UiT The Arctic University of Norway

Faculty of Health Sciences

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

An *in vitro* and *in silico* study of bacteriocins

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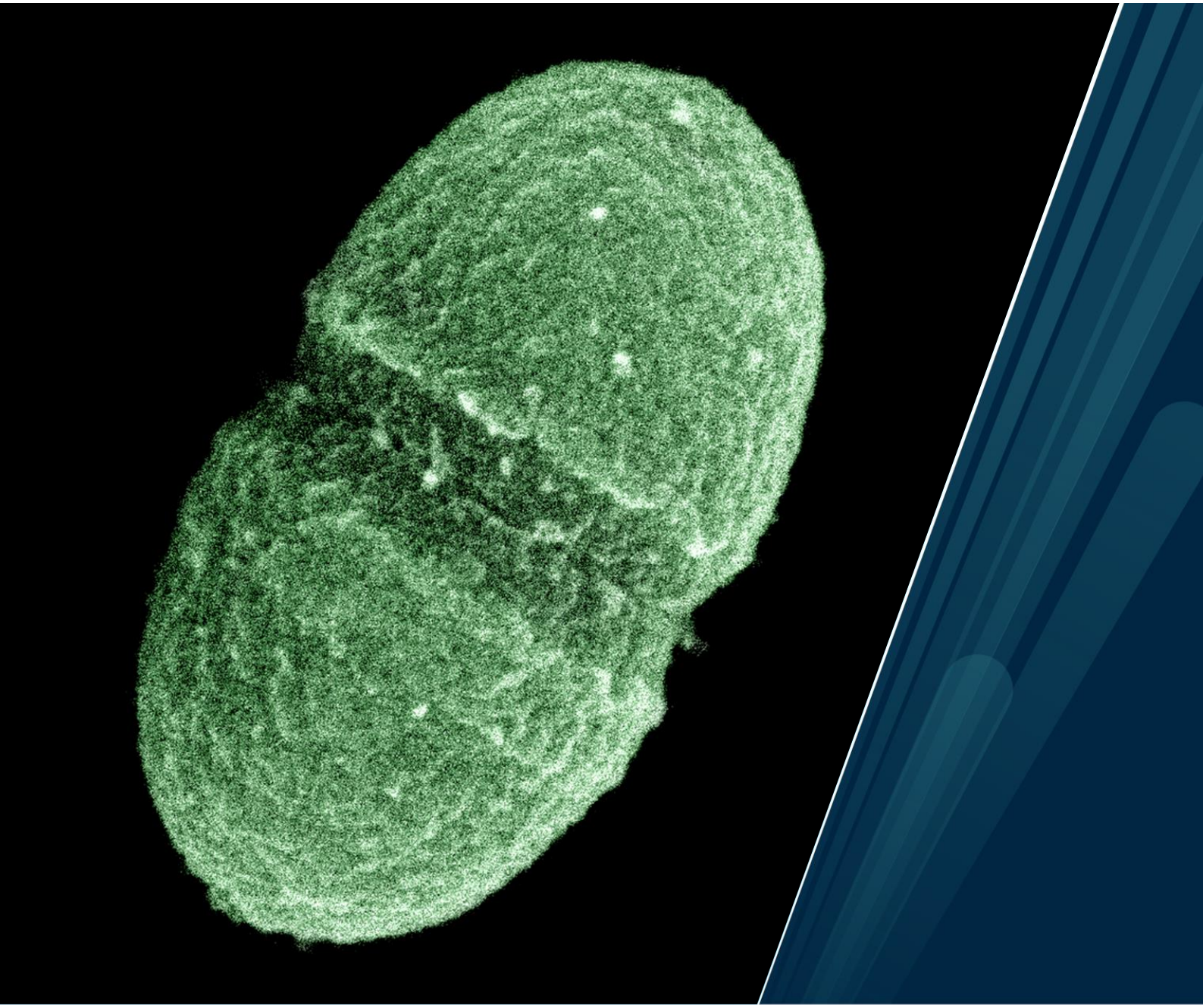

Preface

This thesis aimed to characterize the *in vitro* competition between commensal and clinical *Enterococcus faecium* strains with respect to growth inhibition exerted by bacteriocins produced by the respective strain groups. Previous unpublished studies by Wagner et al. suggested that known and characterized bacteriocins could not explain every interaction between commensal and clinical *E. faecium* strains. To supplement the laboratory assays used in this study, bioinformatical methods were applied.

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Finally, thank you Miriam Grgic for supporting and bearing with me throughout this process.

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Daniel Engi

Abstract

Invasive infection by multidrug-resistant *Enterococcus faecium* is increasingly becoming a healthcare concern. Few studies have addressed whether and how clinical *E. faecium* strains are able to outcompete commensal strains. This thesis aimed to characterize the *in vitro* competition between commensal and clinical *E. faecium* strains with respect to growth inhibition exerted by bacteriocins produced by the respective strain groups. A combination of laboratory assays and bioinformatical methods were applied toward this goal. Laboratory assays consisted of competitively growing commensal and clinical *E. faecium* strains, and using unmodified and modified supernatants from the same strains to investigate the nature of growth inhibition-mediating agents. The bioinformatical methods consisted of genetic relationship analyses and ribosomally synthesized post-translationally modified peptide-mining. It was shown that clinical *E. faecium* strains generally outcompete commensal *E. faecium* strains *in vitro*, though the commensal *E. faecium* strains that inhibit the growth of clinical *E. faecium* strains most often, do so very strongly. Furthermore, some commensal and clinical *E. faecium* strains possibly encoding novel bacteriocins were identified. Finally some commensal *E. faecium* strains that were resistant to the growth-inhibiting mechanisms of most epidemic and hospital-associated clinical *E. faecium* strains were found.

Abbreviations

BHI	Brain Heart Infusion
CFU	Colony-forming Unit
cgMLST	Core Genome Multilocus Sequence Typing
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EU	European Union
K-res	Norwegian National Advisory Unit on Detection of Antimicrobial Resistance
LAB	Lactic Acid Bacteria
MDR	Multidrug-resistant
MLST	Multilocus Sequence Typing
MWCO	Molecular weight cut-off
NCBI	National Center for Biotechnology Information
NORM	Norwegian Surveillance System for Antibiotic Resistance in Microbes
PBS	Phosphate-buffered Saline
RefSeq	Reference Sequence
SNP	Single Nucleotide Polymorphism
ST	Sequence Type

VRE collection	Vancomycin-resistant *E. faecium* from 2010–2015 in Norway
VRE	Vancomycin-resistant Enterococci
VREfm	Vancomycin-resistant *Enterococcus faecium*
WGS	Whole Genome Sequencing

Table of contents

1. Background

1.1 *Enterococcus*

Enterococcus is a genus of gram-positive, facultative anaerobic bacteria typically arranged as diplococci or in short chains.[1] They are a member of the order Lactobacillales, commonly known as lactic acid bacteria (LAB), due to having lactic acid as the major metabolic end-product of carbohydrate fermentation.[2]

Enterococci can be found naturally in water, soil, plants, food products and in the gastrointestinal tracts of a wide variety of animals, including humans.[3-6] The *Enterococcus* genus is sturdy and can survive under a variety of adverse conditions.[1, 7, 8] Its constituent members can grow in temperatures between 10–45 °C, they can survive temperatures up to 60 °C for 30 minutes, and they can tolerate hypo- and hypertonic milieus, desiccation, acidic and alkaline pH-values, long bouts of starvation and exposure to detergents, heavy metals and oxidants.[1, 8-11]

While mostly acting as commensal bacteria in the gut, some enterococcal species are opportunistic pathogens.[12, 13] Pathogenic enterococci were first described in a case study of endocarditis in 1899,[7] and more recent evidence shows that *Enterococcus* spp. cause intraabdominal- and intrapelvic infections, urinary tract infections, surgical wound infections, endocarditis, meningitis and bacteremia, especially in immunocompromised individuals.[14-16] Almost all infections in humans caused by enterococci are caused by *E. faecalis* and *E. faecium*, and are most often hospital-associated.[5, 17, 18] *E. faecalis* has always been the most common enterococcal cause of infection, though *E. faecium* is increasingly taking over that role.[19] Enterococci grow on inanimate surfaces and are in many intensive care units among the most common causative agents in infections involving medical equipment such as urinary catheters, central lines and ventilators.[20]

Enterococci are found to have intrinsic as well as acquired antimicrobial resistance to many antibiotics, including but not limited to aminoglycosides, ß-lactams, cephalosporins, chloramphenicol, glycopeptides, macrolides, streptogramins, tetracyclines, trimethoprim-sulfamethoxazole and quinolones.[9, 16, 17] They possess a vast arsenal of antibacterial-

resistance genes frequently associated with mobile genetic elements.[21] Moreover, enterococci have been shown to have the ability to exchange resistance determinants with other gram-positive bacteria, e.g. *Staphylococcus aureus*.[22, 23]

Data from the Surveillance Atlas of Infectious Diseases by the European Centre for Disease Prevention and Control (ECDC) indicates that whereas the proportion of invasive isolates of *E. faecalis* sensitive to aminopenicillins, high-level gentamicin and vancomycin in 30 European Union/European Economic Area (EU/EAA) countries (including Norway) generally declined from 2015 to 2019, the proportion of invasive isolates of *E. faecium* resistant to vancomycin (vancomycin-resistant *E. faecium,* VREfm) in the same countries nearly doubled (Table 1).[24]

Table 1. Susceptibility of clinically isolated enterococcal species to aminopenicillins, high-level gentamicin and vancomycin in 30 EU/EEA countries. Table based on data from the Surveillance Atlas of Infectious Diseases by ECDC.[24]

	Enterococcus faecium				*Enterococcus faecalis*			
	Percentage of invasive isolates resistant, all ECDC countries		Percentage of invasive isolates resistant, Norway		Percentage of invasive isolates resistant, all ECDC countries		Percentage of invasive isolates resistant, Norway	
Year	2015	2019	2015	2019	2015	2019	2015	2019
Aminopenicillins	89.0	89.3	86.5	75.1	2.1	0.9	0.5	0
High-level gentamicin	44.5	39.9	43.9	51.3	31.5	24.7	9.8	12.1
Vancomycin	9.5	17.5	0	1.0	0.6	0.8	0	0

In the Annual Epidemiological Report for 2019 by ECDC, with few exceptions, national percentages of high-level aminoglycoside resistance and vancomycin resistance in *E. faecium* were higher than for *E. faecalis*. In the same report, many bacterial species–antimicrobial group combinations displayed geographical gradients. However, for VREfm, no discernable geographic gradient was evident (Fig. 1).[25]

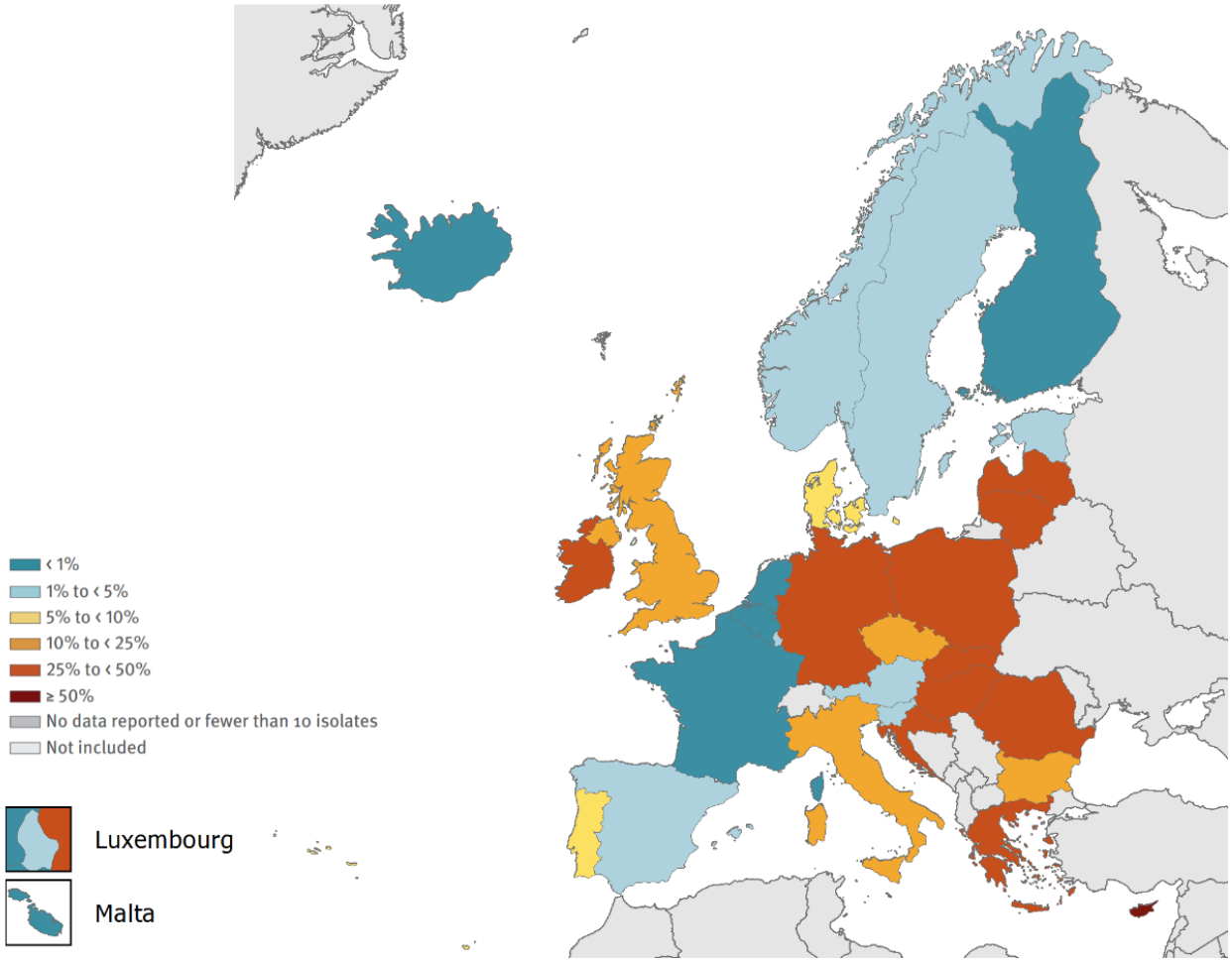

Figure 1. Proportion of invasive isolates of *E. faecium* resistant to vancomycin by country, EU/EEA, 2019. Figure from Annual Epidemiological Report for 2019 by ECDC[25] with reprint permission under a Creative Commons license.

1.2 *Enterococcus faecium*

Human and animal lineages of *E. faecium* diverged around 3000 years ago, coinciding with a period of increased urbanization, domestication of animals and development of hygienic measures. Around 80 years ago, the nosocomial lineage of *E. faecium* emerged, coinciding with the introduction of antibiotics. *E. faecium* came to the forefront in the late 1980s, and quickly became a leading cause of nosocomial infection.[26]

E. faecium is divided into clade A1, A2 and B (Fig. 2). Strains from clade A1 cause the majority of infections and include the epidemic hospital-associated strains. Strains from clade A2 cause sporadic infections in humans. Strains from clade B are considered commensal strains in humans, though they infrequently cause infection.[27] Nearly all clinically isolated

strains belong to clade A1, which has a significantly higher mutation rate than the other clades.[26] Clade B strains were found to outcompete clade A strains as persistent colonizers of the gastrointestinal tract in a murine model, barring antibiotic use.[28] However, VREfm, if introduced, starts to dominate the intestinal microbiome if antibiotics are concurrently administered, and large-scale colonization of the intestine with VREfm precedes VREfm bacteremia in humans.[29]

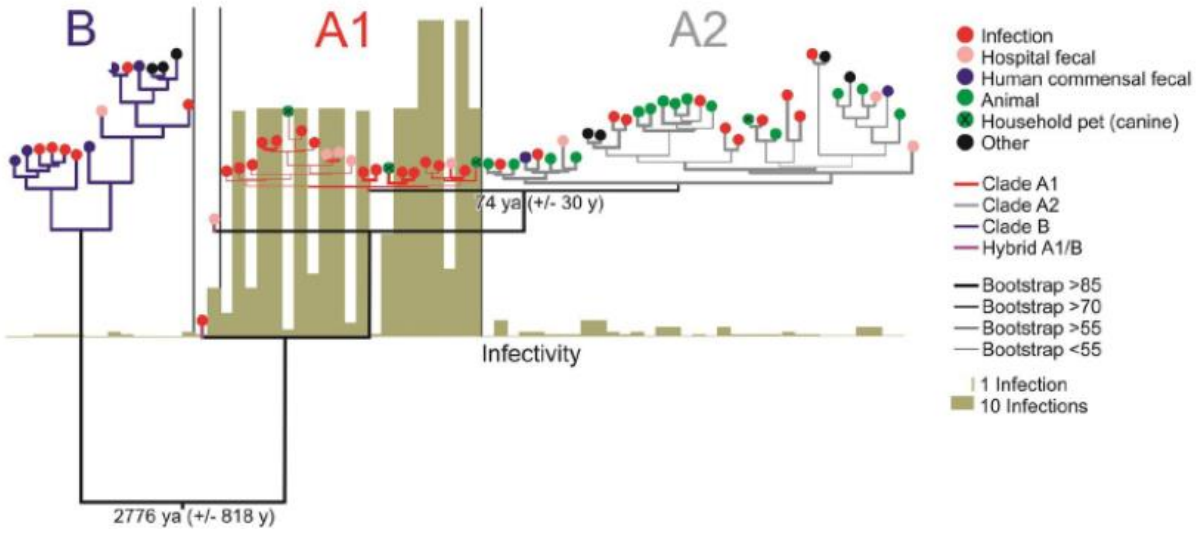

Figure 2. Clade structure of *E. faecium*. Single nucleotide polymorphism (SNP)-based phylogenetic tree based on DNA sequences of 1344 single-copy core genes in 73 *E. faecium* strains. The isolation sources of the strains are indicated. The dates for the split between the clades are estimated (ya, years ago). The infectivity score reflects the number of strains of a particular sequence type (ST), in the multilocus sequence typing (MLST) database (heading 1.3), isolated from infection. The clades are color-coded as follows: Clade B in dark blue, clade A1 in red and clade A2 in gray. Original figure from Lebreton et al. with reprint permission under a Creative Commons license;[26] modified figure by Wagner et al.[30]

VREfm infections are increasingly taxing healthcare providers globally, and VREfm is now classified as a pathogen with high priority in the World Health Organization's priority list for research and development of new antibiotics for antibiotic-resistant bacteria.[31] Some strains of *E. faecium* are found to be resistant to newer agents used to treat VRE and to last-resort antibiotics such as daptomycin, linezolid and quinupristin/dalfopristin, further complicating treatment.[9, 32-36] Additionally, some strains are becoming increasingly resistant to hand sanitizers, thus increasing their infectivity.[37, 38] For the aforementioned reasons alone, the further study of *E. faecium* is both warranted and necessary.

