UNIVERSITY OF LIVERPOOL

Title of thesis

Investigating antimicrobial resistance by competition of *Staphylococcus* species

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Abstract

Staphylococcus aureus can invade the human skin microflora and cause many serious infections as an opportunistic pathogen. The anterior nares are its major niche where it colonises with diverse bacteria that interact and compete for the same resources and binding sites. The study of microbial competition and invasion increases our understanding of species dynamics and how they contribute to evolution of antibiotic resistance and impacts on human health. However, the genetic drivers in the evolution of competition and resistance remains unclear in the *S. aureus* niche.

A deferred growth inhibition assay was used to determine the antimicrobial activities of coagulase- negative *Staphylococcus* (CoNS) species, including *S. capitis* and *S. lugdunensis,* which were determined to have novel genes for Biosynthetic Gene Clusters (BGS). Two species competition (co-culture) was explored to simulate the competition environment among species isolated from human skin and those species with novel BGS. Experimental evolution of interference competition was used to identify mutations selected during co-culture. Illumina platform genome resequencing of *S. aureus* clones identified multiple single nucleotide polymorphisms (SNPs) including several candidate resistance genes.

In co-culture with the unidentified antimicrobial producer *S. capitis* 47, *S. aureus* strains SH1000 and 184 evolved mutations in the repressor gene *cro/cI* that was previously demonstrated to regulate an efflux transporter, supporting that *S. aureus* might use this efflux pump system to survive this antimicrobial activity in competition with other producer species. During serial passages of *S. aureus* SH1000 and 184 strains, further mutations were selected in the *agr* and *sigB* genes that reduced pigment production. In a separate coculture investigation with the epifadin antimicrobial producer *S. epidermidis* IVK83**,** *S. aureus* USA300 showed experimentally evolved resistance with mutation in the two-component system (TCS), *desK* gene. The DesKR TCS was recently implicated in epifadin resistance during a study in our research group at Liverpool. This study extends our knowledge into the strain USA300.

A further co-culture competition was performed with *S. aureus* USA300 and antimicrobial producer *S. lugdunensis* 1007. While confirming existing data that *S. aureus* could not evolve resistance to lugdunin but there were selected mutations that supported increased fitness.

The *S. aureus* mutations identified in the Cro/CI repressor, DesKR TCS and those in response to a lugdunin producer indicate there are an array of mechanisms concerned with interspecific competition. Commensal bacteria are an important resource of molecules and antimicrobials to study for preventing staphylococcal infections.

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List of Abbreviation

agr	Accessory gene regulator
AIP	Autoinducer peptide
AMP	Antimicrobial peptides
BHI	Brain Heart Infusion
BraRS	Bacitracin resistance associated Sensor/Regulator
CAMPs	Cationic antimicrobial peptides
CoNS	Coagulase-negative staphylococci
ESP	Endoserine peptidase
et al.	Et alia (and others)
Fig.	Figure
GraRS	Glycopeptides resistance associated- regulator and sensor
Indels	Insertions or deletions of a number of nucleotides in a DNA sequence
LB	Luria Bertani Broth
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PSM	phenol soluble modulin
Sar	Staphylococcal accessory regulator
SNP	Single nucleotide polymorphism
TCSs	Two component systems
WT	Wild type

Unites and measurements

CFU	Colony forming units
cm	Centimetres
g	Gram
h	Hour
Kb	kilobase
kDa	Kilodalton
mM	Millimolar
mm	Millimetres
ng	Nanogram
^{o}C	Degree Celsius
rcf	Relative centrifugal force
rpm	Revolutions per minute
sec (s)	Second
v/v	Volume per volume
w/v	weight per volume
µg	Microgram
µl	Microlitre

