2 strains (see 5.2).

All clinical strains belonging to ST203 (09_K59-68, 08_K60-31, 07_1-H-4 and 42-45) showed very high inhibition towards commensal strains (Figure 10B). *E. faecium* ST203 strains represent epidemic clones in hospitals causing VRE bacteremia since they encode the *vanB* gene for being resistant towards vancomycin [61].

5.1.2 Relation of *in vitro* to *in vivo* competition studies of *E. faecium*

Montealegre *et al.* described *in vitro* and *in vivo* competition studies of *E. faecium* commensal clade B with clinical clade A1 and revealed clade B having a competitive advantage over clade A1 strains [28]. Compared to this study some contrasts can be outlined. According to the *in vitro* bacterial competition assays from this study clinical strains inhibited commensal strains more frequently and with higher inhibition (Figure 10). Though, commensal clade A2 strains were used which are genomically more distant towards clade B strains [23]. Also, *E. faecium* clade B strains were suggested to be renamed as *E. lactis* [23]. Furthermore, experimental methodologies differed and less than 10 strains per clade were chosen [28] compared to this study which included a wide range of strains with 25 clade A2 and 50 clade A1 strains with different positions in the phylogenetic tree. Also, Wagner and Engi indicated inhibition of clade B strains by clade A1 strains, but with a higher number of experimental samples [51]. Because a wide spectrum of samples is covered in this study compared to Montealegre *et al*. different interaction results can be explained as well [28]. The isolation site of all clinical strains from this study was mainly blood from hospitalized patients in Norway, whereas Montealegre *et al.* used closely related clinical A1 strains isolated specifically from endocarditis patients in the US [28]. Commensal strains were not only sampled from feces like in this study, but also from wounds or gut and might therefore not count as true commensals [28]. However, the here presented *in vitro* study does not represent the natural habitat of the GI-tract or especially not the complex gut habitat which is rich in various bacterial species and other microbes [28]. This particularly could explain the contradicting results between the *in vivo* murine GI-tract model experiments and *in vitro* experiments from this study. Commensal *E. faecium* strains could have an evolutionary colonization advantage towards clinical strains in the gut, since they are known for their ability to adhere to the mucus layer while clinicals might lose this ability due to the cost of acquiring resistance [36, 38]. Therefore, in antibiotic absent *in vivo* models like in Montealegre *et al*. the ability of commensals naturally outcompeting clinicals might be higher. If a higher number of commensals is present due to a lack of antibiotics, they may exert more inhibition. This could explain why commensals can also overtake the GI-tract again once a patient leaves the hospital [28]. *In vitro* experiments do not respect the mucosal habitat and

commensals might not benefit from the advantage of adherence to the mucus layer anymore so clinical strains can significantly carry out more inhibition [36, 38]. Still, 49% of the commensal competitors carried out inhibition in this *in vitro* study suggesting commensal strains acquired different strategies as well to compete against clinical strains like secreting antimicrobials (see 5.2). Furthermore, also commensal *E. faecium* can cause disease in immunosuppressed patients [6]. Lebreton *et al.* described a case where a commensal lineage encoded vancomycin resistance causing bacteremia in an immunocompromised patient [62]. In the clinical strain collection of this study, isolated from blood, six strains belonged to clade B indicating they are commensals (indicated in 4.2). These clinical strains which clustered with clade B mostly carried out low inhibition towards A2 strains, whereas A2 strains could inhibit them in most cases *in vitro* (Figure 10). Montealegre *et al.* described a predominance of clade B strains over clade A2 strains in *in vivo* models, though the experimental setup and number of strains differed [28]. Thus, considering the competitional behavior in the context of the complex gut habitat and not only within the narrow experimental setup of this study might be essential for future studies.

Biofilm formation Another reason why commensals could overgrow clinical *E. faecium* strains in germ-free GI-tract mouse models is their ability to form microcolonies which act within biofilm formation [38]. This was found to be encoded in specific genes which contribute to enterococcal biofilm formation *in vitro* [38]. This could give them a colonization advantage in a healthy gut environment being resilient to environmental changes [38]. Exchanging genetic material, including AMR genes, within these microcolonies could be advantageous for clinical strains as well and antibiotics could be tolerated within the biofilm formation due to metabolic inactive cells [38]. *E. faecium* biofilm formation is associated with disease too, e.g. endocarditis and bacteremia [63].