1.3 Multilocus sequence typing (MLST)

MLST is a method in molecular biology that allows for the intraspecies comparison of single-nucleotide polymorphisms (SNPs) in several housekeeping genes, which are genes that are typically constitutively expressed with products that are required for the maintenance of basal cellular function. These genes have a relatively slow rate of mutation as compared to non-housekeeping genes.[39-41] MLST enables both the subclassification of a species and the estimation of the degree of relatedness between different isolates. It is especially useful in identifying and monitoring clonal outbreaks in epidemiological settings.[42] In the case of *E. faecium,* seven housekeeping genes are used for the MLST scheme.[42]

While MLST holds advantages over other methods,[42] it suffers disadvantages as well. *E. faecium s*trains with deleted alleles at one of the seven housekeeper gene positions (making them untypable by MLST) have been isolated and characterized. These deletions have occurred independently in *E. faecium* on at least three occasions.[43] In addition, next-generation sequencing has given rise to core genome MLST (cgMLST), which has much higher resolution than traditional MLST and uses genome-wide allele classification from typically 1500-4000 genes that are present in the majority, if not all members of a species. cgMLST has been predicted to supplant traditional MLST in the future,[44] and a cgMLST scheme for *E. faecium* has been developed, using 1,423 core genes.[45] For now, the availability of MLST data exceeds that of cgMLST data for *E. faecium*.

According to the PubMLST database as of May 2021, the three most frequently clinically isolated sequence types (STs) of *E. faecium* in Europe are ST78, ST17 and ST18, in order of decreasing frequency.[46] The Norwegian *E. faecium* collection consists of samples from the Tromsø 7 study, the Vancomycin-resistant *E. faecium* from 2010–2015 in Norway (VRE) collections, and the Norwegian Surveillance System for Antibiotic Resistance in Microbes (NORM) 2008 and NORM 2014 samples. The most frequently isolated STs of clade A in the Tromsø 7 study are 32, 22 and 52, while 94 and 116 dominate clade B isolates. In the VRE and NORM collections, globally dispersed hospital-associated clade A1 clones are dominant, the STs of which are 17, 18, 80, 117, 192, 202 and 203 (personal communication Janice; Al-Rubaye et al., unpublished; Hegstad et al., unpublished).

1.4 Bacteriocins

1.4.1 Background

Bacteriocins are a diverse group of ribosomally synthesized antimicrobial peptides that inhibit the growth of or kill other species.[47-49] They usually target closely related species, though some have a wider range of targets.[50, 51] The first bacteriocin, colicin, was identified in *E. coli* and was described in 1925.[50, 52] In 1928, Nisin A, a representative and well-studied LAB bacteriocin, was identified.[53] Since then, many more bacteriocins have been characterized, and the current version of BACTIBASE indexes 230 bacteriocins as of June 2021, 206 of which are isolated from gram-positive bacteria.[54]

Interest in bacteriocins stems, among other reasons, from their use as food preservatives and their potential as candidates for healthcare therapy.[55-57] Many LAB are generally considered safe, and many are classified as "Generally Recognized as Safe" by the United States Food and Drug Administration for use as food additives,[50] though the genus *Enterococcus* has not obtained this status.[58] It is likely that humanity has benefited from bacteriocins in food since cheese and fermented foods were first introduced some 8000 years ago, as they play an important role in inhibiting the growth of spoilage-causing bacteria.[50, 51] The bacteriocin Nisin was first marketed as a food additive in England in 1953, and is now approved for use in over 48 countries. It is currently on the European food additive list as number E234.[51, 59]

As multidrug-resistant (MDR) invasive bacterial species are increasingly becoming a concern, many look to bacteriocins as possible substitutes for or additions to antibiotic treatment in the fight against infection.[49, 56, 60] A wide variety of different bacteriocins have been shown to have significant effects against many clinically important pathogens, including MDR strains, both *in vitro* and *in vivo.*[49, 50, 55, 61, 62] Furthermore, synergistic effects between bacteriocins and antibiotics have been shown.[55] Bacteriocins have also shown promise in combating cancer cells[63, 64] and treating acne,[65-69] atopic dermatitis and peptic ulcers, in addition to having many other (potential) uses.[70]

Bacteriocins, unlike many antibiotics, are genetically encoded and ribosomally synthesized,[71, 72] thus having the advantage of being directly amenable by genetic engineering.[73] As our knowledge of molecular biology increases, this fact might ease the

process of searching for and producing natural and synthetic bacteriocins, thereby circumventing the costly and time-consuming process of antibiotic discovery and engineering.[74] In the future, bacteriocins might also enable individually designed treatment by host-microbe-specific targeting.

Commensal enterococcal strains are able to confer to the host protection against pathogens, as described e.g. for *E. faecalis* bacteriocins.[75] *E. faecium* strains have a variety of known bacteriocins,[9] most of which have been described in commensal strains with regards to probiotics and food production,[76-81] while the knowledge on bacteriocins in nosocomial strains is very limited. However, infections are almost exclusively caused by nosocomial *E. faecium* strains.[26] Therefore, it stands to reason that nosocomial *E. faecium* might inhibit or outcompete commensal strains. In a preliminary study, Wagner et al. investigated whether this hypothesis held true by competitively culturing nosocomial and commensal strains. Here, it was found that nosocomial strains, including VRE, can inhibit the growth of commensal strains. Furthermore, the presence of known bacteriocins was investigated in the genomes of the respective strains and it was observed that known and characterized bacteriocins could not explain every interaction between nosocomial and commensal strains (unpublished results, Wagner et al.). Fully understanding the importance of bacteriocins in the competition between clinical and commensal strains of *E. faecium* may contribute to understanding the overgrowth of clinical—hereunder MDR—isolates in hospitalized patients.

1.4.2 Classification

Though several classifications schemes for bacteriocins have been proposed and adapted,[82] one current classification scheme for bacteriocins from gram-positive bacteria classifies bacteriocins into three classes. Simplified, class I bacteriocins are comprised of peptides < 5 kDa that undergo substantial post-translational enzymatic modification; class II bacteriocins are comprised of peptides between 5 and 10 kDa that do not undergo substantial post-translational enzymatic modification; and class III bacteriocins are comprised of peptides > 30 kDa. Class I and II bacteriocins are heat-stable, whereas class III bacteriocins are heat-labile (some authors have removed class III bacteriocins and reclassified them as

bacteriolysins).[51] Class I and II bacteriocins have several subclassifications based on properties such as primary structure, spatial organization, mechanism of action and co-synthesis with other peptides.[50, 51, 55, 57]

Bacteriocins can also be classified genetically by using consensus sequences. A consensus sequence is the most common sequence of nucleotides at specified positions within a gene or, equivalently, the most common sequence of amino acids at specified positions within a peptide or protein.[83] For instance, the consensus sequence for class IIa bacteriocins (a subclass of the class II bacteriocins) is contained within the N-terminal region and is represented by YGNGV(X)C(X)4C(X)V(X)4A, where Y, G, N, V, C and A refer to specific amino acids, 4 refers to number of repeats of the following amino acid, and (X) refers to any unspecified amino acid.[55] Consensus sequences have been found that allow the classification of more than 70 % of known bacteriocins on a genetic basis, and may also provide a methodology to classify new and unclassified bacteriocins.[55, 84]

1.4.3 Synthesis, immunity, mode of action and resistance development

Bacteriocins are usually synthesized with a leader peptide that must be cleaved off before they become mature (active),[51] though leaderless bacteriocins have also been described.[85] This cleavage takes place either during transport through the cell membrane or by membrane-anchored proteases outside the cell. Transport through the cell membrane is usually handled by a dedicated transporter, though the general secretion pathway of the cell is used in some cases. During transport through the cell membrane, other maturation processes occur, e.g. dehydration of amino acid residues and cyclization of the bacteriocin.[50, 51]

Common for all bacteriocins is that the producing cells must have immunity to their own bacteriocins by specific immunity mechanisms, lest the bacteriocins attack the producing cells.[51] Several mechanisms of immunity have been described, e.g. scavenger proteins that remove bacteriocins, the altering of recognition targets, competitive receptor antagonism and secretion of bacteriocins by membrane pumps.[50, 51, 57, 86] The genes encoding

bacteriocins and the genes encoding specific immunity mechanisms are usually closely related.[51]

Bacteriocins as a group target a wide range of cellular pathways and structures and can be both bacteriostatic and bactericidal, depending on their modes of action. Well-characterized modes of action include 1) permeabilization of the cell membrane by pore formation leading to cell death either through dissipation of the proton motive force or by direct leakage of cellular contents; 2) inhibition of cell wall synthesis; 3) lysis of the cell wall; 4) inhibition of enzymes; 5) inhibition of protein synthesis; and 6) inhibition of nucleic acid synthesis.[51, 55, 57, 87-91]

Bacteria that do not produce a specified bacteriocin and therefore do not produce a specific immunity protein against the same bacteriocin may develop resistance by a variety of mechanisms, including changes in cell membrane composition or individual constituents, changes in cell wall composition, charge or individual constituents, and changes in target gene expression.[57]

2. Aim

The aim of this study was to characterize the interaction between clinical and commensal *E. faecium* strains by competitively culturing 50 commensal and 50 clinical strains *in vitro* and comparing the amount of growth inhibition exerted between the two groups. Furthermore, this study aimed to investigate the attributes of the inhibitory compounds from the strains exerting the most growth inhibition. Finally, bioinformatic methods were to be used to screen for the presence of known bacteriocins to supplement the laboratory findings and possibly detect interactions unexplained by current knowledge.

3. Materials and methods

The aseptic technique described by Bykowski et al. was used throughout all laboratory assays.[92] The analyses of all laboratory assays were designed to minimize the amount of false positives, consequently increasing the probability of false negatives.

3.1 Bacterial strains

49 commensal bacterial strains were selected from the Tromsø 7 collection (Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res)/Hegstad et al., unpublished). One strain from the Netherlands was included (strain E1007, provided by Paganelli et al.).[93] All selected commensal strains belong to clade B.

37 clinical strains were selected from the Norwegian Surveillance System for Antibiotic Resistance in Microbes (NORM) 2008 and 2014 collections (K-res/Al-Rubaye et al., unpublished), whereas 13 clinical strains were selected from the Vancomycin-resistant *E. faecium* in Norway (VRE) collection from 2010 to 2015 (K-res/Al-Rubaye et al., unpublished).

The strains were selected based on having a wide spread of differing STs as defined by MLST. A full list of selected strains is included in Appendix 1.

For long term storage, bacteria were stored at -80 °C in glycerol stock containing brain-heart infusion (BHI), Milli-Q water (filtered and deionized water) and glycerol. Unprotected freezing usually results in cell death, therefore cryoprotectants are used. Glycerol is one such compound, and works by reducing the amount of ice formed at any given temperature by increasing the total number of solutes, while also having low toxicity even though it penetrates bacterial cells.[94]

3.2 Incubation media and diluents

Three different agar media were used: Blood agar, brain heart infusion (BHI) agar and Mueller Hinton agar II, the latter only used due to periods of lack of availability of blood agar;

it provided suboptimal growth for harvesting purposes of many selected *E. faecium* strains as compared to blood agar. BHI broth was used as liquid medium, and 0.85 % NaCl in water was used as a diluent. In lack of this diluent, phosphate-buffered saline (PBS 1x) was used. When not being incubated, growth media and diluents were kept in a cold room or refrigerator at 4 °C. All media was provided by the Section for Infection Control, Education, Method Development and Production, Department of Microbiology, University Hospital of Northern Norway, Tromsø, Norway.

3.3 Competitive *E. faecium* growth

The selected commensal and clinical *E. faecium* strain groups were alternately used as competitors and indicators on BHI agar to investigate the presence and extent of growth inhibition exerted between commensal and clinical *E. faecium* strains. Competitors and indicators were defined as follows: Competitors were plated directly from an overnight BHI broth culture, whereas indicators were taken directly from blood agar and were diluted in 0.85 % NaCl. Consequently, cell densities in competitors were much higher.

Frozen bacterial stock was plated on blood agar and incubated at 37 °C for 16-24 hours. Blood agar was used because it provides ample nutrients for growth of *E. faecium* and because it facilitates visualization of colonies due to the contrast between the red agar and the white or slightly silverish *E. faecium* colonies. After incubation, the plates were individually observed for growth and contamination (e.g. heterogenous growth in the form of size and color discrepancies). If axenic cultures were obtained, aliquots of bacterial colonies from the blood agars were inoculated as suspension cultures in 5 mL BHI broth and the resulting cultures were incubated at 37 °C for 12-16 hours on a shaking rack at 220-225 RPM. The shaking of liquid cultures prevents precipitation and aggregation of bacteria, equalizes the distribution of nutrients and facilitates oxygenation of the solution.

The following day, indicators were made by inoculating bacteria from blood agar in 5 mL 0.85% NaCl to a McFarland standard of 0.5 using a McFarland densitometer, taking care to disaggregate the bacteria properly to obtain accurate readings. After reaching a McFarland standard of 0.5, the bacterial solutions were diluted 1:10 with 0.85 % NaCl or PBS 1x if 0.85 %

NaCl was not available. The McFarland standard uses a solution of either barium sulfate or latex particles as controls to measure the turbidity of a solution. 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 *Escherichia coli* (ATCC 25922). This method of concentration standardization is used in antimicrobial disk susceptibility testing,[95] except the final 1:10 dilution, which was added to reach optimal indicator densities for detecting competitive growth inhibition, based on prior testing of *E. faecium* competition by Wagner et al. (unpublished).

Each BHI agar plate was then plated by placing it on top of a spiral plater, dipping a cotton-tipped applicator into a diluted bacterial solution and transferring the absorbed liquid to the agar by drawing an equal-armed cross on the agar. The spiral plater was turned on, rotating the agar plate, the cotton-tipped applicator was placed in the origin of the circle formed by the agar and was slowly moved to the perimeter and back to the origin. Care was taken to evenly cover the agar. The resulting indicators were left to dry for 5 minutes. BHI agar was used because it provides ample nutrients for the growth of *E. faecium* and does not contain erythrocytes or hemoglobin in appreciable quantities to produce visible hemolysis or hemedigestion, thus eliminating interference from the color changes seen due to these processes, making the analysis step less complicated and standardized across all strains.

Finally, 10 µL of competitors from overnight cultures were streaked on top radially using inoculation loops, starting at the perimeter and moving toward the origin to prevent cross-contamination of competitors. The resulting competitive growth BHI agar plates were incubated for 16-24 hours at 37 °C.

The following day, each agar plate was visually inspected, and the results were noted. A black disc was used as an underlay to better visualize growth inhibition. Fig. 3 illustrates a completed competitive growth dish.

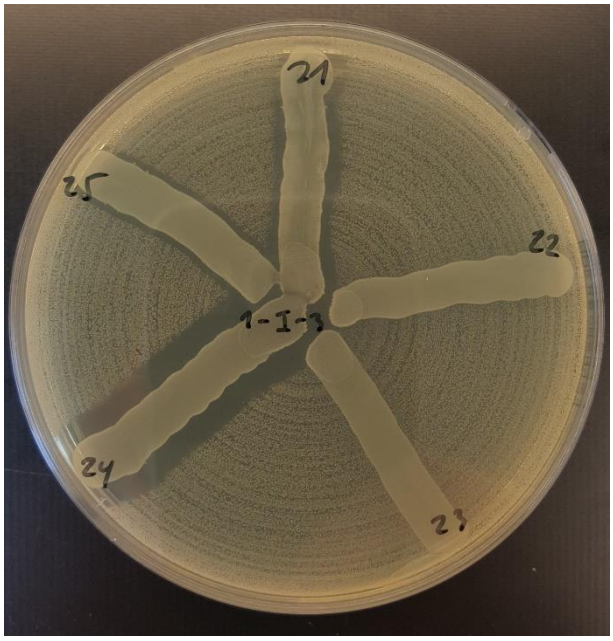

Figure 3. Completed competitive growth dish with clinical indicator 1-I-3 and commensal competitors 21-25. Inhibition zones exerted by the competitors range from absent (22 and 23), narrow (25), intermediate (21), to wide (24).

Inhibition zones were defined as areas around streaks of competitors which displayed no growth of indicators, and were subdivided based on size (Table 2). The widths of the inhibition zones were not rigorously defined or measured because the aim was not to quantify the magnitude of growth inhibition per se, rather to dichotomously define growth inhibition and noninhibition to select the most growth-inhibiting strains from each group for further study.

Table 2. Definitions of inhibition zones in competitive growth of *E. faecium* strains.

Designation	Definition
No inhibition	No visible growth inhibition. The indicators that were in close vicinity to competitors grew completely juxtaposed to the competitors and were uniform with the segments of the indicators that were free of competitors.
Possible inhibition	Possible growth inhibition. Slightly darker areas around the competitors were present. However, there was still evidence of macroscopic indicator growth juxtaposed to the competitors. This was reported as noninhibition in the results.
Definite inhibition zone	Width of inhibition zone around competitor narrow.
Definite inhibition zone +	Width of inhibition zone around competitor intermediate.
Definite inhibition zone ++	Width of inhibition zone around competitor large.
Definite inhibition zone +++	Width of inhibition zone around competitor very large.

After results from the competitive growth were obtained, the 10 strains from each strain group that inhibited the most strains from the opposing group (henceforth designated top strains) acted as competitors against all strains of their own group, using the previously described methodology.

3.4 Growth inhibition exerted by *E. faecium* supernatants

Supernatants from the top strains of the competitive growth were used to look for non-whole cell-mediated growth inhibition against all strains from the opposing group. The methodology of the competitive growth was used, with the additional step of centrifuging 1 mL of the overnight cultures at 13,000 RPM for 5 minutes to separate the soluble and

insoluble fractions of the liquid cultures. Care was taken not to resuspend the sedimented bacteria when the supernatants were pipetted into sterile Eppendorf tubes. 10 µL of competitor supernatants were subsequently pipetted onto BHI agar plates prepared with indicators using the same method as during competitive growth. In addition, control plates were made without indicators to examine the sterility of the supernatants.

As the supernatants were not sterile, a classification scheme similar to but more rigorous than the one used for competitive growth was devised. Growth inhibition was measured as the distance between the external border of growth inside the unfiltered competitor supernatants and the border of the indicators at the point of maximum distance. Furthermore, to standardize measurements to enable comparison between strains within this assay, measurements were done perpendicularly to convex surfaces of the growth within the competitor supernatants. Where it was not possible to delineate the borders of the competitors, e.g. if there was no growth of either strain within the competitor supernatant or there were only a few spread CFUs, the interaction was defined as growth inhibition with the smallest defined inhibition zone, as there was clear growth inhibition but no method of ascertaining the extent of the inhibition.