Chapter 1: General Introduction

1.1 Overview of *Staphylococcus aureus* and skin infections

Staphylococcus aureus is a Gram-positive, facultatively anaerobic bacterium with a diameter of ~ 0.8 μm that was first isolated from infected patients in Scotland in 1880 by Sir Alexander Ogston (Guo et al*.,* 2020). The moist squamous epithelium of the anterior nares is the primary habitat of this bacterium, with 25 to 50% of the human population persistently colonised with *S. aureus* (Goyal et al., 2019). *S. aureus* can be found as part of the microbial flora of the human skin (Brown and Horswill, 2020). Clinically*, S. aureus* causes many serious infections as an opportunistic pathogen. In the UK and USA, *S. aureus* is the most common cause of skin and soft tissue infections (SSTIs) (Larsen et al*.,* 2008; Hindy et al*.,* 2021; Juwita et al*.,* 2022). It is responsible for causing ~ 76 % of all SSTIs, which lead to 500,000 hospital visits per year in USA (Methicillin-Resistant *S. aureus* Infections among Patients in the Emergency Department, 2007; Houghland, 2009). *S. aureus* isolates are frequently multi-drug-resistant and are responsible for nosocomial infections. Even though the development of antibiotic resistance is a natural process for bacteria to protect themselves from factors that threaten survival, the clinical overuse of the antibiotic methicillin in the 1960s led to the emergence of strains resistant to a wide range of beta-lactam antibiotics (Panlilio et al., 1992). Therapeutically, these strains, named methicillin-resistant *S. aureus* (MRSA), have reduced treatment options, requiring the search for new antibiotics and therapies.

1.2 The Staphylococci

Staphylococci are commensals or opportunistic pathogens that colonise human skin and mucous membranes (Parlet et al*.,* 2019). According to the ability to coagulate plasma there are two primary categories of staphylococci, coagulase-positive (CoPS) and coagulase-negative staphylococci (CoNS) (Smith and Andam, 2021). CoPS are pathogenic bacteria that produce

the coagulase enzyme, which converts plasma fibrinogen to fibrin (Heilmann et al., 2019), whereas CoNS do not produce this enzyme and were previously thought to be non-pathogenic infections. In addition to *S. aureus,* which is a member of CoPS, the CoNS are a diverse collection of species that are partly characterised genetically and functionally. CoNS has shown antimicrobial activity that contributes to the human skin microflora, with varying frequencies of species, including *S. epidermidis, S. warneri, S. hominis, S. capitis,* and *S. lugdunensis* (Parlet et al., 2019; Bin Hafeez et al., 2021).

1.2.1 *S. epidermidis*

S. epidermidis is the most frequently recovered staphylococcal species in individuals. The anterior nares, conjunctiva, axillae, and toe webs are among the wet body surfaces where this bacterium is highly abundant (Becker et al.,2014). Though CoNS are typically described as mild or non-pathogenic commensals in humans, *S. epidermidis* is a major cause of nosocomial infections (Otto, 2009; Kollef et al., 2021). According to research, biofilm development is the primary method used by *S. epidermidis* to colonise and infect prosthetic and medical devices (Le et al., 2018). The overall evolution of the *S. epidermidis* genome and its interactions with other Staphylococci, particularly *S. aureus*, are specifically covered by whole-genome analyses (Zhang et al., 2003). However, more studies are needed (Argemi et al., 2019). *S. epidermidis* has reported galidermin and epidermin antimicrobials that inhibit some other Gram-positive bacterial species (Ebner et al., 2018).

1.2.2 *S. warneri*

S. warneri is one of the CoNS isolates and is commonly found on healthy human skin, in the mouths of animals, and in the anterior nares. This bacterium is typically not thought to be harmful; however, it has been identified in several illnesses, including endocarditis of the

native and artificial valves and urinary tract infections (Noshak et al., 2020). Although more studies are needed to understand the mechanism of *S. warneri* pathogenesis, the most common treatment for *S. warneri* meningitis is vancomycin (Azimi et al., 2020). *S. warneri* also showed antimicrobial activity where Nukacin ISK-1 produced by *S. warneri* ISK-1 could inhibit *S. aureus* MM30 (Kawada -Matsuo et al*.,* 2013; Arii et al*.,* 2019).