Nutrient acquisition Depletion of microbes in the GI-tract usually is carried out indirectly by the mucosal immune system or directly via competition with other microbes [38]. Therefore, the fitness of *E. faecium* can change immediately with the presence or absence of different microbes in the gut [38]. As already introduced in Figure 5, antibiotic treatment favors the overgrowth of Gram-positive bacteria in the gut including *E. faecium* due to killing of Gram-negatives by antibiotics [12]. This additionally leads to more nutrient availability and in general to more available resources or increasing colonization of *E. faecium* to act pathogenic [38]. Clinical strains could also have a colonization advantage then, considering that higher numbers of them are more likely to exert inhibition of commensal strains. Additionally, Lindenstrauss *et al.* described that most upregulated genes within the enterococcal

transcriptome of GI-tract colonization in mice encoded for nutrient transport and metabolism including the four-gene complex PTS (phosphotransferase system) which allows an increased carbohydrate uptake [38, 64]. PTS are known to be upregulated in clinical strains of *E. faecium* and could play a role in GI-tract colonization during hospitalization [28, 38]. Therefore, it could be hypothesized clinical strains can succeed over commensal strains since their ability to acquire nutrients is higher. The growth medium used in this study, BHI, might give a growth advantage to some strains used here and might thus have influenced the observed results.

In general, almost all the mechanisms *E. faecium* uses to colonize are encoded in the core genome, since these microbes evolved these mechanisms millions of years ago when naturally colonizing the GI-tract after terrestrialization [38]. Their accessible genome contributes to colonization advantages and makes clinical strains to threatening pathogens [38].

5.2 Involvement of secreted compounds in interaction

E. faecium is known to inhibit other species like *Listeria monocytogenes* or *Staphylococcus aureus* with the help of antimicrobials e.g. bacteriocins [65]. Intraspecies inhibition and resulting colonization between clinical and commensal strains mediated via bacteriocins is described as well [66]. In general, several studies describe the genomic differences between clinical and commensal strains and their resulting impact on the fitness of *E. faecium* [38]. Chromosomally integrated bacteriophages in VRE strains were described to give a colonization advantage in the GI-tract over commensal strains in antibiotic treated mice [67]. Furthermore, plasmids are exchanged as well between strains carrying essential genes which helps them gain competitive advantages [38]. Kommineni *et al.* confirmed in an *in vivo* mouse model the presence of a bacteriocin encoding conjugative plasmid (pPD1) benefit *E. faecalis* strains to outcompete strains lacking this plasmid including clinical strains [66]. pPD1 plasmids are common within *E. faecalis* and can give strains a colonization advantage in the GI-tract [66]. Therefore, enterococci producing bacteriocins could be beneficial in replacing clinical strains during infection [66]. However, it has been shown that MGEs encoded on plasmids can be exchanged between and within clades [20]. Therefore, bacteriocins might be swapped to both clade A1 and A2 strains [20]. Commensal clade A2 and B strains contribute to clade A1 adaption in hospitals over time as they act as gene reservoirs [20].

Bacteriocins are usually more enriched in clinical strains than commensal strains of *E. faecium* [68, 69]. This confirms generated results of bacteriocin frequency in commensal A2 strains with BAGEL4 database (Figure 12) in comparison to generated hits within clinical A1 strains from

a previous study by Wagner and Engi [51] (Figure 13 Appendix). Only approximately half of the bacteriocins found in clinical A1 strains were found within commensal A2 strains, though Enterocin A and Enterolysin A were found in both clinicals and commensals most frequently. Enterocin A is frequently found and isolated from several *E. faecium* strains, especially from food [46]. Enterocin A is not only active against *Enterococcus*, but also against *Lactobacillus, Pediococccus* spp. and *Listeria* spp. [46]. Some bacteriocins predicted in this study, were mostly described as being isolated from *E. faecalis* [46], like Enterolysin A which has a broad-spectrum activity against pathogenic and non-pathogenic bacteria and degrades cell walls [47, 70]. Enterocin P was described as being produced by *E. faecium* and shows broad antimicrobial activity for example against *Enterococcus, Lactobacillus, Pediococcus* spp. and some food-borne pathogens like *S. aureus* [46].