Fig. 4 illustrates a completed dish with unfiltered supernatant competitors. Growth inhibition for unfiltered supernatants is defined in Table 3.

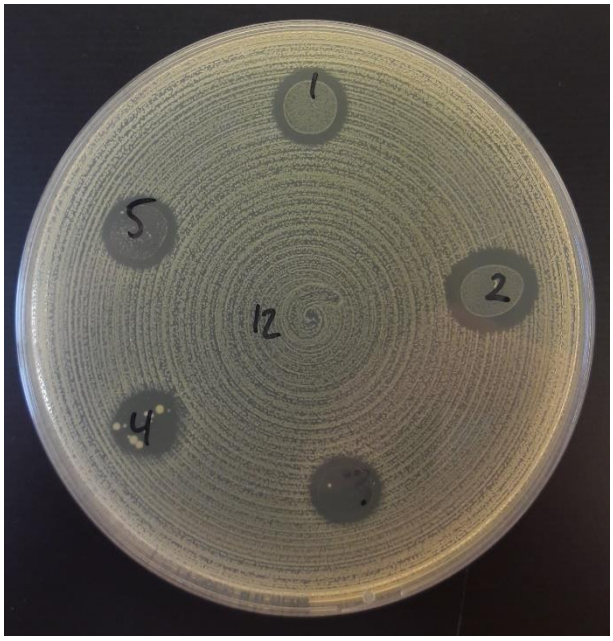

Figure 4. Completed unfiltered supernatant dish with commensal indicator 2E-9-F and unfiltered supernatants from the five clinical competitors that exerted the most growth inhibition during competitive growth. Growth is observed within all competitor supernatants, with varying densities and morphologies. For competitor supernatants 1-3 and 5, it is possible to quantify the growth inhibition by measuring the distance between the external border of the growth within the competitor supernatants and the border of the surrounding indicator. For competitor supernatant 4, the border of the competitor is ill-defined.

Table 3. Definitions of inhibition zones using unfiltered supernatants.

Designation	**Definition**
-	No visible macroscopic inhibition zone.
0	Nonzero inhibition zone < 1 mm.
1	Inhibition zone 1 to < 2 mm.
2	Inhibition zone 2 to < 3 mm.
3	Inhibition zone 3 to < 4 mm.
4	Inhibition zone 4 to < 5 mm.

To obtain cell-free supernatants, the supernatants were filtered after centrifugation. 0.22 µm sterile filters were used to filter the supernatants prior to pipetting 10 µL onto the

indicators. The filter size was chosen due to availability and previous experience by Wagner. Fig. 5 illustrates a completed dish with filtered supernatants. Growth inhibition was measured within competitor supernatants, and was subdivided based on indicator densities (Table 4).

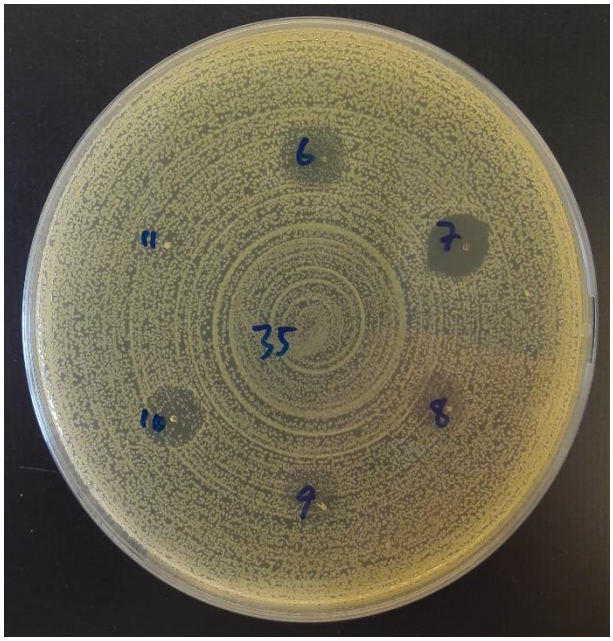

Figure 5. Filtered supernatants with commensal indicator 2E-6-F and clinical competitor supernatants from five of the top clinical strains as defined by competitive growth (marked 6-10). Additional clinical competitor supernatant (marked 11) included for confirmation of previous results produced by Wagner et al. (unpublished). Growth inhibition exerted by competitor supernatants range from absent (11), weak (8 and 9), intermediate (6 and 10) to complete (7).

Table 4. Definitions of growth inhibition using filtered supernatants, upconcentrated supernatants and heat-treated supernatants.

Designation	Definition
Absent	No growth inhibition of indicators by competitor supernatants.
Possible inhibition	Possible growth inhibition of indicators by competitor supernatants. The areas inside competitor supernatants were darker than the surrounding indicators, but there were no reductions in indicator densities or CFU sizes. This designation was reported as noninhibition in the results.
Weak[α]	Clear growth inhibition with reduced indicator densities and/or CFU sizes within competitor supernatants. Ample indicator growth.
Intermediate	Clear growth inhibition with reduced indicator densities and/or CFU sizes within competitor supernatants. Intermediate indicator growth.
Strong	Clear growth inhibition with reduced indicator densities and/or CFU sizes within competitor supernatants. Scanty indicator growth.
Complete	Clear growth inhibition with no growth within competitor supernatants.

α: Defined as inhibition for filtered supernatants and noninhibition for upconcentrated and heat-treated supernatants.

To investigate whether the loss of growth inhibition from competitive growth to the filtered supernatants was dependent on solute concentrations, filtered supernatants were upconcentrated. 14 mL of filtered competitor supernatants were added to tubes containing 3 kDa molecular weight cut-off (MWCO) filters. The MWCO filter size was chosen based on a preliminary search of known *E. faecium* bacteriocins, which suggested a range of 4.63–37.3 kDa.

The MWCO tubes were subsequently centrifuged at 8000 g for approximately 3.5 hours, or until the volume of upconcentrated supernatants reached approximately 2.8 mL, an approximate 5x increase in the solute concentrations compared to the filtered supernatants. It was not possible to reach an exact upconcentration value for all strains as they filtered at different rates while having to be centrifuged together for logistic reasons. Therefore, a minimum upconcentration of 5x was ensured, while several supernatants reached higher upconcentrations (range: 5–10x). Due to the nonsterile nature of the MWCO tubes (a hole for

air passage was situated in the lids to allow for pressure equalization), another filtration step with 0.22 µm filters was added.

Finally, the resulting solutions were used as upconcentrated competitor supernatants. The flow-throughs, which are the fractions of supernatants that passed through the MWCO filters and thus consisted of solvent and solutes < 3 kDa, were also filtered using 0.22 µm filters before being used as competitor supernatants.

Additionally, to investigate whether the growth inhibition-mediating agents of the upconcentrated supernatants were resistant to degradation by heat, upconcentrated supernatants were heat-treated at 100 °C for 10 minutes before being used as competitors.

Fig. 6 illustrates a completed dish with upconcentrated, flow-through and heat-treated supernatants. Growth inhibition was defined as for the filtered and upconcentrated supernatants (Table 4).

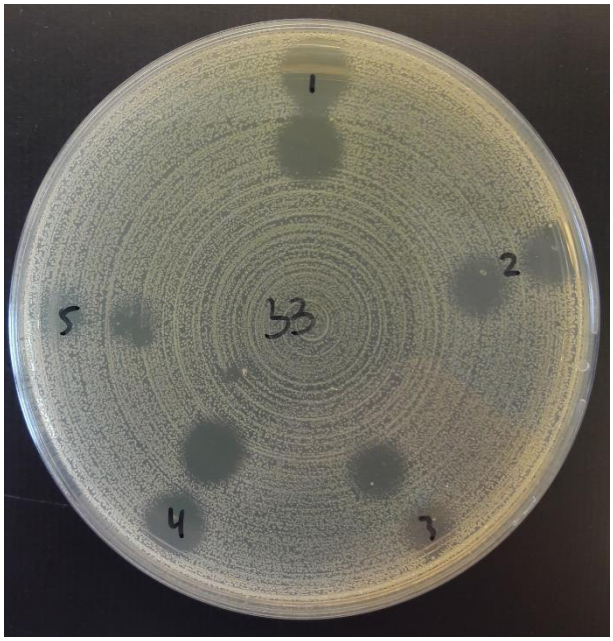

Figure 6. Completed upconcentrated supernatant and heat-treated supernatant dish with clinical indicator 1-D-5 and competitor supernatants from five of the top commensal strains from competitive growth. Heat-treated supernatants closest to the perimeter; upconcentrated supernatants in the middle; flow-through supernatants closest to the center. Upconcentrated supernatants and heat-treated supernatants 1-5 clearly exert growth inhibition. Growth inhibition by flow-through not seen in 1-3 and 5. Possibly seen in 4, though most likely contamination due to the small area and the CFU at the center of the inhibition zone.

3.5 Genetic relationships of selected *E. faecium* strains

To investigate the genetic relationships between selected *E. faecium* strains, four phylogenetic trees were constructed from whole genome sequencing (WGS) data in this study: 1) A phylogenetic tree of all selected strains with the addition of *E. faecium* reference genomes from the the Reference Sequence (RefSeq) database maintained by the National Center for Biotechnology Information (NCBI);[96] 2) a phylogenetic tree of selected commensal strains; 3) a phylogenetic tree of selected clinical strains; 4) a phylogenetic tree of all selected strains. All trees were made using the program Parsnp[97] with the following parameters: 1) Average nucleotide identity (detects the DNA conservation of the core genome) and DNA content (calculated proportion of DNA shared between genomes) were the main properties used for tree synthesis; 2) SNPs located in calculated regions of recombination (based on tests by the PhiPack software package)[98] were ignored in the construction of the trees; 3) a reference genome was randomly chosen from the available WGS data.

FigTree[99] was used for visualization, and the trees were rooted at the midpoint, i.e. at the midpoint between the two longest branches of the trees. All trees were constructed by Janice (unpublished).

3.6 Detecting known bacteriocins in selected *E. faecium strains* using WGS data

Bacteriocins are a subset of ribosomally synthesized post-translationally modified peptides (RiPPs). Having full access to the WGS data of all selected strains, the WGS of each strain was run through Bagel4,[100] a RiPP miner, in search of bacteriocins. RiPP mining was carried out to increase the chances of finding inhibitory interactions that were not explained by contemporary knowledge, opening the possibility of detecting and/or characterizing novel inhibitory compounds in the future.

In short, Bagel4 translates the WGS to one large protein spanning the entire genome, starting from the first identified start codon. Once it has done this, it shifts its reading frame and repeats this process. For any given double-stranded DNA sequence, there are a total of six reading frames,[101] resulting in six genome-spanning proteins. It then screens the six proteins obtained against a fixed list for the occurrence of specific sequence motifs, and also uses a basic local alignment search tool[102] to compare the obtained sequences with

sequences in the annotation and core peptide databases.[100] At this point, Bagel4 has determined areas of interest if they are present, which may contain bacteriocins. The areas of interest are then analyzed in detail using Glimmer3,[103] a program based on an interpolated Markov model approach, before results are returned. Simplified, the interpolated Markov model scoring algorithm in Glimmer3 calculates the likelihood of an amino acid sequence being coding, as opposed to non-coding.

4. Results

4.1 Relationships of selected *E. faecium* strains

Strains were chosen to adequately represent the diversity of *E. faecium* by selecting 34 different STs of commensal strains and 29 different STs of clinical strains, with 50 strains in each group. Their relationships were analyzed through the construction of phylogenetic trees. All trees were constructed by Janice (unpublished).

To examine the extent to which the selected strains were representative of the *E. faecium* population, a phylogenetic tree comparing all selected strains with the addition of closed *E. faecium* genomes in the RefSeq database was constructed using WGS data. The majority of the RefSeq genomes come from clinical isolates and belong to clade A1. This revealed the genetic diversity of the selected strains. Though the selected strains did not cover all internal nodes, they were spread throughout the tree, ensuring that the selection was adequately representative of *E. faecium* strains (Fig. 7).

Two additional phylogenetic trees were constructed to investigate the relationships within the commensal and clinical strain groups, respectively. Commensal strains were diverse with respect to their core genomes and DNA identities (Fig. 8), and all clustered with the clade B strains of the RefSeq database (Fig. 7). The core genomes and DNA identities in clinical strains were also diverse. Furthermore, their distribution was much more skewed, with 44 strains clustering to one main branch (clade A) and six strains to the other (clade B) (Fig. 7 and Fig. 9).

Finally, a phylogenetic tree of all selected strains was constructed to investigate the relationship between commensal and clinical strains. 44 clinical strains clustered on a main branch with no commensal strains. Interestingly, the six clinical strains that clustered to their own branch in the clinical strain phylogenetic tree, now clustered with the commensal strains (Fig. 10).

Subsequent reported results adopt the ordering in the phylogenetic trees of commensal and clinical strains (Fig. 8 and Fig. 9, respectively) to determine if relatedness affected interaction patterns.

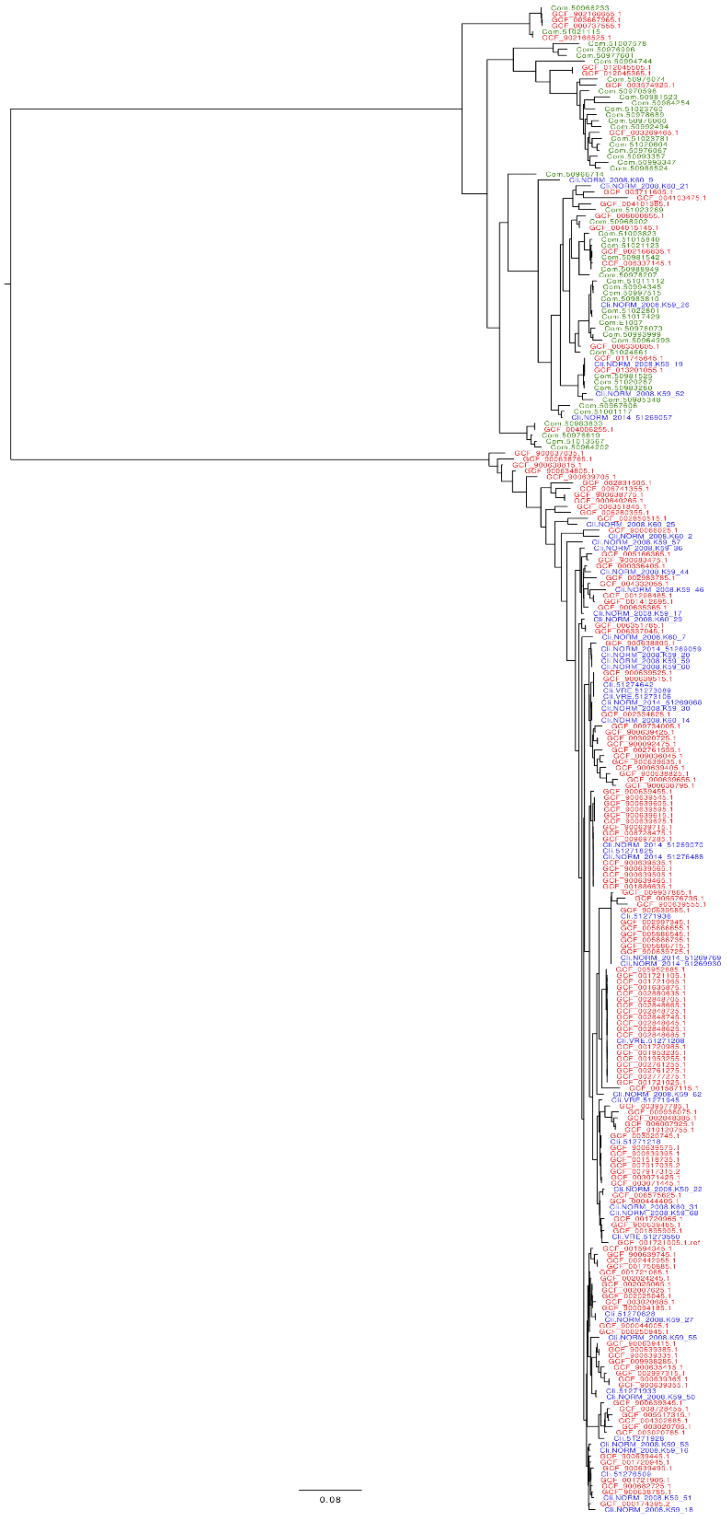

Figure 7. Midpoint-rooted phylogenetic tree with selected strains and closed *E. faecium* genomes from the RefSeq database. Strains are colored as follows: Commensal strains in green, clinical strains in blue and RefSeq reference strains in red. The line segment at the bottom visualizes the horizontal branch length equal to 0.08 substitutions/site. The majority of the RefSeq genomes come from clinical isolates and belong to clade A1 (bottom), while the commensal isolates cluster with clade B isolates at the top of the tree.