1.2.3 *S. hominis*

S. hominis, like other CoNS isolates, is a regular part of the typical human skin microbiota (Noshak et al., 2020). In addition to *S. warneri*, *S. hominis* constitutes 14% of all CoNS isolates in body fluids such as urine, semen, and prostatic secretions (Azimi et al., 2020). Although *S. hominis* rarely develops novobiocin resistance, this organism is a potential pathogen and can cause various infections, such as infective endocarditis, in immunocompromised patients (Azimi et al., 2020; Noshak et al., 2020). Hominicin is among the antimicrobials produced by *S. hominis* MBBL 2-9 against *S. aureus* ATCC 25923 and CCARM 3501 (Kim et al., 2010).

1.2.4 *S. capitis*

It was discovered that sebaceous glands were surrounded by *S. capitis* on the scalp and forehead after adolescence (Becker et al.,2014). *S. capitis* has also been described in clinical infections, and multidrug resistance has emerged as a crucial issue in its infections. Analysis of whole genome sequencing data confirmed that *S. capitis* can have antimicrobial activity against Gram-positive bacteria, including *S. aureus* (Argemi et al., 2019). For example, *S. capitis* TE8 isolated from human skin showed antimicrobial activity against *Micrococcus luteus, S. aureus,* and *Bacillus subtilis*. Using antiSMASH, RAST server, and BAGEL3 software, whole genome sequencing of *S. capitis* TE8 identified four separate gene clusters

with the ability to encode antimicrobial peptides, including gallidermin, epidermicin, and phenol-soluble modulins (PSM) (Kumar et al.,2017).

1.2.5 *S. lugdunensis*

S. lugdunensis is an essential component of the typical flora of the skin and primarily found in the pelvic and perineum areas, the lower extremities, and the axillae. However, it is less common to find it in the anterior nares than *S. aureus* (Becker et al.,2014; Bieber and Kahlmeter, 2010). Although *S. lugdunensis* is thought to be a prominent pathogen in human infections, its pathogenicity is most likely lower than that of *S. aureus* (Argemi et al.,2017). Interestingly, *S. lugdunensis* showed its ability to produce an antimicrobial named lugdunin that could inhibit *S. aureus* (Zipperer et al., 2016). Thus, this antimicrobial in combination with others that CoNS produced such as galidermin, hominicin, epidermin, and capidermycin can further our understanding of species dynamics and how they contribute to the evolution of antibiotic resistance and its impact on human health (Zipperer et al., 2016; Lynch et al., 2019; Lynch et al., 2021). However, the genetic drivers of competition and resistance to AMPs in the *S. aureus* niche remain unknown.

1.3 The skin microbiome

Human skin is a physiological barrier that protects the host from opportunistic infections while maintaining internal homeostasis. This barrier is densely populated with a diversity of microorganisms such as bacteria, fungi, and viruses that interact and compete for colonisation and survival (Byrd et al., 2018; Ndhlovu et al., 2022). This microbiome is active on the skin and represents a direct influence on human health. Beneficial bacteria colonise the skin, acting as a physical barrier to limit the invasion of pathogens such as *S. aureus* which is the most responsible bacteria for skin infection. To maintain a healthy balance of these relations, the skin possesses several mechanisms to control the microbiome. Among these mechanisms are

skin glands, water content, pH, and expression of AMPs. For instance, the eccrine sweat glands secrete water that contribute to regulation of skin temperature and have a role in the acidification of the skin via lactate release (Byrd et al., 2018; Fölster-Holst, 2022). Normal skin's surface pH ranges from 4.5 to 6.0, which is not sufficient to completely kill bacteria such as *S. aureus*, whose optimal growth pH is 7.0 to 7.5. Skin pH is one of the critical factors involved in the wound healing process. For instance, it can affect the matrix metalloproteinase activity, fibroblast activity, keratinocyte proliferation, microbial proliferation, biofilm formation, and immunological responses (Kuo et al., 2020). While AMPs are produced either by keratinocyte cells or skin microbes. Recent research has revealed that the diversity and dynamism of human skin microbes may be in fierce competition, implying that colonising bacteria may use a range of techniques to overcome competitors (Ndhlovu et al., 2022). Thus, the expression of AMPs and how skin microbial members interact or protect themselves from these AMPs, particularly, *S. aureus,* has received great interest from researchers (Herman and Herman, 2018; Parlet et al., 2019). This interest stems from the difficulties in treating *S. aureus* infections*,* mainly skin infections, and how difficult these are to treat. There is an immediate requirement for new methods to circumvent *S. aureus'* resistance mechanisms and the requirement to discover new antibiotics (Bin Hafeez et al., 2021). Despite several active research projects addressing critical virulence and resistance mechanisms of *S. aureus*, the systems that drive bacterial fitness, competition, and proliferation in microorganisms are still poorly understood (Bitrus et al., 2018; Goyal et al., 2019).