This as well is in line with the results of the supernatant assays since most strains showed no inhibition when treated with proteinase K and still inhibition when treated with heat, indicating *E. faecium* strains secreted heat-stable proteinaceous compounds e.g. bacteriocins (Figure 11) [65]. Most bacteriocins are heat-stable, however, Enterolysin A, a heat-labile bacteriocin (class III) was predicted via BAGEL4 as well (Figure 12). Clinical supernatant competitors of clade A1 might encode additional non-proteinaceous and some heat-unstable antimicrobials according to Figure 11A. A logical pattern between the competitive interactions of the supernatant assays and found bacteriocins in BAGEL4 hardly reveals throughout the results, since the presence of bacteriocins can not necessarily be associated with clear inhibition. Therefore, it can be hypothesized commensal strains most likely encode unknown antimicrobials or novel bacteriocins which are not annotated in the BAGEL4 database yet. Different antimicrobials produced by gut commensals are described to contribute to pathogen extinction within the gut [71, 72]. Short-chain fatty acids (e.g. butyric, acetic and propionic acids) can decrease pathogen colonization (like *Salmonella* spp. and *E. coli* O157) via inhibiting virulence gene expression or lowering the pH in specific regions [72-74]. Using the BAGEL4 database limits this study given that it only includes known bacteriocins and unknown ones cannot be identified. The lack of strong bacteriocin databases represents a fundamental issue [75]. Databases which focus on new tools and algorithms to identify bacteriocins could be a solution [75].

Strains 01_T7EF-51021120 and 25_T7EF-51010478 from commensal clade A2 showed constant inhibition towards clinical A1 strains indicating highly competitive commensal strains might carry bacteriocins targeting more clinical strains to a higher degree. Supernatant assays

of these strains revealed proteinaceous, heat-stable secreted compounds. However, strain 25_T7EF-51010478 shows five more bacteriocins than strain 01_T7EF-51021120 (Figure 12). This indicates that bacteriocins might act to different degrees in competition towards different strains, since a strain only encoding Enterocin A could carry out similar inhibition results as a strain encoding six bacteriocins. On the other hand, it might be unknown bacteriocins and their immunity genes were involved. Additionally, competitors represented a much higher density than indicators in the experimental setup of this study. Higher cell densities can increase bacteriocin activity, since a higher amount of bacteriocins is produced [76]. Therefore, a competitor strain more likely produces a higher amount of bacteriocins than the less dense population of the indicator strain and outcompetes the indicator strain. This should be considered in future studies and represents a limitation of the method used.

Bacteriocins are usually produced under stressful growth conditions like limitations of nutrients, changes in optimal pH and optimal temperature [43, 76]. Growth rates are slower and bacteriocin production is increased when lacking resources and energy [43, 76]. Optimal *in vitro* growth conditions favoring high growth rates of *E. faecium* and therefore the availability of metabolites for bacteriocin synthesis is reduced since they are demanded for growth [43, 76]. This could explain why some bacterial strains did not show any inhibition, since the strain itself was grown under optimal conditions without any stressors present like in a natural gut habitat with other competing microbials or dynamic growth conditions. Therefore, if bacteriocins still were produced, degradation of them during the supernatant preparation might have influenced the results as well. In addition, since bacteriocin production is costly for bacteria, their production may be dependent on the presence of competitors [36]. Quorum-sensing regulated bacteriocin production could be hypothesized, the mechanism where a specific population density must be present to achieve sending signals for antimicrobial production [36]. To take this into account, co-culture supernatant assays were conducted, where two strains were grown together, however, supernatants extracted from co-culture did not show an increased inhibition in pilot experiments (data not shown).

The phylogenetic relations within the commensal and within the clinical clade revealed similar inhibition patterns of strains being related to each other (Figure 10). This pattern was more consistent within the clinical phylogeny (Figure 10A). Concluding, more related strains also have a similar genome carrying out similar inhibition patterns and encoding similar bacteriocins which can be slightly visible in bacteriocin prediction as well (Figure 12) [21].

6 Conclusion

It has been disclosed that both clinical and commensal *E. faecium* strains are able to outcompete strains of the other clade *in vitro*. Clinical clade A1 strains carried out inhibition towards commensal clade A2 strains with a higher frequency. Most of these clinical strains were associated with globally trending STs causing nosocomial *E. faecium* outbreaks underlining the clinical relevance.

Several commensal clade A2 strains showed high inhibition towards clinical A1 strains and were resistant to inhibition of clinicals emphasizing commensals having colonization advantages once hospitalization ends. This potentially could contribute to new approaches in research.

Most of the inhibitory interactions between commensal and clinical *E. faecium* were shown to be proteinaceous and heat-stable secreted agents, indicating mostly bacteriocins mediate inhibitory processes during enterococcal competition. Still, some strains possibly encode unknown bacteriocins or other types of secreted compounds.

All in all, the use of a wide range of bacterial samples spread over the phylogenetic tree highly strengthens this study and stands out compared to other studies.

7 Outlook

Nosocomial *E. faecium* infections highly contribute to AMR associated mortality rates worldwide [6, 12]. Clinical *E. faecium* developed strategies to outcompete commensal *E. faecium*, which are naturally a part of the human microbiome within the gut, to cause disease in immunocompromised patients [13, 28].