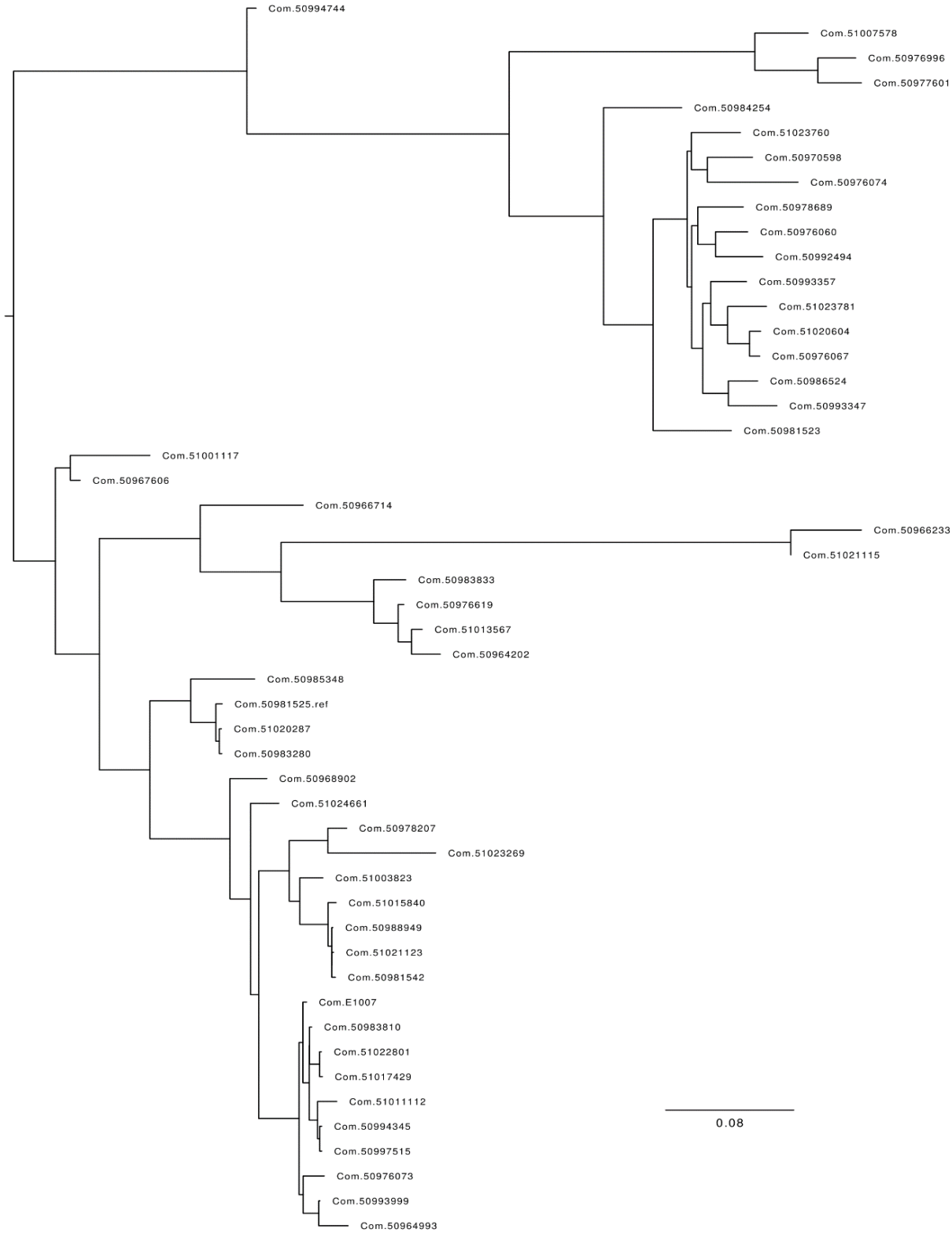

Figure 8. Midpoint-rooted commensal strain phylogenetic tree. The line segment at the bottom right visualizes the horizontal branch length equal to 0.08 substitutions/site.

Figure 9. Midpoint-rooted clinical strain phylogenetic tree. The line segment at the bottom visualizes the horizontal branch length equal to 0.7 substitutions/site.

Figure 10. Midpoint-rooted phylogenetic tree of selected commensal and clinical strains. Strains are colored as follows: Commensal strains in blue, clinical strains in green. The line segment at the bottom visualizes the branch length equal to 0.09 substitutions/site.

4.2 Clinically isolated *E. faecium* strains encode more bacteriocins

To screen for the presence of known bacteriocin genes in selected strains, Bagel4[100] was used. 93 unique amino acid sequences (Appendix 2) encoding 29 bacteriocins were found across all selected strains. Many of the unique amino acid sequences encoded the same bacteriocins. Bacteriocin sizes ranged from 21 to 412 amino acids.

Clinical strains generally encoded more bacteriocins. Table 5 summarizes data from bacteriocin screening. An overview of bacteriocins in the selected strains is presented in Fig. 11.

Table 5. Bacteriocin count in selected *E. faecium* strains.

Unique bacteriocins	Selected commensal strains	Selected clinical strains	Top commensal strains[ß]	Top clinical strains[ß]
Mean per strain[α]	2.28	3.82	4.2	5.9
Range[α]	0–6	0–11	3–5	3–11
Total[α]	72	191	42	59
Strains without bacteriocins	9	2	0	0

α: Duplicate bacteriocins in the same strains are not included.
ß: The 10 competitors that inhibited the growth of indicators of the opposing group most frequently during competitive growth.

Commensal strains did not encode the following eight bacteriocins: Acidocin LF221B (Gassericin K7B), Enterocin B, Enterocin Nkr-5-3B, Enterocin Q, Enterocin X (chain alpha), Enterocin X (chain beta), ggmotif, and Hiracin JM79. One bacteriocin was encoded in more than half the commensal strains: Enterolysin A (68 %). Nine of the top commensal strains encoded Enterocin L50a, whereas seven encoded MR10B, neither of which the clinical strains encoded.

Clinical strains did not encode the following nine bacteriocins: Bacteriocin 31, Bavaricin MN, Carnocin CP52, EntA, Enterocin L50a, Enterocin L50b, MR10A, MR10B, and Pediocin. Three bacteriocins were encoded in more than half the clinical strains: Bacteriocin IIc (52 %), Enterocin A (80 %) and Enterolysin A (76 %).

Two of the top commensal strains encoded three bacteriocins unique to the commensal strains, while the other eight strains encoded two such bacteriocins. Three of the top clinical strains did not encode any bacteriocins that were unique to the clinical strains. In the remaining seven clinical strains, one strain encoded four bacteriocins unique to clinical strains, one strain encoded two, and five strains encoded one such bacteriocin.

14 commensal strains and six clinical strains encoded duplicates of one bacteriocin. Most of these encoded two or three duplicates of one bacteriocin, whereas the maximum was found in clinical strain 3-E-9, encoding 5 duplicates of Enterolysin A. This strain did not inhibit the growth of any commensal strains during competitive growth. No strains encoded duplicates of more than one type of bacteriocin.

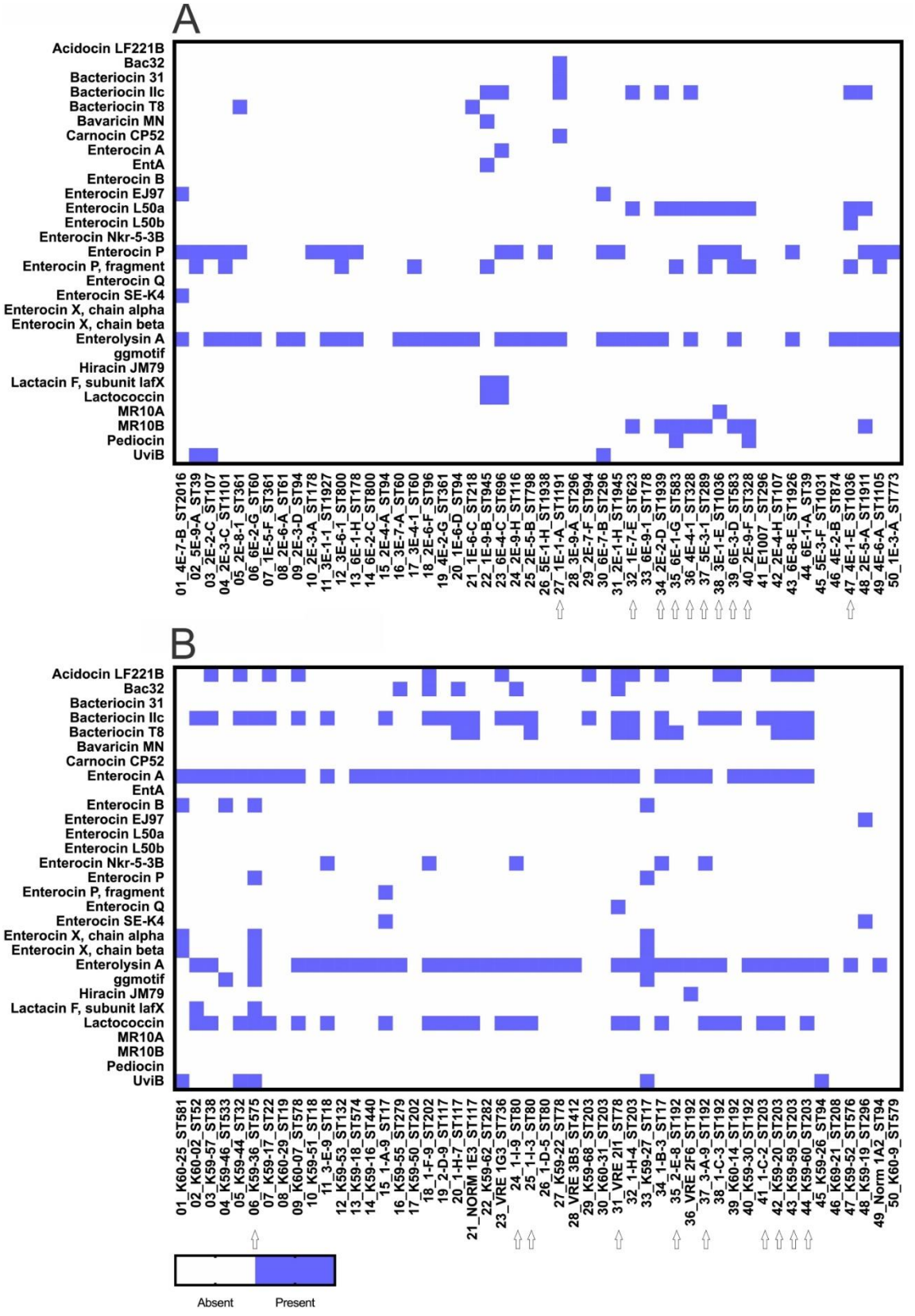

Figure 11. Bacteriocins in commensal (A) and clinical (B) strains. X-axis, bacteriocin; y-axis, strain. Presence of bacteriocins is marked in blue. Strains are sorted by their order in the commensal (Fig. 8) and clinical (Fig. 9) phylogenetic trees. The strains that inhibited the most strains of the opposing group during competitive growth are marked with arrows.

4.3 Clinical *E. faecium* strains generally outcompete commensal strains *in vitro*

To investigate inhibitory interactions between commensal and clinical *E. faecium* strains, they were grown competitively. Commensal competitors inhibited the growth of clinical indicators in 29 % of 2500 interactions (range: 0–100 %), compared to growth inhibition in 54 % of 2500 interactions with clinical competitors (range: 0–96 %). The results of competitive growth are summarized for growth inhibition exerted by commensal and clinical competitors in Fig. 12 and Fig. 13, respectively.

Interestingly, there were many more commensal competitors which did not inhibit any clinical indicators (64 %), than clinical competitors which did not inhibit any commensal indicators (34 %). However, 14 % of commensal competitors inhibited the growth of all clinical indicators, whereas none of the clinical competitors inhibited the growth of all commensal indicators.

There was a clear tendency toward all-or-nothing growth inhibition, i.e. a strain in question would inhibit either most or all the strains in the opposing group, or inhibit few if any strains in the opposing group. Of the 18 commensal competitors that exerted growth inhibition, 77.8 % of these inhibited more than 90 % of the clinical indicators. Of the 33 clinical competitors that exerted growth inhibition, 78.8 % of these inhibited the growth of more than 90 % of the commensal indicators.

The 10 strains from each group that inhibited the most strains from the opposing group, regardless of the amplitude of growth inhibition, were designated top strains. The top commensal strains inhibited the complete set of clinical strains in 98.6 % of all interactions, whereas the top clinical strains inhibited the complete set of commensal strains in 96 % of all interactions. All top clinical strains belong to clade A1.

Comparing the growth inhibition between the top strains of each group against each other, the commensal strains inhibited the clinical strains in 98 % of interactions, and clinical strains inhibited commensal strains in 100 % of interactions. There was a clear tendency, however, toward the commensal strains inhibiting the clinical strains more strongly: 54 % of the top commensal strains inhibited the top clinical strains with the widest inhibition zone defined, versus 9 % in the opposite direction.

Commensal strains 4E-2-B and 3E-7-A were markedly resistant to being inhibited by clinical strains, only being inhibited by one top clinical strain, K59-36. This strain was the only top clinical strain with an ST not commonly isolated in Europe or Norway. Acting as indicators against all clinical competitors, 4E-2-B was inhibited in 8 % of interactions and 3E-7-A in 4 %. These two strains inhibited no clinical strains when acting as competitors, and both had only one bacteriocin, Enterolysin A, which all top clinical strains encoded. Another commensal indicator that was markedly resistant to being inhibited by clinical competitors was 6E-4-C, being inhibited in 28 % of all interactions, though its growth was inhibited by nine top clinical strains. This strain, too, exerted no growth inhibition upon any clinical indicator when acting as a competitor. However, 6E-4-C had 6 bacteriocins. None of the clinical strains displayed similar resistance patterns.

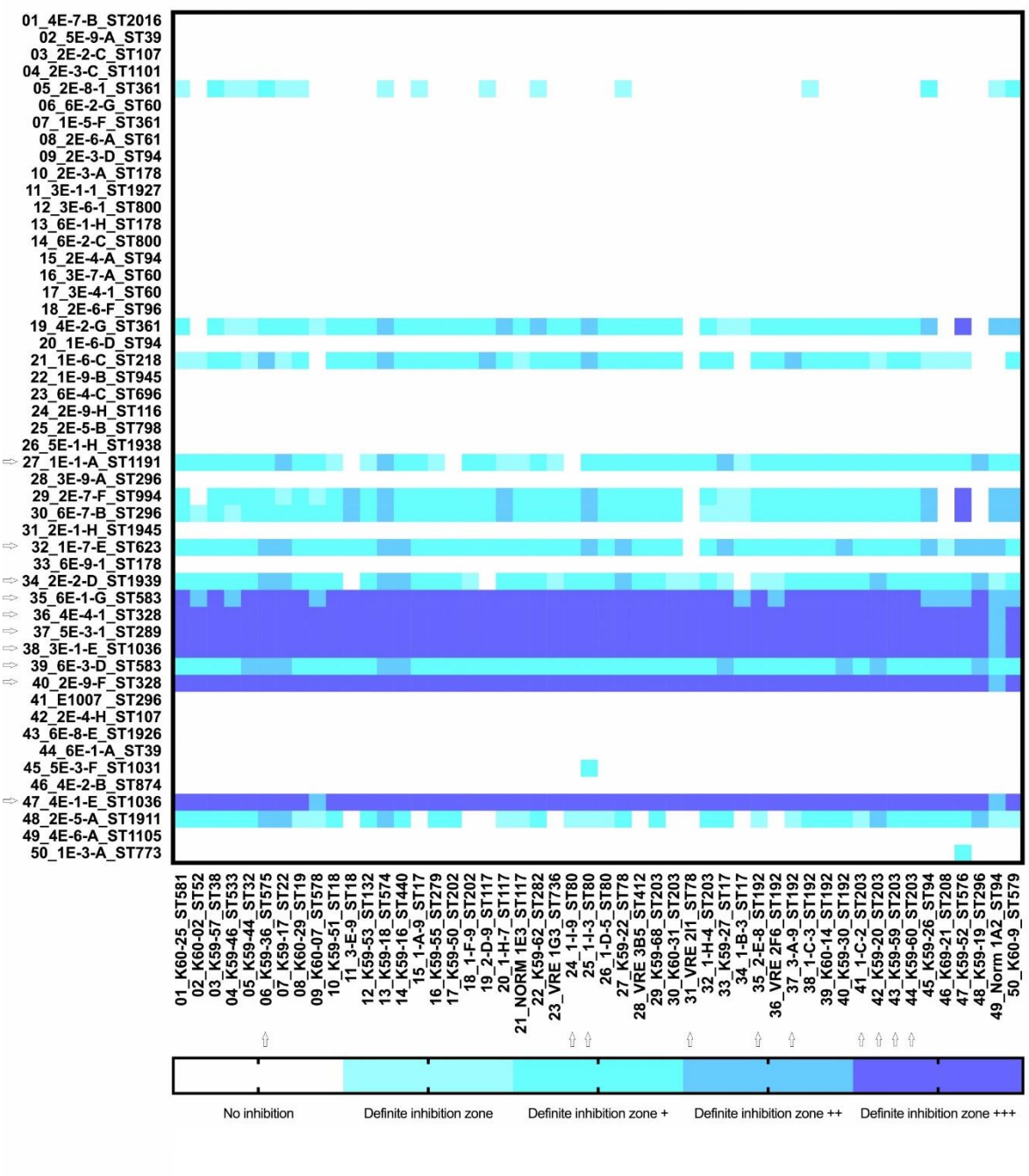

Figure 12. Growth inhibition exerted by commensal competitors. X-axis, clinical strains; y-axis, commensal strains. Strains are sorted by their order in the commensal (Fig. 8) and clinical (Fig. 9) phylogenetic trees. The 10 strains of each group that exerted growth inhibition most frequently (top strains) are marked with arrows. All colored squares denote growth inhibition. Inhibition zones are defined in Table 2.

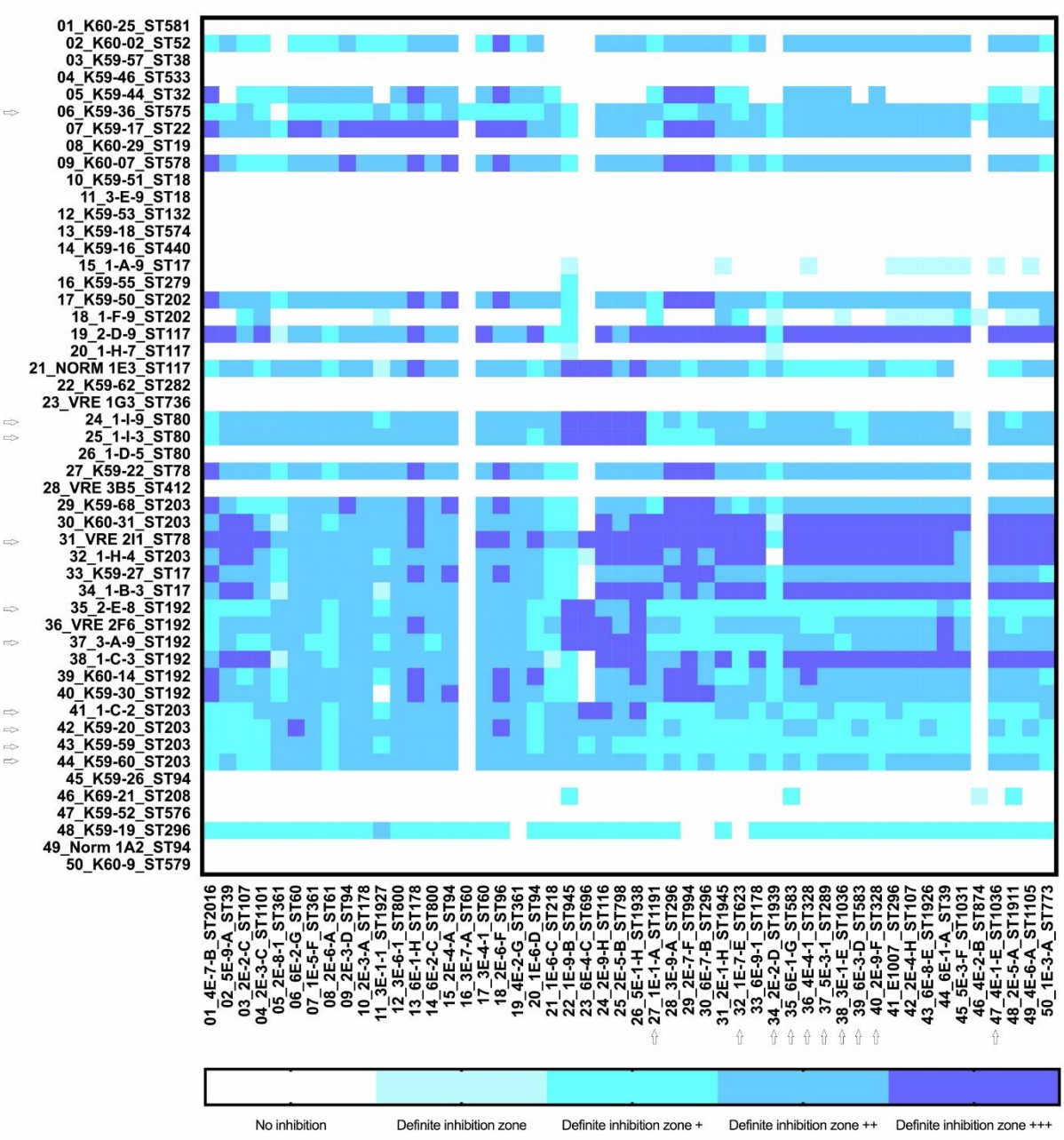

Figure 13. Growth inhibition exerted by clinical competitors. X-axis, commensal strains; y-axis, clinical strains. Strains are sorted by their order in the commensal (Fig. 8) and clinical (Fig. 9) phylogenetic trees. The 10 strains of each group that exerted growth inhibition most frequently (top strains) are marked with arrows. All colored squares denote growth inhibition. Inhibition zones are defined in Table 2.