1.4 Bacteriocins as novel antimicrobial peptides (AMPs)

Many bacteria produce AMPs traditionally known as bacteriocins (Cotter et al*.,* 2005). These bacteriocins are a highly heterogeneous array of molecules made by bacteria and used to interfere with competition and can inhibit growth or kill competitors (Wang et al*.,* 2020). Due to the ability of bacteriocins to inhibit or kill some antibiotic-resistant strains such as MRSA,

bacteriocins are intriguing applicants for further research as antimicrobial agents for use in healthcare settings (Mahlapuu et al., 2016; Zheng et al., 2017). Studying AMPs and their mode of action may provide us with new strategies to combat important pathogens (Wang et al., 2020). However, to limit the development of resistance and loss of efficacy, further research is needed to clarify the antimicrobial mechanisms of AMPs (Newstead et al., 2020). There are some cases in which some bacteriocins remain uncharacteristic, however, most bacteriocins produced by Gram-positive bacteria are divided into four categories: (I) lantibiotics (I-A and I-B), (II) non-lantibiotics, (III) large-sized bacteriocins (sometimes known as bacteriolysins), and (IV) bacteriocins with a unique structure (Bin Hafeez et al., 2021).

1.5 Lantibiotics

1.5.1 Class I-A

1.5.1.1 Nisin, epidermin, gallidermin, and Pep5

Nisin, epidermin, gallidermin, and Pep5 are small peptides less than 5 kilodaltons (kDa) in size classified under class I-A lantibiotics that involve post translationally modified amino acids lanthionine and methyllanthionine (Kawada-Matsuo et al., 2013, Simons et al., 2020). I-A lantibiotics are flexible, elongated, stable to heat peptides containing ~19-38 amino acids that have positive charge and show inhibitory activity against many Gram-positive bacteria (Bin Hafeez et al., 2021). Nisin, epidermin and gallidermin are proposed to have a mode of action that involves interaction with the cell membrane of bacteria via a peptidoglycan precursor molecule called lipid II as binding sites. This interaction occurs through electrostatic interactions with phospholipid headgroups resulting in membrane dissipation (Korobov et al., 2022). Nisin is produced by *Lactococcus lactis subsp* that can inhibit Gram-positive and Gram-negative bacteria (de Arauze et al., 2009). Gallidermin and epidermin are generated by *S. epidermidis* Tü3298 and *S. gallinarum*, respectively, while Pep5 is produced by various *S.*

epidermidis. These antimicrobials act against a variety of Gram-positive bacteria (Bierbaum et al., 1996; Götz et al. 2014; Ebner et al., 2018). PeP5 acts mostly by creating pores in bacterial membranes. This action leads to an influx of small molecules, potential dissipation of the membranes, and finally stopping cellular biosynthesis (Wang et al*.,* 2020).

1.5.2 Class I-B

1.5.2.1 Lacticin 481, salivaricins and cytolysin.

Lacticin 481, cytolysin, and salivaricins are classified under lantibiotic group I-B and have the same structure as lantibiotic group I-A. However, they are negatively charged, globular, and inflexible peptides that block key enzymes in the targeted bacteria, as with Lacticin 481 that produced by *L. lactis subsp*. Nukacin ISK-1 is believed to belong to the Lacticin 481 group (Sashihara et al., 2000, Simons et al*.,* 2020; Knerr et al*.,* 2013). Nukacin ISK-1 antimicrobial is produced by *S. warneri* that inhibit *S. aureus* (Kawada-Matsuo et al*.,* 2013). Salivaricins work as an antimicrobial compound via binding to lipid II and are then followed by pore formation in the cytoplasmic membrane or interference with cell wall synthesis, resulting in bacterial cell damage. *Streptococcus salivarius* produces salivaricins, which are active against oral streptococci and other bacterial species in the mouth area (Mansur and Ahmed, 2022). Cytolysin, on the other hand, sticks to cell membranes and makes holes in them, which leads to the osmotic lysis of the cell (Simons et al*.,* 2020). Cytolysin can be produced by a large member of Gram-positive bacteria (Van et al*.,*2013).