Continuous research is needed in the future to fully understand the dynamics between commensal and clinical *E. faecium* clades [28]. This study does not consider the real habitat of the GI-tract environment, thus further *in vivo* studies are needed. In future competition studies, high-throughput screenings of isolated clinical and commensal samples in mice could be interesting. Though, higher experimental workloads and ethical precautions must be considered. Different isolation sites could be interesting as well e.g. for clinicals directly from wounds or diseased patients as well as samples from different hospitals in Europe. Due to immigration and travel new strains might be present as well in Norway.

Finding new antibacterial strategies besides antibiotics is essential in fighting AMR [50]. Bacteriocins represent potential candidates for antimicrobial drugs [50]. Many bacteriocins are specifically active against clinical strains including AMR strains [50]. As shown in this study, many commensal *E. faecium* which showed inhibition towards clinical *E. faecium*, could potentially be studied as an antibacterial alternative for *E. faecium* infections. Probiotic effects of commensal enterocins or bacteriocins of other species represents a potential therapy option against clinical *E. faecium* strains and could influence human health in a positive way [48]. Isolated strains from food were described to survive within the GI-tract or carry out inhibition towards specific pathogens or modulate bigger microbial communities [48, 77]. Marine animals in remote ecologies, like the Arctic or Antarctic, could be studied to identify novel bacteriocins in the future [47]. Enterococci isolated from marine animals possibly can serve as probiotics as well and were proven to show high potential [78, 79]. Furthermore, bacteriocins have a low toxicity towards the host since they act direct towards the pathogen [50]. Bioengineering of bacteriocins might be considered too, since their peptide structure could be synthesized or manipulated, and their clinical functions could be improved [50]. Resistant development is possible, but much less as with traditional antibiotics and strategies could be introduced to avoid this [50]. Future perspectives for this study might be bacteriocin isolation and determination with the help of mass spectrometry or HPLC (high-performance liquid chromatography) to identify specific bacteriocins and test them individually for probiotic performances.

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Appendix

Table 1: List of selected *E. faecium* strains of clade A1, A2 and B. Vancomycin-res. = Vancomycin-resistant

ID (freeze number)	Source	ST	Year	Country	Isolation site	Vancomycin-res.	Ampicillin-res.
Clade A1							
K60-25	Hospitalized patient	581	2008	NOR	Blood	No	n.a. *
K60-2	Hospitalized patient	52	2008	NOR	Blood	No	No
K59-57	Hospitalized patient	38	2008	NOR	Blood	No	No
K59-46	Hospitalized patient	533	2008	NOR	Blood	No	No
K59-44	Hospitalized patient	32	2008	NOR	Blood	No	No
K59-36	Hospitalized patient	575	2008	NOR	Blood	No	No
K59-17	Hospitalized patient	22	2008	NOR	Blood	No	No
K60-29	Hospitalized patient	19	2008	NOR	Blood	No	Yes
K60-7	Hospitalized patient	578	2008	NOR	Blood	No	Yes
K59-51	Hospitalized patient	18	2008	NOR	Blood	No	Yes
3-E-9	Hospitalized patient	18	2012	NOR	Blood	Yes	Yes
K59-53	Hospitalized patient	132	2008	NOR	Blood	No	Yes
K59-18	Hospitalized patient	574	2008	NOR	Blood	No	Yes
K59-16	Hospitalized patient	440	2008	NOR	Blood	No	Yes
1-A-9	Hospitalized patient	17	2015	NOR	Feces	Yes	Yes
K59-55	Hospitalized patient	279	2008	NOR	Blood	No	Yes
K59-50	Hospitalized patient	202	2008	NOR	Blood	No	Yes
1-F-9	n.a.	202	2011	NOR	Urine	Yes	Yes
2-D-9	Hospitalized patient	117	2014	NOR	Blood	No	Yes
1-H-7	Hospitalized patient	117	2013	NOR	Feces	Yes	Yes
NORM 1-E-3	Hospitalized patient	117	2014	NOR	Blood	No	Yes