16.3 % of all interactions displayed mutual growth inhibition, in which commensal competitors would inhibit clinical indicators and be inhibited by the same strains when their roles were reversed. These interactions are visualized in Fig. 14.

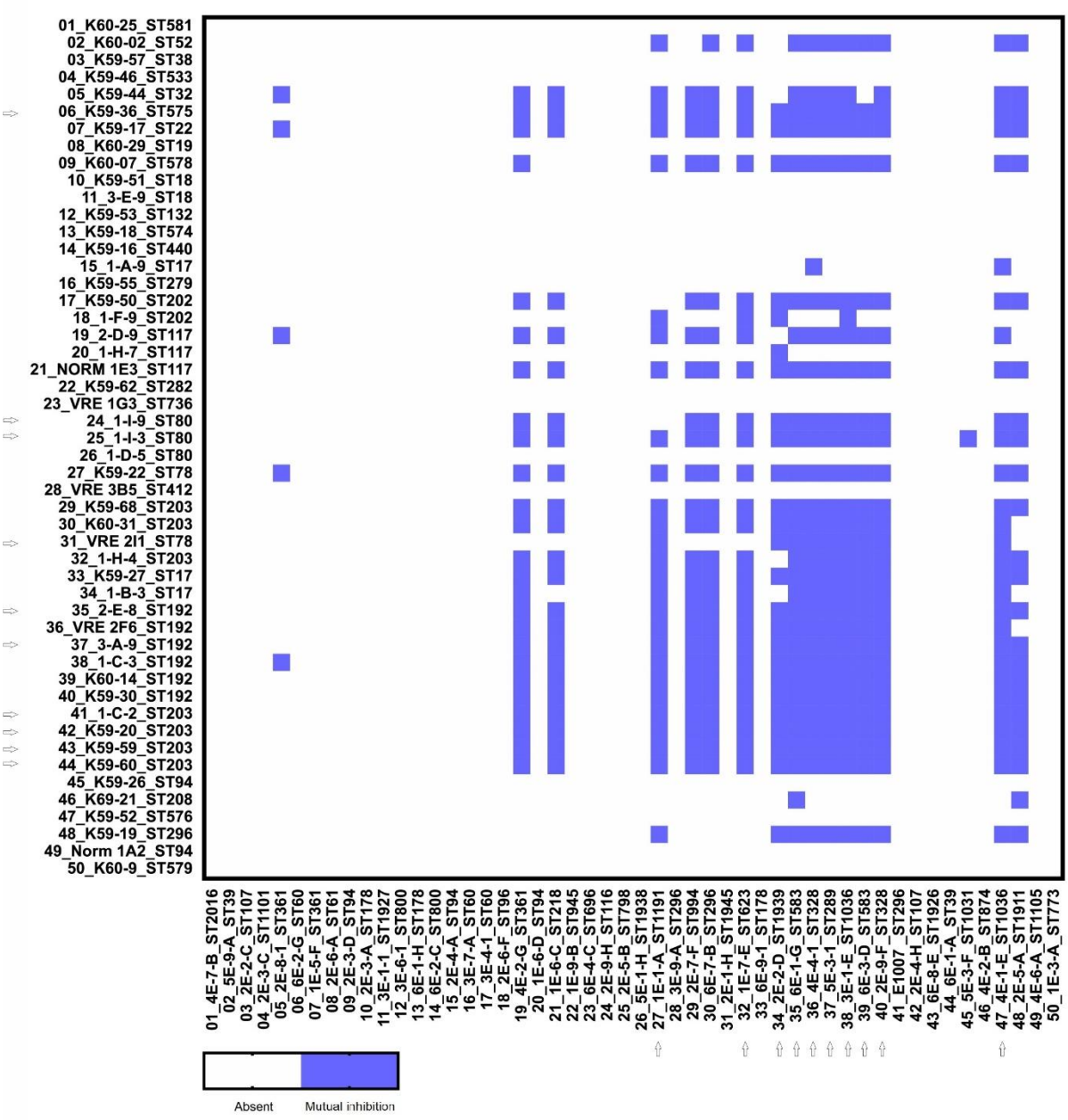

Figure 14. Mutual growth inhibition. X-axis, commensal strains; y-axis, clinical strains. Strains are sorted by their order in the commensal (Fig. 8) and clinical (Fig. 9) phylogenetic trees. The 10 strains of each group that exerted growth inhibition most frequently during competitive growth (top strains) are marked with arrows. Marked in blue are interactions where both strains inhibited the growth of the other, depending on which strain was the competitor.

To differentiate whether the growth inhibition exerted by the top strains from each group onto all strains in the opposing group was universal or target-specific, the top strains from each group were used as competitors against all strains of their own group. Top commensal competitors inhibited commensal indicators in 91.2 % of interactions, and top clinical competitors inhibited clinical indicators in 81.8 % of interactions. Interestingly, six of the top

commensal competitors inhibited the growth of their own indicators. None of the clinical strains exhibited the same phenomenon. Five of the six competitors that inhibited the growth of their own indicator clustered closely together in the commensal phylogenetic tree (Fig. 8). Interactions between commensal and clinical strains are visualized in Fig. 15.

4E-2-B and 3E-7-A, commensal strains that were resistant to inhibition exerted by clinical competitors, were both inhibited to various degrees by eight top commensal strains. 6E-4-C, the last commensal strain to show resistance to being inhibited by clinical competitors, was inhibited by all top commensal competitors.

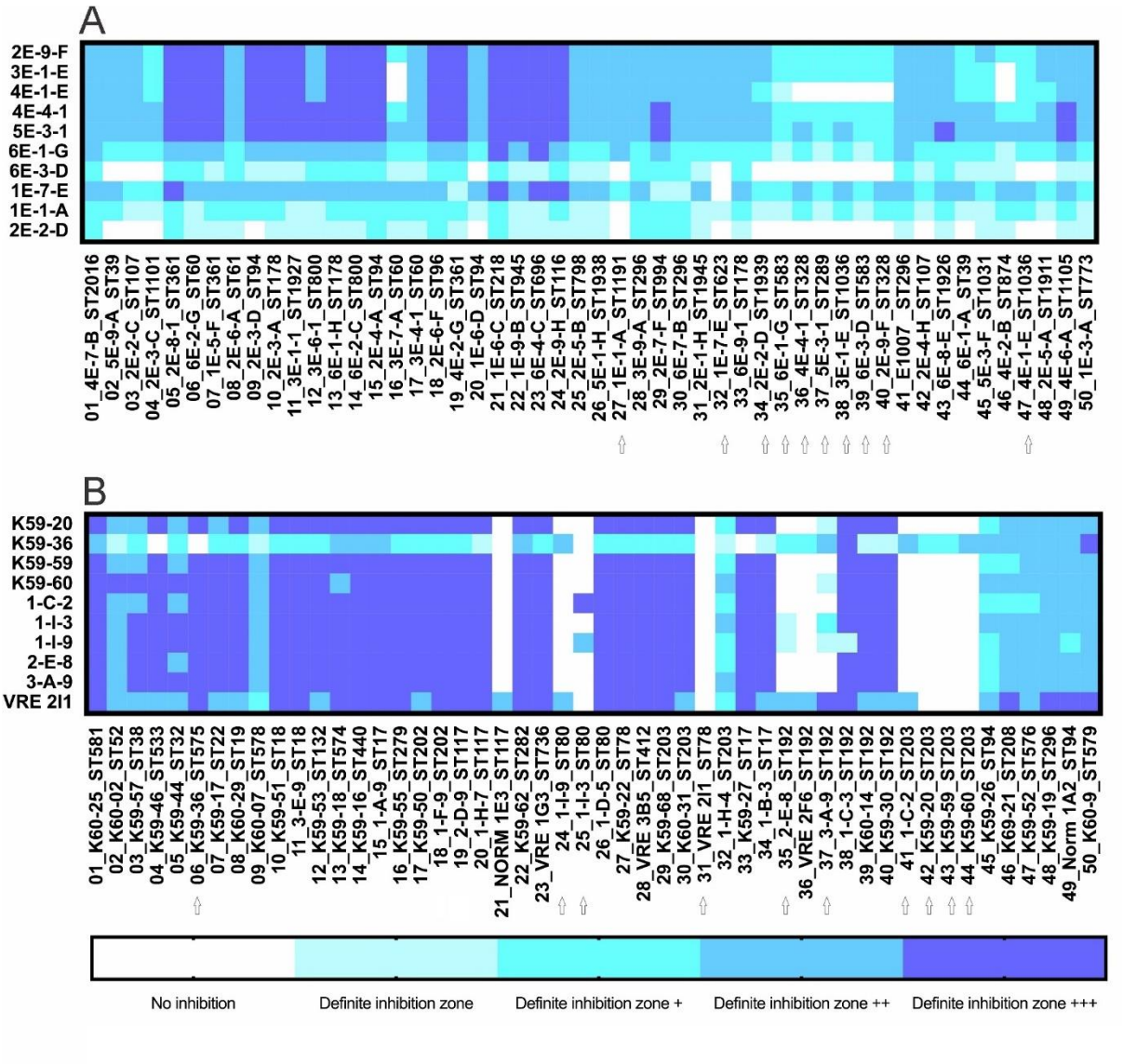

Figure 15. Growth inhibition exerted by top commensal strains onto all commensal strains (A), and by top clinical strains onto all clinical strains (B). X-axes, all strains of group as indicators; y-axes, top strains of group as competitors. Strains on the x-axes are sorted by their orders in their respective phylogenetic trees; strains on the y-axes are sorted top-to-bottom by how many strains of the opposing group they inhibited the growth of, in order of decreasing frequency of growth inhibition. All colored squares denote growth inhibition. The indicators which inhibited the most strains of the opposing group as competitors during competitive growth are marked with arrows. Inhibition zones are defined in Table 2.

4.4 Agents secreted by *E. faecium* often mediate growth inhibition

To examine whether growth inhibition was mediated by secreted agents, e.g. bacteriocins, unfiltered competitor supernatants were used. All unfiltered supernatants displayed growth in control dishes without indicators, though less dense than the growth displayed during competitive growth. Top commensal competitors inhibited the growth of

clinical indicators in 70.8 % of interactions, whereas top clinical competitors inhibited the growth of commensal indicators in 93 % of interactions (Fig. 16). These were reductions from competitive growth by 28.2 % and 3.1 %, respectively. The widest inhibition zones were observed with clinical competitors.

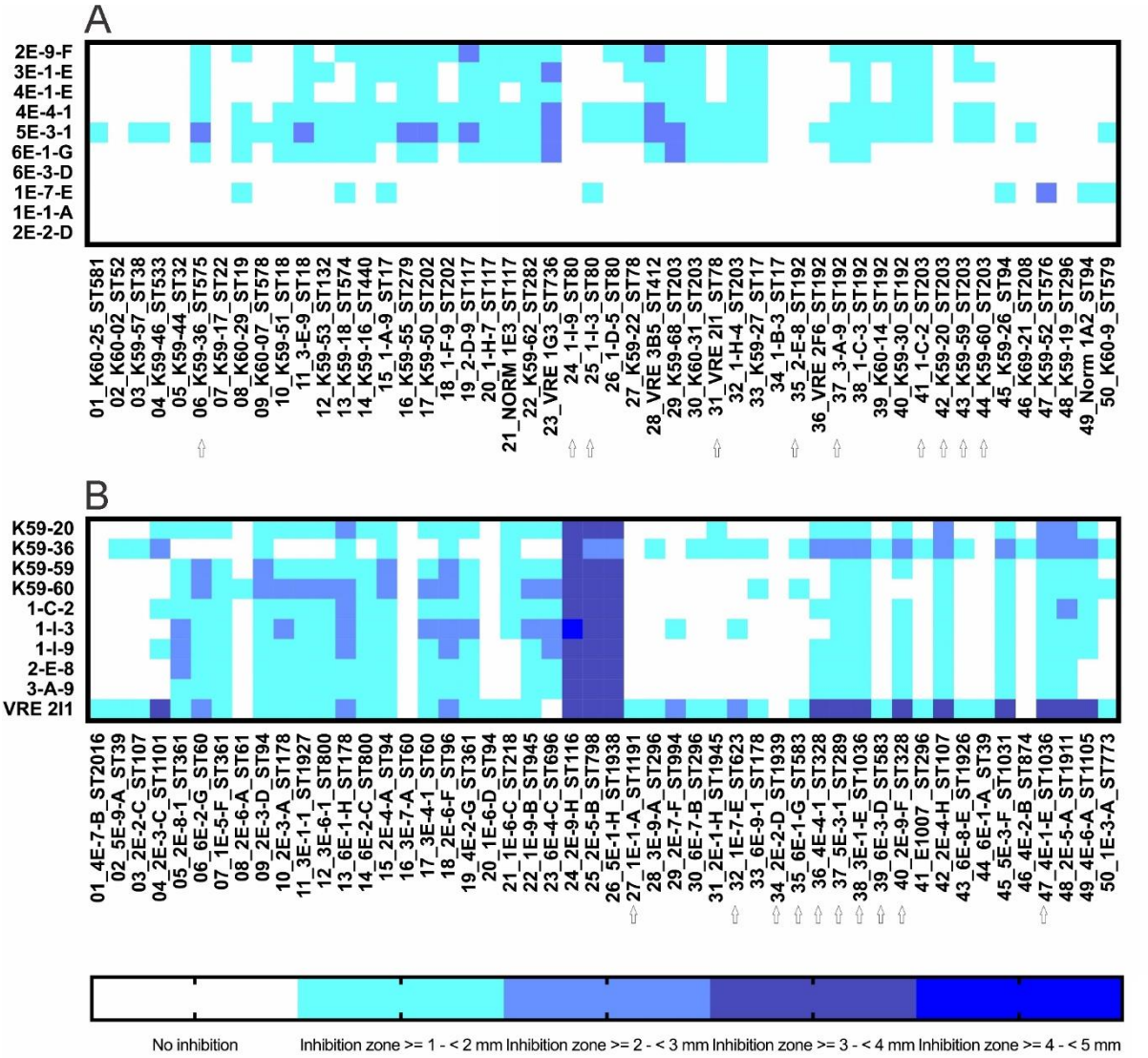

Figure 16. Unfiltered supernatants with commensal (A) and clinical (B) competitors. X-axes, indicators; y-axes, competitors. Strains on the x-axes are sorted by their order in their respective phylogenetic trees; strains on the y-axes are sorted top-to-bottom by how many strains of the opposing group they inhibited the growth of during competitive growth, in order of decreasing frequency of growth inhibition. All colored squares denote growth inhibition. The strains that inhibited the most strains of the opposing group during competitive growth are marked with arrows. Inhibition zones are defined in Table 3.

The unfiltered supernatants demonstrated the necessity of removing residual bacteria to obtain cell-free supernatants. Therefore, a filtration step was added, resulting in the filtered supernatants. The filtered supernatants displayed a marked decrease in the proportion of growth inhibition as compared to the competitive growth and the unfiltered supernatants. The top commensal filtered competitor supernatants inhibited clinical indicators in 3.4 % of all interactions, whereas the top clinical filtered competitor supernatants inhibited commensal indicators in 30 % of all interactions (Fig. 17). These were reductions as compared to the unfiltered supernatants by 95.2 % and 67.8 %, respectively.