1.5.3 Class II non-lantibiotics

1.5.3.1 IIa, IIb, IIc and IId

There are four subclasses in class II bacteriocins, including IIa, IIb, IIc and IId (Elalem, 2021). Class II bacteriocins include less than 10 kDa of unchanged (non-lanthionine) amino acids.

These bacteriocins can also be heat-stable peptides that act by causing pore formation in the cell membrane (Simons et al., 2020). Disulfide-containing linear peptides with comparable amino acid sequences that have potent antilisterial effects, such as pediocin PA-1, leucocin A, acidocin A, and enterocin P, are found in subclass IIa. *Pediococcus acidilactici* PAC1.0 produces the bacteriocin Pediocin PA-1, which could kill other *Pediococcus* bacteria (Chikndas et al., 1993). *Leuconostoc gelidum* UAL 187 produces leucocin A. This bacteriocin, which is plasmid-encoded, inhibits *Listeria monocytogenes*, *Enterococcus. faecalis*, and other lactic acid bacteria (Hastings et al., 1991; Allison et al., 1995). Produced by the *Lactobacillus acidophilus* TK9201, Acidocin A acts efficiently against Listeria monocytogenes and other closely related lactic acid bacteria such as *L. monocytogenes* (Kanatani et al., 1995). While enterocin P is produced by *Enterococcus faecium* P13 which displays an inhibitory activity against *L. monocytogenes* (Cintas et al., 1997; Herranz et al., 2001).

Subclass IIb bacteriocins, on the other hand, are made up of two equally sized peptide subunits that function as a single unit (Simons et al., 2020; Elalem, 2021). These two peptides are essential for antimicrobial activity. lactococcin G and Q and plantaricin EF and JK, NC8, thermophilin 13, and lactacin F are examples of two-peptide bacteriocins (Nissen-Meyer et al., 2009). Subclass IIc bacteriocins consist of two short cyclic peptides known as the N- and C-terminals that are linked by a peptide bond. The bacteriocin subclass IId is one of the last uncharacterised bacteriocins in the class and includes all the remaining uncharacterized bacteriocins in group II, such as lactoxin A, B, 972, enterocin L50, and lacticin Q. In general, all class II subclasses act by permeating the cell membrane, making the target bacterial cell more permeable, resulting in cell damage (Bin Hafeez et al., 2021).

1.5.4 Class III and Class IV bacteriocins

Class III includes heat sensitive and macro peptides of more than 30 kDa that include several members (Elalem, 2021; Dai et al., 2021). Among these, zoocin A is produced by *Streptococcus equi* subsp. *zooepidemicus* 4881 against other streptococcal species (Heath et al., 2004). Lysostaphin is produced by *S. simulans* staphylococci that acts against staphylococcus and break down peptidoglycans in bacterial cell walls (Schindler et al., 1964; Bastos et al., 2010). Enterolysin A is another class of III bacteriocins. This antimicrobial is produced by *Enterococcus faecalis* LMG2333/DPC5280 or II/1 that has been shown to kill *L. lactis ssp* (Khan et al., 2013). Finally, heleveticin M is produced by *Lactobacillus crispatus* that has inhibitory effect against *S.aureus*, *S. saprophyticus*, and *Enterobacter cloacae* (Sun et al., 2018).

Class IV bacteriocins are structurally unique as they include specific lipids or carbohydrates. These specific molecules, such as lactocin 27 is produced by *Lactobacillus helveticus* strain LP27 against *L. helveticus* strain LS18 (Upreti et al., 1975). Pediocin SJ-1 produced by *Pediococcus acidilactici* LMG235 that shows activity against *L. monocytogenes* (Rodeiguez et al., 2002). Plantaricin S or leuconocin S are other type of class IV bacteriocins that can damage the bacterial cell membrane. Each class of these bacteriocins has enzymatic activity, such as endopeptidase, which causes bacterial cell wall disorder (Bin Hafeez et al., 2021).