K59-62	Hospitalized patient	282	2008	NOR	Blood	No	Yes
VRE 1-G-3	n.a.	736	2011	NOR	Urine	Yes	Yes
1-I-9	n.a.	80	2012	NOR	Urine	Yes	Yes
1-I-3	Hospitalized patient	80	2014	NOR	Blood	No	Yes
1-D-5	Hospitalized patient	80	2014	NOR	Blood	No	Yes
K59-22	Hospitalized patient	78	2008	NOR	Blood	No	Yes
VRE 3-B-5	Hospitalized patient	412	2013	NOR	Feces	Yes	n.a
K59-68	Hospitalized patient	203	2008	NOR	Blood	No	Yes
K60-31	Hospitalized patient	203	2008	NOR	Blood	No	Yes
VRE 2-I-1	Hospitalized patient	78	2014	NOR	Feces	Yes	Yes
1-H-4	Hospitalized patient	203	2014	NOR	Blood	Yes	Yes
K59-27	Hospitalized patient	17	2008	NOR	Blood	No	Yes
1-B-3	n.a.	17	2010	NOR	Urine	Yes	Yes
2-E-8	Hospitalized patient	192	2011	NOR	Feces	Yes	Yes
VRE 2-F-6	Hospitalized patient	192	2011	NOR	Feces	Yes	Yes
3-A-9	Hospitalized patient	192	2010	NOR	Feces	Yes	Yes
1-C-3	Hospitalized patient	192	2014	NOR	Blood	No	Yes
K60-14	Hospitalized patient	192	2008	NOR	Blood	No	Yes
K59-30	Hospitalized patient	192	2008	NOR	Blood	No	Yes
1-C-2	Hospitalized patient	203	2014	NOR	Blood	No	Yes
K59-20	Hospitalized patient	203	2008	NOR	Blood	No	Yes
K59-59	Hospitalized patient	203	2008	NOR	Blood	No	Yes
K59-60	Hospitalized patient	203	2008	NOR	Blood	No	No
K59-26**	Hospitalized patient	94	2008	NOR	Blood	No	No
K60-21**	Hospitalized patient	580	2008	NOR	Blood	No	No
K59-52**	Hospitalized patient	576	2008	NOR	Blood	No	No
K59-19**	Hospitalized patient	296	2008	NOR	Blood	No	Yes
NORM 1-A-2**	Hospitalized patient	94	2014	NOR	Blood	No	No
K60-9**	Hospitalized patient	579	2008	NOR	Blood	No	No

Clade B							
2E-8-1	Non-hospitalized person	1945	2014/15	NOR	Feces	No	No
6E-1-H	Non-hospitalized person	116	2014/15	NOR	Feces	No	No
4E-2-G	Non-hospitalized person	1938	2014/15	NOR	Feces	No	No
1E-6-C	Non-hospitalized person	1031	2014/15	NOR	Feces	No	No
5E-1-H	Non-hospitalized person	696	2014/15	NOR	Feces	No	No
1E-1-A	Non-hospitalized person	1926	2014/15	NOR	Feces	No	No
2E-7-F	Non-hospitalized person	296	2014/15	NOR	Feces	No	No
6E-7-B	Non-hospitalized person	296	2014/15	NOR	Feces	No	No
1E-7-E	Non-hospitalized person	218	2014/15	NOR	Feces	No	No
2E-2-D	Non-hospitalized person	178	2014/15	NOR	Feces	No	No
6E-1-G	Non-hospitalized person	96	2014/15	NOR	Feces	No	No
4E-4-1	Non-hospitalized person	94	2014/15	NOR	Feces	No	No
5E-3-1	Non-hospitalized person	2016	2014/15	NOR	Feces	No	No
3E-1-E	Non-hospitalized person	361	2014/15	NOR	Feces	No	No
6E-3-D	Non-hospitalized person	361	2014/15	NOR	Feces	No	No
2E-9-F	Non-hospitalized person	361	2014/15	NOR	Feces	No	No
4E-1-E	Non-hospitalized person	60	2014/15	NOR	Feces	No	No
2E-5-A	Non-hospitalized person	94	2014/15	NOR	Feces	No	No
4E-6-A	Non-hospitalized person	178	2014/15	NOR	Feces	No	No
Clade A2							
T7EF-51012792	Non-hospitalized person	59	2014/15	NOR	Feces	No	No
T7EF-51007961	Non-hospitalized person	247	2014/15	NOR	Feces	No	No
T7EF-51024681	Non-hospitalized person	2027	2014/15	NOR	Feces	No	No
T7EF-50994001	Non-hospitalized person	69	2014/15	NOR	Feces	No	No
T7EF-50965510	Non-hospitalized person	512	2014/15	NOR	Feces	No	No
T7EF-51025019	Non-hospitalized person	1952	2014/15	NOR	Feces	No	No