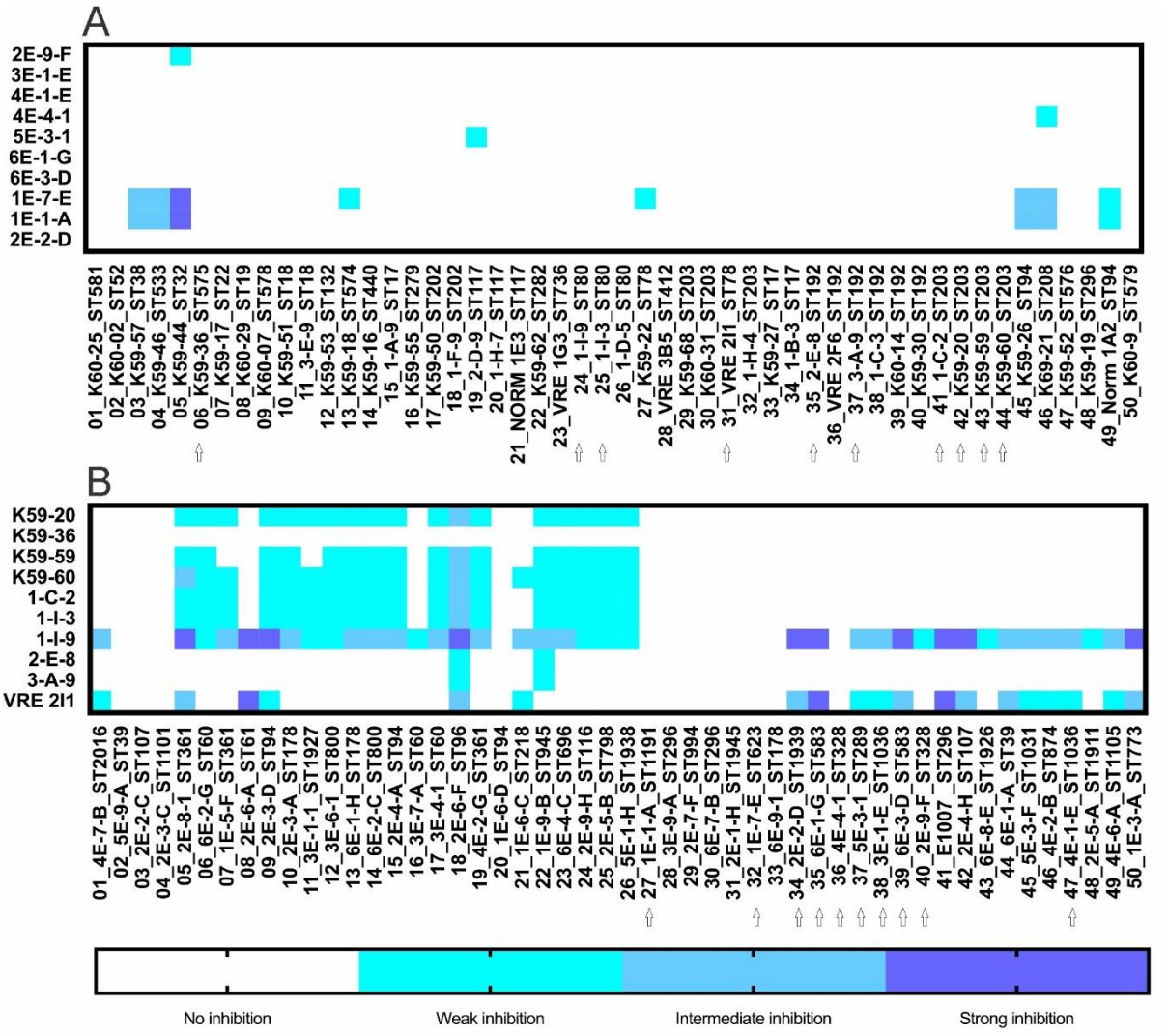

Figure 17. Filtered supernatants with commensal (A) and clinical (B) competitor supernatants. X-axes, indicators; y-axes, competitors. X-axes, indicators; y-axes, competitors. Strains on the x-axes are are sorted by their order in their respective phylogenetic trees; strains on the y-axes are sorted top-to-bottom by how many strains of the opposing group they inhibited the growth of during competitive growth, in order of decreasing frequency of growth inhibition. All colored squares denote growth inhibition. The strains that inhibited the most strains of the opposing group during competitive growth are marked with arrows. Inhibition zones are defined in Table 4.

To investigate whether growth inhibition was lost between competitive growth and filtered supernatants due to low solute concentrations, the supernatants were upconcentrated to a minimum of five times the original solute concentrations. The MWCO filter size of 3 kDa was chosen based on a preliminary search of known *E. faecium* bacteriocins, which suggested a range of 4.63–37.3 kDa.

The upconcentrated supernatants from the top commensal competitors inhibited the growth of clinical indicators in 37.4 % (range: 0-88 %) of all interactions, while the upconcentrated supernatants from the top clinical competitors inhibited the growth of commensal indicators in 40.2 % (range: 4–96 %) of all interactions. This was an increase in frequency of growth inhibition compared to the filtered supernatants by 1,000 % for upconcentrated commensal competitor supernatants and 34 % for upconcentrated clinical competitor supernatants. Furthermore, these increases might be underestimates, as the results from upconcentrated supernatants interpreted the weakest inhibition definition from the filtered supernatants as noninhibition to further decrease the probability of false positives.

Two of the upconcentrated top commensal supernatants did not inhibit the growth of any clinical indicators, whereas all upconcentrated clinical competitor supernatants inhibited at least two commensal indicators. Flow-through supernatants exhibited no growth inhibition.

To investigate whether the growth inhibition-mediating agents of the upconcentrated supernatants were resistant to degradation by heat, upconcentrated supernatants were heat-treated at 100 °C for 10 minutes. Heat-treating the supernatants yielded a decrease in growth inhibition exerted by commensal and clinical upconcentrated competitor supernatants by 3.2 % and 23.9 % respectively, as compared to the upconcentrated supernatants.

A summary of results from the upconcentrated supernatants and heat-treated supernatants is presented in Fig. 18.

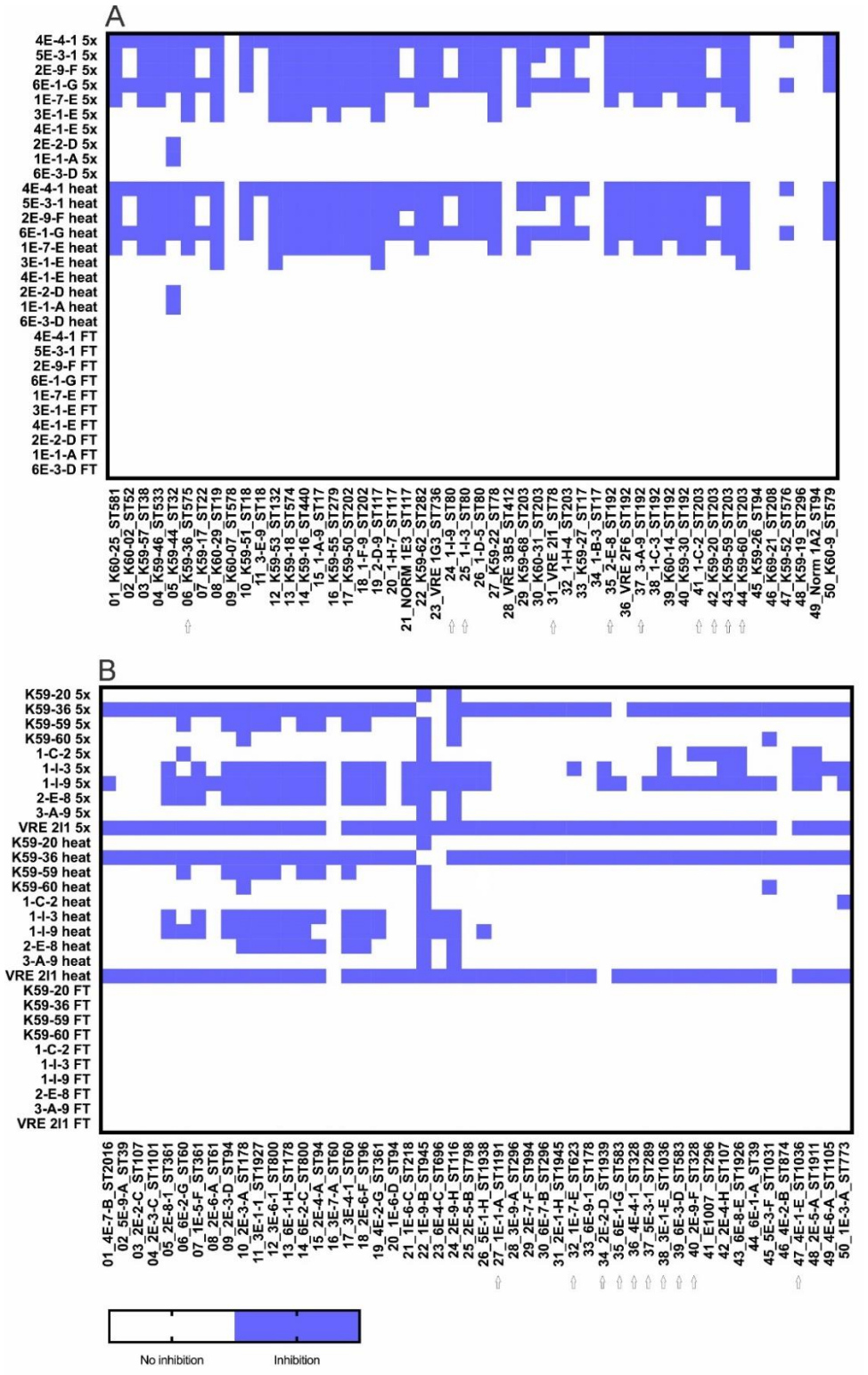

Figure 18. Upconcentrated and heat-treated supernatants from commensal (A) and clinical (B) competitors against indicators of the opposing group. The strains that inhibited the most strains of the opposing group during competitive growth are marked with arrows. X-axes, indicators; y-axes, competitors; 5x, upconcentrated supernatants; heat, heat-treated supernatants; FT, flow-through supernatants. Interactions displaying growth inhibition are marked in blue, and correspond to designations intermediate, strong and complete in Table 4.

4.5 *E. faecium* strains potentially encoding novel bacteriocins

To detect candidate strains possibly encoding unknown bacteriocins, data from RiPP mining and competitive growth were combined to look for growth inhibition exerted by strains that encoded no known bacteriocins.

Of the nine commensal strains in which no bacteriocins were detected, seven inhibited none of the clinical strains. One commensal competitor (5E-3-F) inhibited one clinical indicator (1-I-3, a member of top clinical strains), whereas one commensal competitor (2E-7-F) inhibited 46 clinical indicators.

Of the two clinical strains in which no bacteriocins were detected, K60-09 inhibited no commensal strains, whereas K69-21 inhibited four commensal strains.

5. Discussion

5.1 Bacteriocins in *E. faecium*

While *E. faecium* strains have a variety of known bacteriocins,[9] most have been described in commensal strains with respect to probiotics and food production.[76-81] Broadening our understanding of *E. faecium* bacteriocins and their role in growth inhibition might lead to understanding the mechanisms of overgrowth of clinical—hereunder MDR—isolates in hospitalized patients, and possibly to discovery of novel treatments for invasive *E. faecium* infection.

A wide array of bacteriocins were found in the WGS data, displaying the diversity of antimicrobial peptides in both commensal and clinical *E. faecium* strains. However, no genetic expression data was collected, therefore it cannot be known whether the bacteriocins encoded by the selected *E. faecium* strains were the actual growth inhibition-mediating agents in the laboratory assays, as several other classes of growth-inhibiting agents in (LAB) bacteria have been described.[104-106] Many of the unique amino acid sequences mined, coded for the same bacteriocins, demonstrating SNPs, larger deletions and substitutions while presumably retaining function. Moreover, bacteriocin sizes varied widely, showcasing the wide spectrum of possible bacteriocin configurations.

Clinical *E. faecium* strains generally encoded more bacteriocins than commensal strains, and there were more commensal strains than clinical strains which completely lacked known bacteriocins, both of which are novel discoveries. Furthermore, each strain group of *E. faecium* had several unique bacteriocins, which might relate to *E. faecium* strains adapting to niches and having differing optimal plasmid configurations in hospital environments.[107, 108]

Both strain groups had several members that encoded at least one duplicate bacteriocin, which might lead to increased bacteriocin expression. Synergistic expression of duplicate genes have been described,[109] i.e. the encoding of two gene duplicates may increase gene expression more than two-fold. The range of bacteriocins in top strains varied widely, and some strains encoded as few as three bacteriocins. This might indicate that some bacteriocins are more potent against particular *E. faecium* strains than others.[110]

Whereas some strains exhibited inhibition despite not encoding any known bacteriocins, none of these strains placed as top strains, therefore their supernatants were not investigated further. Commensal strains 5E-3-F and 2E-7-F, and clinical strain K69-21 may encode hitherto unknown bacteriocins, though further studies are needed.

Some commensal strains barely encoding any bacteriocins were markedly resistant toward growth inhibition exerted by clinical strains, without being able to inhibit the growth of those strains. Bacteriocin target site mutations have been described,[57] though it is not known whether these commensal strains encoded such mutations.

5.2 Clinical *E. faecium* strains generally outcompete commensal strains

E. faecium strains are known to inhibit the growth of several gram-positive bacterial species.[111] However, intraspecies growth inhibition has not been well characterized. As demonstrated by competitive growth, clinical *E. faecium* strains inhibited the growth of commensal strains more frequently. This contradicted the *in vitro* results of Montealegre et al.,[28] though their sample sizes were small and their methodologies differed. While commensal and clinical *E. faecium* strains have differing core genes,[26] the phylogenetic tree of selected clinical and commensal strains (Fig. 10) revealed that six of 50 clinical strains clustered with commensal strains. This indicated the highly unusual possibility of infection by clade B strains,[27] though it is possible that they would cluster with the rest of the clinical strains using different parameters.

There were many interactions during competitive growth in which a competitive pairing would display mutual growth inhibition depending on which strain was the competitor. This phenomenon was likely a result of the study design, as the competitor densities were much higher than that of the indicators. If two strains in competition each secreted bacteriocins that the opposing strain did not have immunity to, it stands to reason that the denser population would produce higher concentrations of bacteriocins, resulting in visible growth inhibition of the less dense population. It is possible that the indicators inhibited the growth of or killed the competitors on a cellular level, and that the effect sizes were simply too small to be detected by the methodology used.

In all strains tested by competitive growth, there was a tendency toward all-or-nothing growth inhibition, meaning that a strain in question would inhibit the growth of either most or all of the strains in the opposing group, or inhibit the growth of few if any strains in the opposing group. Delving further into analysis of growth inhibition for each interaction cross-referenced with bacteriocins possessed by both the commensal and clinical strains would likely (partly) elucidate the reason for this. However, the limited amount of time afforded to this thesis precluded the option of doing this, as there were simply too many unique data points to analyze.

The competition between the top commensal and top clinical strains (as defined by their abilities to inhibit the growth of strains of the opposing group) revealed that while the top clinical strains inhibited top commensal strains slightly more frequently, top commensal strains exhibited much larger inhibition zones against all clinical strains (including the top clinical strains) than they did in the majority of competitive growth against commensal strains. It is possible that these commensal strains could confer upon the host protection against invasive infection by clinical *E. faecium* strains, as has been described for *E. faecalis* strains.[75] As bacteriocins usually target closely related species,[50, 51] this might explain the overgrowth of clinical VRE when antibiotics are administered.[29] Antibiotics would target the commensal *E. faecium* strains while sparing the clinical strains due to their antimicrobial resistance, and other bacterial species would not target clinical *E. faecium* strains with their antimicrobial peptides. The majority of the top commensal strains clustered closely together on the phylogenetic tree, suggesting a core genome basis for their inhibitory mechanisms, possibly a result of chromosomal genes or preferential uptake of mobile genetic elements.[108] Whereas the possibility exists that top commensal strains encode bacteriocins that target clinical strains to a higher degree, their growth inhibition-mediating agents must be isolated, purified and investigated further before any conclusions can be drawn.

All top clinical *E. faecium* strains belong to clade A1,[46] and it seems reasonable that their propensity to inhibit the growth of commensal *E. faecium* strains contributes to their success as epidemic hospital-associated strains.[27]

Interestingly, only the commensal strains had members which inhibited all strains of the opposing group. Additionally, the top commensal strains inhibited the top clinical strains

much more strongly. RiPP mining revealed that bacteriocins Enterocin L50a and MR10B dominated in the top commensal strains, none of which were encoded in clinical strains. There is a possibility that these bacteriocins are more potent than the bacteriocins unique to the clinical strains, or that they act synergistically, as has been described for Enterocin L50a with Enterocin L50b and MR10A with MR10B.[112, 113] Though further studies are required, the fact that the top commensal strains inhibited the top clinical strains much more strongly lends credibility to this hypothesis.

No clinical strain inhibited all commensal strains, even though seven of the top clinical strains encoded at least one bacteriocin unique to the clinical strain group. Because the top commensal strains encoded less bacteriocins per strain than the top clinical strains, the proportion of produced bacteriocins unique to the producing strain group could be higher in commensal strains, explaining this effect.

Six of the top commensal strains inhibited the growth of their own diluted strains. As all strains were incubated concurrently at equal time intervals, however, it is not possible to rule out that this effect was simply due to higher cell densities in the relevant strains, as post-incubation densities of competitors were not measured.

Two commensal strains that were markedly resistant to being inhibited by clinical strains were found. These strains were inhibited by only one of the top clinical strains. The clinical strain in question did not encode any bacteriocins that were not present in at least one of the other top clinical strains, and it was the only top clinical strain with an uncommonly isolated ST type in Europe and Norway. Furthermore, the two commensal strains were inhibited by 80 % of top commensal strains. Finally, these strains did not inhibit the growth of any clinical strains. These facts indicate that some commensal strains might be resistant to being inhibited by specific clinical genetic lineages, possibly by mechanisms other than direct bacteriocin immunity genes.

5.3 Growth inhibition is often mediated by secreted agents

The presence of residual bacteria in unfiltered supernatants demonstrated the necessity of adding a filtration step to obtain cell-free supernatants, as all unfiltered competitor supernatants were contaminated with bacteria.

Interestingly, the growth inhibition exerted by commensal strains decreased much more from competitive growth to unfiltered supernatants, than did the growth inhibition exerted by clinical strains. Due to bacterial cell concentrations in the unfiltered competitor supernatants not being determined, there could have been a large discrepancy between the cell concentrations of commensal and clinical strains in their respective unfiltered competitor supernatants, causing this effect.

Growth inhibition exerted by commensal strains decreased considerably more frequently from competitive growth and unfiltered supernatants to the filtered supernatants. Possible explanations include that the growth inhibition-mediating agents from commensal strains were less potent; that in commensal strains they were more susceptible to proteolysis and degradation; that they were produced in higher quantities in clinical strains; and that there was more directly cell-mediated (as opposed to secreted) growth inhibition by commensal strains.

The upconcentrated supernatants displayed a marked increase in inhibitory interactions for both commensal and clinical strains, as compared to the filtered supernatants. As the solute concentrations were at least five times higher and molecular weights of characterized and mined bacteriocins were known, this was expected. It is possible that some inhibitory compounds were degraded (e.g. by endopeptidases) and therefore failed to exert growth inhibition as compared to the competitive growth and unfiltered supernatants, as there would not be continuous production of growth-inhibiting agents in the filtered and upconcentrated supernatants. Furthermore, none of the methods in this study directly measured the presence of inhibitory compounds, rather they indirectly implicated their existence through observed effects on indicators. For this reason, stating that competitors did not display growth inhibition is not equivalent to stating that competitors produced no growth-inhibiting agents.

The fact that none of the flow-through supernatants exhibited inhibition strongly suggests that all inhibitory effectors in this study were > 3 kDa, which is unsurprising as a preliminary screening of known bacteriocin molecular weights revealed a minimum mass > 3 kDa. However, it is not possible to rule out that inhibitory compounds < 3 kDa did exist, and were simply too dilute to display macroscopic effects.

There was barely any reduction in growth inhibition frequency when heat-treating the commensal upconcentrated supernatants, whereas almost one quarter of inhibitory interactions were lost when doing the same with clinical upconcentrated supernatants. This indicates that clinical strains might have produced more inhibitory agents not classified as class I and class II bacteriocins. Overall, most of the inhibitory activity was conserved after heat-treatment, suggesting that the inhibitory effectors were bacteriocins, or at the very least resistant to heat-treatment.