T7EF-51014738	Non-hospitalized person	101	2014/15	NOR	Feces	No	No
T7EF-50995300	Non-hospitalized person	29	2014/15	NOR	Feces	No	No
T7EF-51024161	Non-hospitalized person	524	2014/15	NOR	Feces	No	Yes
T7EF-50987227	Non-hospitalized person	640	2014/15	NOR	Feces	No	No
T7EF-51021120	Non-hospitalized person	5	2014/15	NOR	Feces	No	Yes
T7EF-51019450	Non-hospitalized person	1971	2014/15	NOR	Feces	No	No
T7EF-50994726	Non-hospitalized person	533	2014/15	NOR	Feces	No	No
T7EF-50967182	Non-hospitalized person	32	2014/15	NOR	Feces	No	No
T7EF-50976613	Non-hospitalized person	22	2014/15	NOR	Feces	No	No
T7EF-51024665	Non-hospitalized person	32	2014/15	NOR	Feces	No	No
T7EF-51010478	Non-hospitalized person	1940	2014/15	NOR	Feces	No	No
T7EF-50980395	Non-hospitalized person	165	2014/15	NOR	Feces	No	No
T7EF-50964995	Non-hospitalized person	1928	2014/15	NOR	Feces	No	No
T7EF-50997139	Non-hospitalized person	52	2014/15	NOR	Feces	No	No
T7EF-51016241	Non-hospitalized person	649	2014/15	NOR	Feces	No	No
T7EF-50999666	Non-hospitalized person	1239	2014/15	NOR	Feces	No	Yes
T7EF-51002286	Non-hospitalized person	1994	2014/15	NOR	Feces	No	Yes
T7EF-51001128	Non-hospitalized person	1982	2014/15	NOR	Feces	No	No
T7EF-50989535	Non-hospitalized person	44	2014/15	NOR	Feces	No	Yes

* n.a. = not available

** *E. faecium* strains which clustered with clade B within the phylogenetic tree, but were isolated in a clinical context

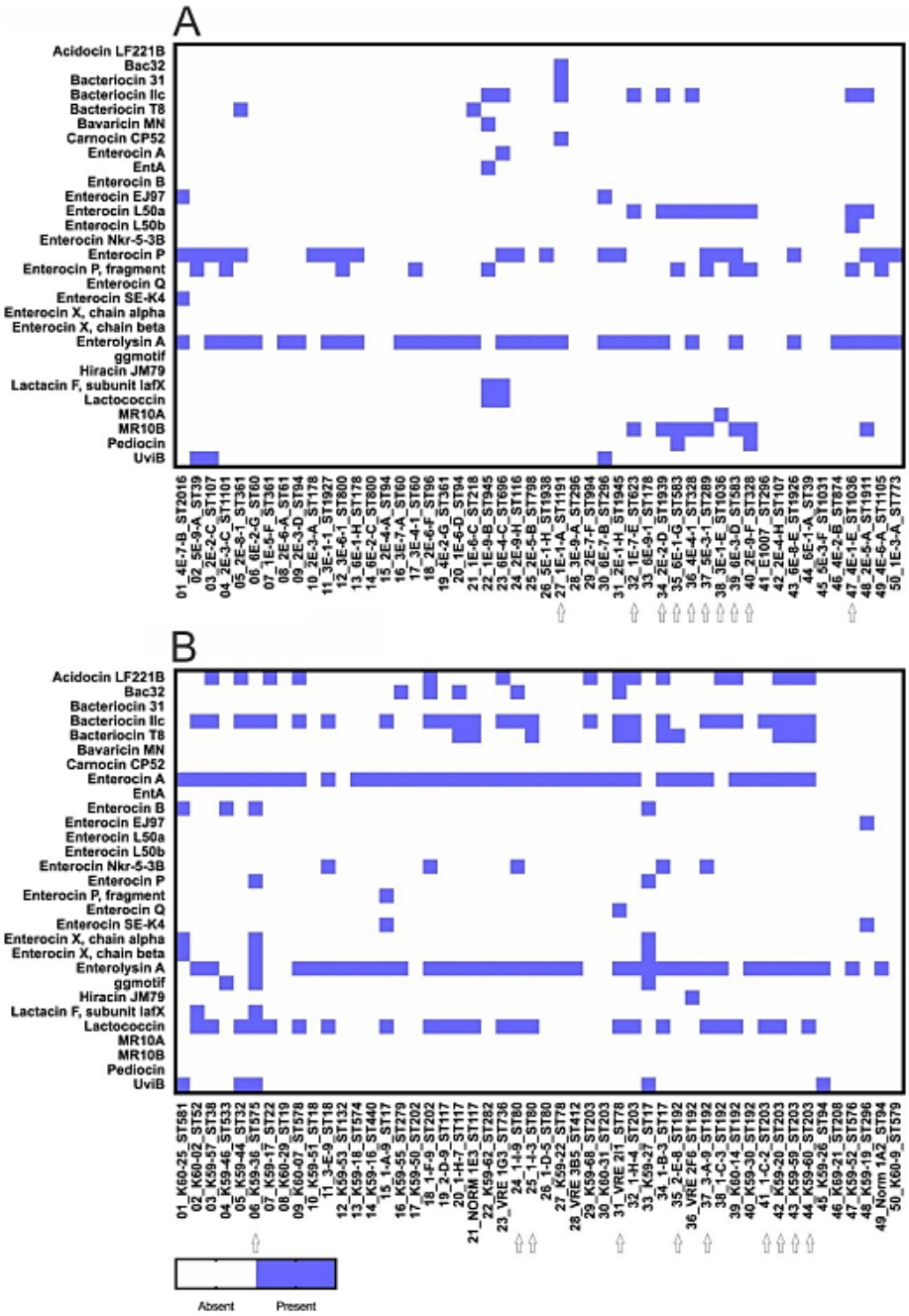

Figure 13: Bacteriocins in commensal and clinical strains (Figure taken from Wagner and Engi [51]). Bacteriocins in commensal (A) and clinical (B) strains. X-axis, bacteriocin; y-axis, strain. Presence of bacteriocins is marked in blue. Strains are sorted by their order in the commensal and clinical phylogenetic trees. The strains that inhibited the most strains of the opposing group during competitive growth of the study of Wagner and Engi are marked with arrows.

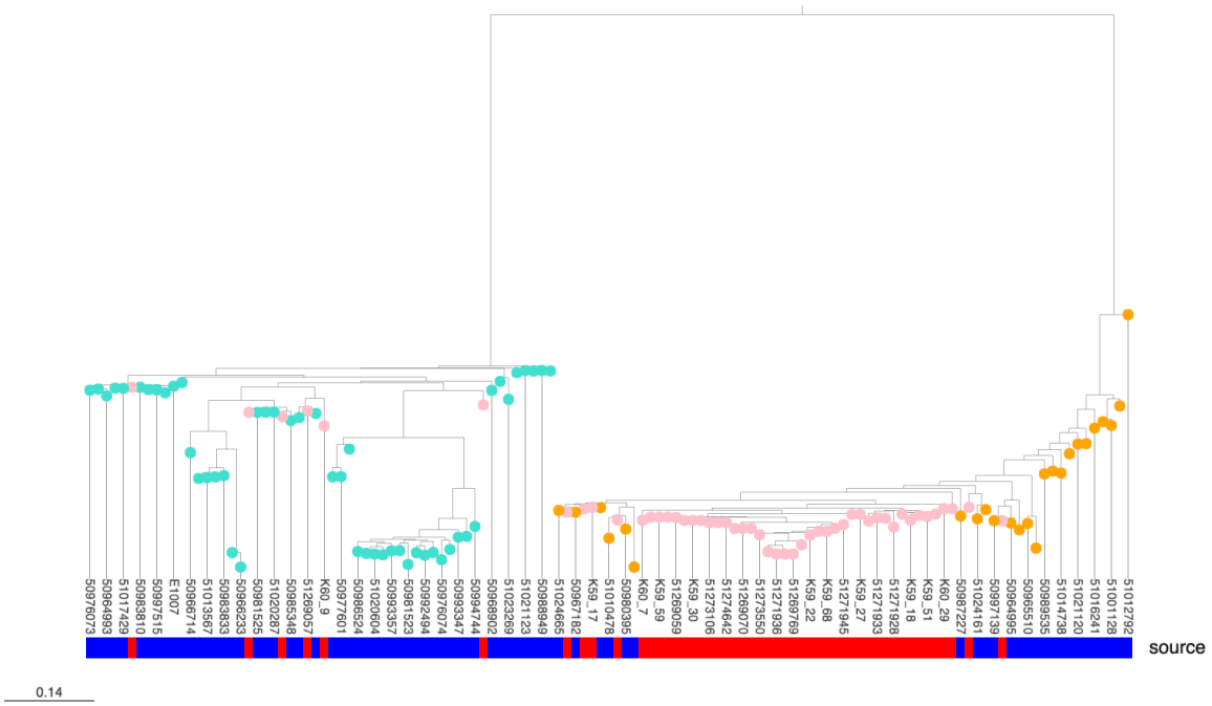

Figure 14: Phylogenetic tree of *E. faecium* representing relations between clade A1 (pink), A2 (orange) and B (blue). Tree made by Anna Pöntinen, K-res, using the program parsnp based on WGS sequences (parameters: parsnp -x -c -o Parnp_com_cli_ncbi -d all_genomes/ -r !). The tree is rooted at the midpoint. The source of bacterial strain is indicated: red = clinical, blue = commensal. For visualization, the application Microreact was used (http://microreact.org) [80].