6. Future perspectives

Due to limited time allotted to this project, some planned methods were not undertaken, while others were ruled out before laboratory assays commenced for the same reason. This section will consider which further analyses could be done, though it will not be a comprehensive overview of all available methods.

6.1 Bacteriocin mining using Neural Bacteriocin Identifier

Neural Bacteriocin Identifier, another RiPP miner, implements the deep recurrent neural network methodology. In a paper by Hamid et al., it is shown that this approach outcompetes alignment-based approaches, such as the one used by Bagel4.[114] Using this miner, it is possible that the sensitivity of RiPP mining would be improved.

6.2 Endopeptidase-treated upconcentrated supernatants

Once it was established that the upconcentrated supernatants still exhibited growth inhibition after being heat-treated, the next step would be to treat the upconcentrated supernatants with endopeptidases to ascertain whether the growth-inhibiting agents were proteins or peptides. Some bacteriocins are shown to be sensitive to degradation e.g. by Proteinase K,[115] whereas others are resistant.[116] It would also be possible to to use mixtures of endopeptidases simultaneously. Most, if not all bacteriocins detected by RiPP mining are well-characterized, therefore one could construct a table of all known interactions with endopeptidases to find novel interactions. One could also predict endopeptidase effects from the amino acid sequences provided by the RiPP mining. This analysis would serve to strengthen the case that the inhibitory effectors in the supernatants that ceased to exhibit growth inhibition after endopeptidase treatment were peptides. In the case of inhibitory activity continuing, the possibility of the growth-inhibiting agents being peptides would persist, as some bacteriocins are resistant to degradation by specific endopeptidases.

Further treatment of supernatants for the purposes of characterization is often done, including adding acids, metal ions, alcohols and salts.[117]

6.3 Protein and peptide purification

Protein purification is a series of procedures intended to isolate a single protein species from complex biological sources, e.g. bacterial cultures. This is usually achieved by separating all proteins from the non-proteinaceous components of a mixture, and subsequently isolating the desired protein species from all other proteins present.[118]

Proteins are often separated from non-proteinaceous components using protein precipitation («salting out»). These methods collectively employ salts to increase the number of ions in solution, which in turn increases the surface charge of proteins, making them aggregate and precipitate out of solution.[119] Salts such as sodium chloride, sodium sulfate, sodium phopshate and ammonium sulfate have been used for these purposes.[120, 121] Other compounds such as trichloroacetic acid and acetone may also be used for precipitation. However, these may denature proteins, rendering further analyses of biochemical activity impossible.[122, 123]

Following the precipitation, the resulting mixture of proteins must be separated to isolate the protein of interest. Other techniques such as ultracentrifugation may be employed, though chromatographic techniques are most commonly used. Chromatographic techniques such as reversed-phase chromatography, ion-exchange chromatography, high-performance liquid chromatography and others, take advantage of physicochemical characteristics of proteins such as size, charge, hydrophobicity and solubility to separate proteins by migration through solid media. Proteins with differing physicochemical characteristics will migrate through solid media at different rates, enabling the separation of protein species.[118, 121]

Peptides, which are too small for precipitation methods, may first be separated from other components of mixtures by methods such as ultracentrifugation or by chromatographic methods,[124, 125] enabling further analyses of smaller bacteriocins.

6.4 Protein and peptide characterization

Techniques such as sodium dodecyl sulphate–polyacrylamide gel electrophoresis, mass spectrometry, X-ray crystallography and nuclear magnetic resonance are often employed to determine bacteriocin characteristics including molar mass, amino acid sequences, structures and post-translational modifications.[126-130] Immunoassays that employ antibodies for detection and quantification of bacteriocins have also been described.[131] All the aforementioned methods could be employed both to confirm that the inhibitory action seen throughout the laboratory assays were results of the bacteriocins found during RiPP mining, and to characterize novel bacteriocins not detected during RiPP mining.

7. Conclusion

It has been shown that clinical *E. faecium* strains generally outcompete commensal *E. faecium* strains *in vitro*. Care must be taken to generalize these results, as *in vivo* settings are complicated by a multitude of factors.

Much of the inhibitory activity seen during competitive growth was mediated by secreted agents. Though the growth inhibition-mediating agents were concentration-dependent, most of them were resistant to heat, strengthening the hypothesis that they were bacteriocins. *E. faecium* strains producing possible novel bacteriocins have been identified, though further research is necessary.

Finally, some commensal *E. faecium* strains were markedly resistant to the inhibitory mechanisms of top clinical *E. faecium* strains, opening possible new avenues for research.

8. References

1. Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10(4):266-78.
2. Bintsis T. Lactic acid bacteria: their applications in foods. J Bacteriol Mycol Open Access. 2018;6(2):89–94.
3. Ateba CN, Lekoma KP, Kawadza DT. Detection of vanA and vanB genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR analysis. J Water Health. 2013;11(4):684-91.
4. Roberts MC, Soge OO, Giardino MA, Mazengia E, Ma G, Meschke JS. Vancomycin-resistant Enterococcus spp. in marine environments from the West Coast of the USA. J Appl Microbiol. 2009;107(1):300-7.
5. Guzman Prieto AM, van Schaik W, Rogers MRC, Coque TM, Baquero F, Corander J, et al. Global Emergence and Dissemination of Enterococci as Nosocomial Pathogens: Attack of the Clones? Frontiers in Microbiology. 2016;7(788).
6. Fortina MG, Ricci G, Mora D, Manachini PL. Molecular analysis of artisanal Italian cheeses reveals Enterococcus italicus sp. nov. International Journal of Systematic and Evolutionary Microbiology. 2004;54(5):1717-21.
7. Maccallum WG, Hastings TW. A CASE OF ACUTE ENDOCARDITIS CAUSED BY MICROCOCCUS ZYMOGENES (NOV. SPEC.), WITH A DESCRIPTION OF THE MICROORGANISM. J Exp Med. 1899;4(5-6):521-34.
8. Sherman JM. THE STREPTOCOCCI. Bacteriol Rev. 1937;1(1):3-97.
9. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection. Boston: Massachusetts Eye and Ear Infirmary; 2014.
10. Sherman JM. The Enterococci and Related Streptococci. J Bacteriol. 1938;35(2):81-93.
11. Giard J-C, Rince A, Benachour A, Hartke A, Laplace J-M, Breton Y, et al. The response to environmental stresses in Enterococcus faecalis. 2003. p. 325-39.
12. Rashid R, Cazenave-Gassiot A, Gao IH, Nair ZJ, Kumar JK, Gao L, et al. Comprehensive analysis of phospholipids and glycolipids in the opportunistic pathogen Enterococcus faecalis. PLOS ONE. 2017;12(4):e0175886.
13. Zou J, Shankar N. The opportunistic pathogen Enterococcus faecalis resists phagosome acidification and autophagy to promote intracellular survival in macrophages. Cell Microbiol. 2016;18(6):831-43.
14. Iaria C, Stassi G, Costa GB, Di Leo R, Toscano A, Cascio A. Enterococcal meningitis caused by Enterococcus casseliflavus. First case report. BMC Infect Dis. 2005;5(1):3.
15. Pintado V, Cabellos C, Moreno S, Meseguer MA, Ayats J, Viladrich PF. Enterococcal meningitis: a clinical study of 39 cases and review of the literature. Medicine (Baltimore). 2003;82(5):346-64.
16. Raza T, Ullah SR, Mehmood K, Andleeb S. Vancomycin resistant Enterococci: A brief review. J Pak Med Assoc. 2018;68(5):768-72.
17. Mundy LM, Sahm DF, Gilmore M. Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev. 2000;13(4):513-22.
18. Moellering RC, Jr. Emergence of Enterococcus as a significant pathogen. Clin Infect Dis. 1992;14(6):1173-6.

19. Top J, Willems R, Blok H, de Regt M, Jalink K, Troelstra A, et al. Ecological replacement of Enterococcus faecalis by multiresistant clonal complex 17 Enterococcus faecium. Clin Microbiol Infect. 2007;13(3):316-9.
20. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, et al. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infection Control & Hospital Epidemiology. 2008;29(11):996-1011.
21. Shepard BD, Gilmore MS. Antibiotic-resistant enterococci: the mechanisms and dynamics of drug introduction and resistance. Microbes and Infection. 2002;4(2):215-24.
22. Murray BE. Beta-lactamase-producing enterococci. Antimicrob Agents Chemother. 1992;36(11):2355-9.
23. Rice LB. Emergence of vancomycin-resistant enterococci. Emerg Infect Dis. 2001;7(2):183-7.
24. European Centre for Disease Prevention and Control: Surveillance Atlas of Infectious Diseases. Available at https://www.ecdc.europa.eu/en/surveillance-atlas-infectious-diseases. Last accessed June 2021.
25. European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2019. Stockholm: ECDC; 2020.
26. Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, et al. Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. mBio. 2013;4(4).
27. Lebreton F, Valentino MD, Schaufler K, Earl AM, Cattoir V, Gilmore MS. Transferable vancomycin resistance in clade B commensal-type Enterococcus faecium. J Antimicrob Chemother. 2018;73(6):1479-86.
28. Montealegre MC, Singh KV, Murray BE. Gastrointestinal Tract Colonization Dynamics by Different Enterococcus faecium Clades. J Infect Dis. 2016;213(12):1914-22.
29. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest. 2010;120(12):4332-41.
30. Wagner T. How to Be a Bad Bug: Virulence Determinants of Enterococcus faecium. Doctoral thesis dissertation, Faculty of Health Sciences, Department of Medical Biology, the Arctic University of Norway, Tromsø, Norway. 2018.
31. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018;18(3):318-27.
32. Gonzales RD, Schreckenberger PC, Graham MB, Kelkar S, DenBesten K, Quinn JP. Infections due to vancomycin-resistant Enterococcus faecium resistant to linezolid. The Lancet. 2001;357(9263):1179.
33. Long JK, Choueiri TK, Hall GS, Avery RK, Sekeres MA. Daptomycin-Resistant Enterococcus faecium in a Patient With Acute Myeloid Leukemia. Mayo Clinic Proceedings. 2005;80(9):1215-6.
34. Munoz-Price LS, Lolans K, Quinn JP. Emergence of Resistance to Daptomycin during Treatment of Vancomycin-Resistant Enterococcus faecalis Infection. Clinical Infectious Diseases. 2005;41(4):565-6.

35. Leavis HL, Bonten MJM, Willems RJL. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol. 2006;9(5):454-60.
36. van Harten RM, Willems RJL, Martin NI, Hendrickx APA. Multidrug-Resistant Enterococcal Infections: New Compounds, Novel Antimicrobial Therapies? Trends Microbiol. 2017;25(6):467-79.
37. Pidot SJ, Gao W, Buultjens AH, Monk IR, Guerillot R, Carter GP, et al. Increasing tolerance of hospital Enterococcus faecium to handwash alcohols. Sci Transl Med. 2018;10(452).
38. Bradley CR, Fraise AP. Heat and chemical resistance of enterococci. J Hosp Infect. 1996;34(3):191-6.
39. Zhu J, He F, Hu S, Yu J. On the nature of human housekeeping genes. Trends Genet. 2008;24(10):481-4.
40. Hounkpe BW, Chenou F, de Lima F, De Paula Erich V. HRT Atlas v1.0 database: redefining human and mouse housekeeping genes and candidate reference transcripts by mining massive RNA-seq datasets. Nucleic Acids Research. 2021;49(D1):D947-D55.
41. Eisenberg E, Levanon EY. Human housekeeping genes are compact. Trends Genet. 2003;19(7):362-5.
42. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, et al. Multilocus sequence typing scheme for Enterococcus faecium. J Clin Microbiol. 2002;40(6):1963-71.
43. Carter GP, Buultjens AH, Ballard SA, Baines SL, Tomita T, Strachan J, et al. Emergence of endemic MLST non-typeable vancomycin-resistant Enterococcus faecium. Journal of Antimicrobial Chemotherapy. 2016;71(12):3367-71.
44. Kimura B. Will the emergence of core genome MLST end the role of in silico MLST? Food Microbiol. 2018;75:28-36.
45. de Been M, Pinholt M, Top J, Bletz S, Mellmann A, van Schaik W, et al. Core Genome Multilocus Sequence Typing Scheme for High- Resolution Typing of Enterococcus faecium. J Clin Microbiol. 2015;53(12):3788-97.
46. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 2018;3:124-.
47. Christopher RC, Phillip SC, Michael SG. Enterococcal Cytolysin: A Novel Two Component Peptide System that Serves as a Bacterial Defense Against Eukaryotic and Prokaryotic Cells. Current Protein & Peptide Science. 2005;6(1):77-84.
48. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology. 2005;3(10):777-88.
49. Cotter PD, Ross RP, Hill C. Bacteriocins — a viable alternative to antibiotics? Nature Reviews Microbiology. 2013;11(2):95-105.
50. Nishie M, Nagao J, Sonomoto K. Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. Biocontrol Sci. 2012;17(1):1-16.
51. Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol. 2005;3(10):777-88.
52. Gratia A (1925) Sur un remarquable example d'antagonisme entre deux souches de colibacille. Compt. Rend. Soc. Biol, 1040-1042.
53. Rogers LA, Whittier EO. LIMITING FACTORS IN THE LACTIC FERMENTATION. J Bacteriol. 1928;16(4):211-29.

54. Hammami R, Zouhir A, Le Lay C, Ben Hamida J, Fliss I. BACTIBASE second release: a database and tool platform for bacteriocin characterization. BMC Microbiol. 2010;10:22.
55. Ahmad V, Khan MS, Jamal QMS, Alzohairy MA, Al Karaawi MA, Siddiqui MU. Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. International Journal of Antimicrobial Agents. 2017;49(1):1-11.
56. van Heel AJ, Montalban-Lopez M, Kuipers OP. Evaluating the feasibility of lantibiotics as an alternative therapy against bacterial infections in humans. Expert Opinion on Drug Metabolism & Toxicology. 2011;7(6):675-80.
57. Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S. Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. Microb Pathog. 2019;128:171-7.
58. Hanchi H, Mottawea W, Sebei K, Hammami R. The Genus Enterococcus: Between Probiotic Potential and Safety Concerns-An Update. Frontiers in microbiology. 2018;9:1791-.
59. European Commission Food Additives Database. Available at https://webgate.ec.europa.eu/foods_system/main/index.cfm?sector=FAD&auth=SANCAS. Last accessed June 2021.
60. Ghosh C, Sarkar P, Issa R, Haldar J. Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. Trends Microbiol. 2019;27(4):323-38.
61. Oh S, Kim SH, Ko Y, Sim JH, Kim KS, Lee SH, et al. Effect of bacteriocin produced by Lactococcus sp. HY 449 on skin-inflammatory bacteria. Food Chem Toxicol. 2006;44(4):552-9.
62. van Staden AD, Brand AM, Dicks LMT. Nisin F-loaded brushite bone cement prevented the growth of Staphylococcus aureus in vivo. Journal of Applied Microbiology. 2012;112(4):831-40.
63. Yaghoubi A, Khazaei M, Hasanian SM, Avan A, Cho WC, Soleimanpour S. Bacteriotherapy in Breast Cancer. Int. 2019;20(23):5880.
64. Soleimanpour S, Hasanian SM, Avan A, Yaghoubi A, Khazaei M. Bacteriotherapy in gastrointestinal cancer. Life Sci. 2020;254:117754.
65. Goodarzi A, Mozafarpoor S, Bodaghabadi M, Mohamadi M. The potential of probiotics for treating acne vulgaris: A review of literature on acne and microbiota. Dermatol Ther. 2020;33(3):e13279.
66. Mottin VHM, Suyenaga ES. An approach on the potential use of probiotics in the treatment of skin conditions: acne and atopic dermatitis. Int J Dermatol. 2018;57(12):1425-32.
67. Deidda F, Amoruso A, Nicola S, Graziano T, Pane M, Mogna L. New Approach in Acne Therapy: A Specific Bacteriocin Activity and a Targeted Anti IL-8 Property in Just 1 Probiotic Strain, the L. salivarius LS03. J Clin Gastroenterol. 2018;52 Suppl 1, Proceedings from the 9th Probiotics, Prebiotics and New Foods, Nutraceuticals and Botanicals for Nutrition & Human and Microbiota Health Meeting, held in Rome, Italy from September 10 to 12, 2017:S78-s81.
68. Kang BS, Seo JG, Lee GS, Kim JH, Kim SY, Han YW, et al. Antimicrobial activity of enterocins from Enterococcus faecalis SL-5 against Propionibacterium acnes, the causative agent in acne vulgaris, and its therapeutic effect. J Microbiol. 2009;47(1):101-9.
69. Cebrián R, Arévalo S, Rubiño S, Arias-Santiago S, Rojo MD, Montalbán-López M, et al. Control of Propionibacterium acnes by natural antimicrobial substances: Role of the bacteriocin AS-48 and lysozyme. Sci Rep. 2018;8(1):11766.
70. Manuel M-L, Marina S-H, Eva V, Manuel M-B, Mercedes M. Are Bacteriocins Underexploited? NOVEL Applications for OLD Antimicrobials. Current Pharmaceutical Biotechnology. 2011;12(8):1205-20.

71. Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnology Advances. 2003;21(6):465-99.
72. Mak P. Chapter 13 - Staphylococcal Bacteriocins. In: Savini V, editor. Pet-To-Man Travelling Staphylococci: Academic Press; 2018. p. 161-71.
73. Gillor O, Nigro LM, Riley MA. Genetically Engineered Bacteriocins and their Potential as the Next Generation of Antimicrobials. Current Pharmaceutical Design. 2005;11(8):1067-75.
74. Wohlleben W, Mast Y, Stegmann E, Ziemert N. Antibiotic drug discovery. Microb Biotechnol. 2016;9(5):541-8.
75. Kommineni S, Bretl DJ, Lam V, Chakraborty R, Hayward M, Simpson P, et al. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. Nature. 2015;526(7575):719-22.
76. Foulquie Moreno MR, Rea MC, Cogan TM, De Vuyst L. Applicability of a bacteriocin-producing Enterococcus faecium as a co-culture in Cheddar cheese manufacture. Int J Food Microbiol. 2003;81(1):73-84.
77. Dundar H. Bacteriocinogenic Potential of Enterococcus faecium Isolated from Wine. Probiotics Antimicrob Proteins. 2016;8(3):150-60.
78. Leroy F, Foulquie Moreno MR, De Vuyst L. Enterococcus faecium RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. Int J Food Microbiol. 2003;88(2-3):235-40.
79. Hugas M, Garriga M, Aymerich MT. Functionality of enterococci in meat products. Int J Food Microbiol. 2003;88(2-3):223-33.
80. Liu H, Zhang L, Yi H, Han X, Gao W, Chi C, et al. A novel enterocin T1 with anti-Pseudomonas activity produced by Enterococcus faecium T1 from Chinese Tibet cheese. World J Microbiol Biotechnol. 2016;32(2):21.
81. Bagci U, Ozmen Togay S, Temiz A, Ay M. Probiotic characteristics of bacteriocin-producing Enterococcus faecium strains isolated from human milk and colostrum. Folia Microbiol (Praha). 2019;64(6):735-50.
82. Rea MC, Ross RP, Cotter PD, Hill C. Classification of Bacteriocins from Gram-Positive Bacteria. In: Drider D, Rebuffat S, editors. Prokaryotic Antimicrobial Peptides: From Genes to Applications. New York, NY: Springer New York; 2011. p. 29-53.
83. Pierce BA. Genetics: A conceptual approach. New York: Freeman. 2012:1144-9.
84. Zouhir A, Hammami R, Fliss I, Hamida JB. A New Structure-based Classification of Gram-positive Bacteriocins. The Protein Journal. 2010;29(6):432-9.
85. Diep DB, Nes IF. Ribosomally synthesized antibacterial peptides in Gram positive bacteria. Curr Drug Targets. 2002;3(2):107-22.
86. Gajic O, Buist G, Kojic M, Topisirovic L, Kuipers OP, Kok J. Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins. J Biol Chem. 2003;278(36):34291-8.
87. Jiang H, Zou J, Cheng H, Fang J, Huang G. Purification, Characterization, and Mode of Action of Pentocin JL-1, a Novel Bacteriocin Isolated from Lactobacillus pentosus, against Drug-Resistant Staphylococcus aureus. Biomed Res Int. 2017;2017:7657190.
88. Wu Y, An J, Liu Y, Wang Y, Ren W, Fang Z, et al. Mode of action of a novel anti-Listeria bacteriocin (CAMT2) produced by Bacillus amyloliquefaciens ZJHD3-06 from Epinephelus areolatus. Arch Microbiol. 2019;201(1):61-6.

89. Lu Y, Yan H, Li X, Gu Y, Wang X, Yi Y, et al. Physicochemical properties and mode of action of a novel bacteriocin BM1122 with broad antibacterial spectrum produced by Lactobacillus crustorum MN047. J Food Sci. 2020;85(5):1523-35.
90. Vasilchenko AS, Vasilchenko AV, Valyshev AV, Rogozhin EA. A Novel High-Molecular-Mass Bacteriocin Produced by Enterococcus faecium: Biochemical Features and Mode of Action. Probiotics Antimicrob Proteins. 2018;10(3):427-34.
91. Riley MA. Molecular mechanisms of bacteriocin evolution. Annu Rev Genet. 1998;32:255-78.
92. Bykowski T, Stevenson B. Aseptic Technique. Current Protocols in Microbiology. 2020;56(1):e98.
93. Paganelli FL, Leavis HL, He S, van Sorge NM, Payré C, Lambeau G, et al. Group IIA-Secreted Phospholipase A2 in Human Serum Kills Commensal but Not Clinical *Enterococcus faecium* Isolates. Infection and Immunity. 2018;86(8):e00180-18.
94. Pegg DE. Principles of Cryopreservation. In: Wolkers WF, Oldenhof H, editors. Cryopreservation and Freeze-Drying Protocols. New York, NY: Springer New York; 2015. p. 3-19.
95. CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard. Eleventh edition. CLSI guideline M02-A11. Wayne, PA: Clinical and Laboratory Standards Institute. 2012.
96. O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, et al. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 2016;44(D1):D733-45.
97. Treangen TJ, Ondov BD, Koren S, Phillippy AM. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biology. 2014;15(11):524.
98. Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. Genetics. 2006;172(4):2665-81.
99. Figtree. Available at http://tree.bio.ed.ac.uk/software/figtree/. Last accessed June 2021.
100. van Heel AJ, de Jong A, Song C, Viel JH, Kok J, Kuipers OP. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Research. 2018;46(W1):W278-W81.
101. Sieber P, Platzer M, Schuster S. The Definition of Open Reading Frame Revisited. Trends Genet. 2018;34(3):167-70.
102. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10.
103. Delcher AL, Bratke KA, Powers EC, Salzberg SL. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics. 2007;23(6):673-9.
104. Piard J, C., Desmazeaud M. Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. Lait. 1992;72(2):113-42.
105. Piard J, C., Desmazeaud M. Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. Lait. 1991;71(5):525-41.
106. van de Guchte M, Ehrlich SD, Maguin E. Production of growth-inhibiting factors by Lactobacillus delbrueckii. J Appl Microbiol. 2001;91(1):147-53.
107. Kim EB, Jin GD, Lee JY, Choi YJ. Genomic Features and Niche-Adaptation of Enterococcus faecium Strains from Korean Soybean-Fermented Foods. PLoS One. 2016;11(4):e0153279.

108. Arredondo-Alonso S, Top J, McNally A, Puranen S, Pesonen M, Pensar J, et al. Plasmids Shaped the Recent Emergence of the Major Nosocomial Pathogen Enterococcus faecium. mBio. 2020;11(1):e03284-19.
109. Loehlin DW, Carroll SB. Expression of tandem gene duplicates is often greater than twofold. Proc Natl Acad Sci U S A. 2016;113(21):5988-92.
110. Geldart K, Kaznessis YN. Characterization of Class IIa Bacteriocin Resistance in Enterococcus faecium. Antimicrob Agents Chemother. 2017;61(4).
111. Vimont A, Fernandez B, Hammami R, Ababsa A, Daba H, Fliss I. Bacteriocin-Producing Enterococcus faecium LCW 44: A High Potential Probiotic Candidate from Raw Camel Milk. Frontiers in Microbiology. 2017;8(865).
112. Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Håvarstein LS. Enterocins L50A and L50B, two novel bacteriocins from Enterococcus faecium L50, are related to staphylococcal hemolysins. Journal of bacteriology. 1998;180(8):1988-94.
113. Martín-Platero AM, Valdivia E, Ruíz-Rodríguez M, Soler JJ, Martín-Vivaldi M, Maqueda M, et al. Characterization of antimicrobial substances produced by Enterococcus faecalis MRR 10-3, isolated from the uropygial gland of the hoopoe (Upupa epops). Appl Environ Microbiol. 2006;72(6):4245-9.
114. Hamid M-N, Friedberg I. Identifying antimicrobial peptides using word embedding with deep recurrent neural networks. Bioinformatics. 2019;35(12):2009-16.
115. Zhang J, Yang Y, Yang H, Bu Y, Yi H, Zhang L, et al. Purification and Partial Characterization of Bacteriocin Lac-B23, a Novel Bacteriocin Production by Lactobacillus plantarum J23, Isolated From Chinese Traditional Fermented Milk. Frontiers in microbiology. 2018;9:2165.
116. Zangeneh M, Khorrami S, Khaleghi M. Bacteriostatic activity and partial characterization of the bacteriocin produced by L. plantarum sp. isolated from traditional sourdough. Food Sci Nutr. 2020;8(11):6023-30.
117. Zhang J, Yang Y, Yang H, Bu Y, Yi H, Zhang L, et al. Purification and Partial Characterization of Bacteriocin Lac-B23, a Novel Bacteriocin Production by Lactobacillus plantarum J23, Isolated From Chinese Traditional Fermented Milk. Frontiers in Microbiology. 2018;9(2165).
118. Walls D, McGrath R, Loughran ST. A digest of protein purification. Methods Mol Biol. 2011;681:3-23.
119. Duong-Ly KC, Gabelli SB. Salting out of proteins using ammonium sulfate precipitation. Methods Enzymol. 2014;541:85-94.
120. Shih YC, Prausnitz JM, Blanch HW. Some characteristics of protein precipitation by salts. Biotechnol Bioeng. 1992;40(10):1155-64.
121. Merlich A, Galkin M, Choiset Y, Limanska N, Vasylieva N, Ivanytsia V, et al. Characterization of the bacteriocin produced by Enterococcus italicus ONU547 isolated from Thai fermented cabbage. Folia Microbiol (Praha). 2019;64(4):535-45.
122. Koontz L. TCA precipitation. Methods Enzymol. 2014;541:3-10.
123. Ge J, Du R, Zhao D, Song G, Jin M, Ping W. Kinetic study of a β-mannanase from the Bacillus licheniformis HDYM-04 and its decolorization ability of twenty-two structurally different dyes. Springerplus. 2016;5(1):1824-.
124. Insuasty Cepeda DS, Pineda Castañeda HM, Rodríguez Mayor AV, García Castañeda JE, Maldonado Villamil M, Fierro Medina R, et al. Synthetic Peptide Purification via Solid-Phase Extraction with Gradient Elution: A Simple, Economical, Fast, and Efficient Methodology. Molecules. 2019;24(7):1215.

125. Irvine GB. High-performance size-exclusion chromatography of peptides. Journal of Biochemical and Biophysical Methods. 2003;56(1):233-42.
126. Lakshmanan R, Kalaimurugan D, Sivasankar P, Arokiyaraj S, Venkatesan S. Identification and characterization of Pseudomonas aeruginosa derived bacteriocin for industrial applications. International Journal of Biological Macromolecules. 2020;165:2412-8.
127. Ekblad B, Kristiansen PE. NMR structures and mutational analysis of the two peptides constituting the bacteriocin plantaricin S. Sci Rep. 2019;9(1):2333.
128. Perumal V, Yao Z, Kim JA, Kim HJ, Kim JH. Purification and Characterization of a Bacteriocin, BacBS2, Produced by Bacillus velezensis BS2 Isolated from Meongge Jeotgal. J Microbiol Biotechnol. 2019;29(7):1033-42.
129. Gor MC, Vezina B, McMahon RM, King GJ, Panjikar S, Rehm BHA, et al. Crystal structure and site-directed mutagenesis of circular bacteriocin plantacyclin B21AG reveals cationic and aromatic residues important for antimicrobial activity. Sci Rep. 2020;10(1):17398.
130. Ortiz-López FJ, Carretero-Molina D, Sánchez-Hidalgo M, Martín J, González I, Román-Hurtado F, et al. Cacaoidin, First Member of the New Lanthidin RiPP Family. Angew Chem Int Ed Engl. 2020;59(31):12654-8.
131. Keren T, Yarmus M, Halevy G, Shapira R. Immunodetection of the bacteriocin lacticin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Applied and environmental microbiology. 2004;70(4):2098-104.

9. Appendix

9.1 List of selected *E. faecium* strains

Listed below are the selected strains, sorted by their order in the clinical and commensal phylogenetic trees. Top strains as defined by competitive growth are marked in red. The six clinical strains that clustered with the commensal strains in the phylogenetic tree are marked by asterisks.

Clinical strains	Commensal strains
01_K60-25_ST581	01_4E-7-B_ST2016
02_K60-02_ST52	02_5E-9-A_ST39
03_K59-57_ST38	03_2E-2-C_ST107
04_K59-46_ST533	04_2E-3-C_ST1101
05_K59-44_ST32	05_2E-8-1_ST361
06_K59-36_ST575	06_6E-2-G_ST60
07_K59-17_ST22	07_1E-5-F_ST361
08_K60-29_ST19	08_2E-6-A_ST61
09_K60-07_ST578	09_2E-3-D_ST94
10_K59-51_ST18	10_2E-3-A_ST178
11_3-E-9_ST18	11_3E-1-1_ST1927
12_K59-53_ST132	12_3E-6-1_ST800
13_K59-18_ST574	13_6E-1-H_ST178
14_K59-16_ST440	14_6E-2-C_ST800
15_1-A-9_ST17	15_2E-4-A_ST94
16_K59-55_ST279	16_3E-7-A_ST60
17_K59-50_ST202	17_3E-4-1_ST60
18_1-F-9_ST202	18_2E-6-F_ST96
19_2-D-9_ST117	19_4E-2-G_ST361
20_1-H-7_ST117	20_1E-6-D_ST94
21_NORM 1E3_ST117	21_1E-6-C_ST218
22_K59-62_ST282	22_1E-9-B_ST945
23_VRE 1G3_ST736	23_6E-4-C_ST696
24_1-I-9_ST80	24_2E-9-H_ST116
25_1-I-3_ST80	25_2E-5-B_ST798
26_1-D-5_ST80	26_5E-1-H_ST1938
27_K59-22_ST78	27_1E-1-A_ST1191
28_VRE 3B5_ST412	28_3E-9-A_ST296
29_K59-68_ST203	29_2E-7-F_ST994
30_K60-31_ST203	30_6E-7-B_ST296

Clinical strains	Commensal strains
31_VRE 2I1_ST78	31_2E-1-H_ST1945
32_1-H-4_ST203	32_1E-7-E_ST623
33_K59-27_ST17	33_6E-9-1_ST178
34_1-B-3_ST17	34_2E-2-D_ST1939
35_2-E-8_ST192	35_6E-1-G_ST583
36_VRE 2F6_ST192	36_4E-4-1_ST328
37_3-A-9_ST192	37_5E-3-1_ST289
38_1-C-3_ST192	38_3E-1-E_ST1036
39_K60-14_ST192	39_6E-3-D_ST583
40_K59-30_ST192	40_2E-9-F_ST328
41_1-C-2_ST203	41_E1007 _ST296
42_K59-20_ST203	42_2E-4-H_ST107
43_K59-59_ST203	43_6E-8-E_ST1926
44_K59-60_ST203	44_6E-1-A_ST39
45_K59-26_ST94*	45_5E-3-F_ST1031
46_K69-21_ST208*	46_4E-2-B_ST874
47_K59-52_ST576*	47_4E-1-E_ST1036
48_K59-19_ST296*	48_2E-5-A_ST1911
49_Norm 1A2_ST94*	49_4E-6-A_ST1105
50_K60-9_ST579*	50_1E-3-A_ST773

9.2 Unique bacteriocin amino acid sequences

The following are the 93 unique amino acid sequences of bacteriocins found in the selected *E. faecium* strains by RiPP mining. They are sorted by length, from longest to shortest.

1.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFIDDGTWIDPLPFLNSSKNRSGEKIWKIKR

2.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLMTILITVYVTLFLILAAGSIDNSDSDSSSGGEVFGEAFTG
EYSEGLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLF
PKGTARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGASLLILGRGGYAAAGITEDS
PYYTGCMSIITSNKLTEYDEFAIKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRP
NGFHDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKV
GDQVKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

3.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFIDDGTWIDPLPFLNSSKK

4.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAMKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNG
FHDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGD
QVKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGAWIDPLPFLNSSKK

5.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKNSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

6.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAMKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNG
FHDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGD
QVKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

7.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGETFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYTG
CMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFH
DGLDFGSVDHQGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

8.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLMTILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSE
GLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSLSAKNDLNFFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGVSLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDELAIKHWGEGGNNNGTITGEWMNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

9.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLMTILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSE
GLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSLSAKNDLNFFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPSSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

10.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILINVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAMKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNG
FHDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGD
QVKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGAWIDPLPFLNSSKK

11.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGETFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYTG
CMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFH
DGLDFGSVDHQGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTGDGTWIDPLPFLNSSKK

12.
MENQDESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGETFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITGDSPYYTG
CMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFH
DGLDFGSVDHPGSEIHAVHVGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSCVKVGDQV
KVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTGDGTWIDPLPFLNSSKK

13.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEG
LPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWSEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

14.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLMTILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSE
GLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSLSAKNDLNFFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGVSLLILGRGGYAAAGITEDSPYYT
GCMSIITTNKLTEYDELAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

15.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGETFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYTG
CMSIITSNKLTEYDEFAIKHWGEGGNNNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFH
DGLDFGSVDHQGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQEHAFTDDGTWIDPLPFLNSSKK

16.
MENQNESLIKQYVKRRAKRRLFLWLFGTSAGLMTILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSE
GLPIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSLSAKNDLNFFGITWFDGCLFPKGT
ARGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGASLLILGRGGYAAAGITEDSPYYT
GCMSIITSNKLTEYDEFAIKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGF
HDGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQ
VKVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

17.
MENQDESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNASVGNKSPGASLLILGRGGYAAAGITEDSPYYTG
CMSIITSNKLTEYDEFAIKHWGEGGNDNGTITGEWTNPFPGSSLDKSSFSGGQLFGTNPGGEFRPNGFH
DGLDFGSVDHPGSEIHAVHGGKVVYVGNPGISGLGACVIVINYDGLNMVYQEFANSTGNSRVKVGDQV
KVGQVIGIRDTAHLHLGFTRMDWRQAQGHAFTDDGTWIDPLPFLNSSKK

18.
MENQDESLIKQYVKRRAKRRLFLWLFGTSAGLITILITVFVTLFLILAAGSIDNSDSDSSSGGEAFTGEYSEGL
PIYKEIKGRGPFSDEIAQYAVGAAVKYKLLPSVILSQYGYESAFGTSASARNDLNYFGITWFDGCLFPKGTA
RGIGGIEGGWYMKFPNSKAAFSYYGFMVATQSNFNACVGNKSPGASLLILGRGGYAAAGITEDSPYYTG
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19.
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20.
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21.
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22.
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23.
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24.
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25.
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26.
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27.
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28.
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29.
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30.
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31.
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32.
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33.
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34.
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35.
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36.
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37.
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38.
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39.
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40.
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41.
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YLMSWNASSRTAKYYYYI

42.
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YLMSWTASSRTAKYYYYI

43.
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44.
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WVQHGPWAPR

45.
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GGAAAGWDARKK

46.
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GGAAAGWDARKK

47.
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GGAAAGWDARKK

48.
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49.
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PWAPRR

50.
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WAPRR

51.
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52.
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MGH

53.
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MGH

54.
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GA

55.
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GA

56.
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57.
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SKCN

58.
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KCN

59.
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60.
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61.
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62.
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63.
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64.
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65.
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66.
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67.
LILGIVLLSVSTLGITVDAATYYGNGVYCNTQKCWVDWSIARSEIVDRGVKAYVNGFTKVLGGVGGR

68.
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69.
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70.
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71.
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72.
VIYNYYEQKNDWRSYIMGAIAKLVAKFGWPIVKKYYKQIMQFIGEGWAINKIIEWIKKHI

73.
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74.
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75.
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76.
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77.
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78.
MKKYNELSKKELLQIQGGIAPIIVAGLGYLVKDAWDHSDQIISGFKKGWNGGRRK

79.
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80.
VTNFGTKVDAVTRSYDNGIYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH

81.
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82.
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83.
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84.
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85.
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86.
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87.
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88.
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89.
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90.
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91.
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92.
MLVNIRGKQTMRTCYLMSWTASSRTAKYYYYI

93.
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