Supplementary data

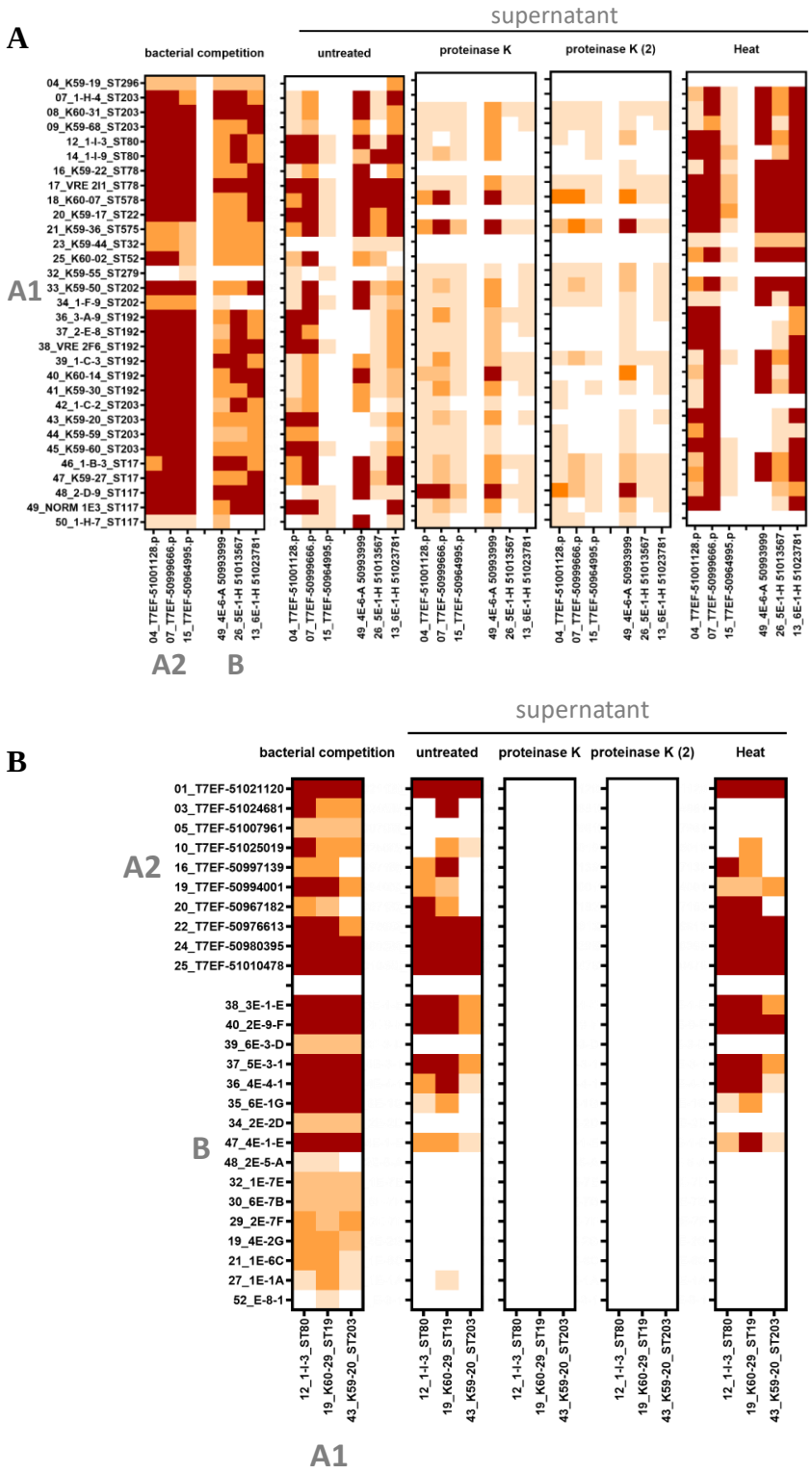

Figure 15: A: Heatmaps of summarizing growth inhibition grades of bacterial competition assays versus supernatant assays with stress treatments (with original Proteinase K treatment of 1 mg/ml and Proteinase K (2) doubled concentration 2 mg/ml and heat) between A: *E. faecium* clade A1 strains (clinical competitors) and *E. faecium* clade A2 and B strains (commensal indicators as lawn). Growth inhibition was exerted by clinical competitors (both bacterial and supernatant competitors) and **B: *E. faecium* clade B/A2 strains (commensal competitors) and *E. faecium* clade A1 strains (clinical indicators as lawn).** Growth inhibition was exerted by commensal competitors (either bacterial or supernatant competitors). All bacterial strains are sorted by their order in the phylogenetic tree. Indicator lawns are always indicated on the x-axis and competitor strains on the y-axis.