1.6 Microbial competition

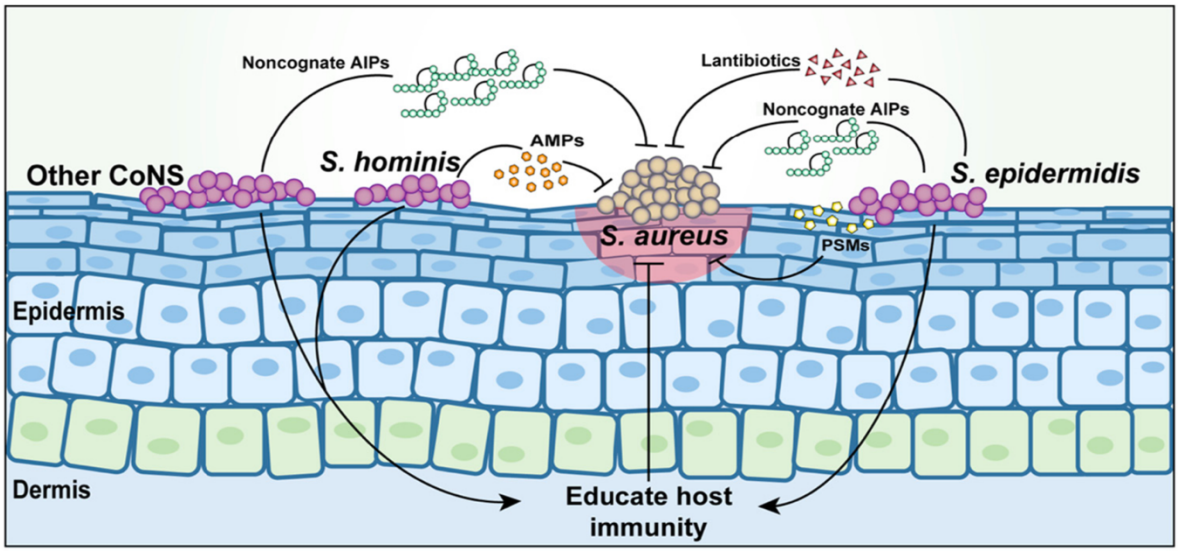
In a neutral environment, microorganisms compete for nutrition and limited space. When two different types of microbes meet, one is likely to be limited by the other, especially if the competition is related to a single resource. With this challenge in mind, microbes must protect themselves to outperform their neighbours (Ghoul and Mitri, 2016). Microbial competition has mostly been studied in *vitro*, either in culture or in biofilms, although the first use of

bacteriotherapy for *S. aureus* skin colonisation was reported (Zipperer et al*.,* 2016). Generally, there are two types of microbial competition: interference competition and exploitative competition (Stubbendieck and Straight, 2016; Raffatellu, 2018). Firstly, interference competition is exemplified by the secretion of specialised metabolites, which have an extensive range of activity. These metabolites include antibiotics, including, cephalosporins and penicillins that can kill microorganisms. Producers are protected by immunity proteins to avoid self-damage where bacteriocins usually target closely related organisms (Dobson et al*.,* 2011; Hassan et al*.,* 2012). Quorum sensing interference competition between bacteria is an example of interference competition when an organism uses specific small molecules to inhibit another organism (O'Loughlin et al., 2013). Secondly, exploitative competition is a mechanism that occurs when organisms compete for a shared nutrient. In this type of competition, the energy resources, for example iron or carbon, of one organism are likely to be limited by another organism in the same niche (Vraspir and Butler, 2009).

1.7 Bacterial competition among CoNS and *S. aureus* on human skin

Healthy skin is densely populated by a diverse group of CoNS as well as other microorganisms that interact and compete for the purpose of colonising and surviving. This healthy balance, however, can be quickly offset by the expansion of the opportunistic pathogen *S. aureus,* which is the main reason for skin infections (Parlet et al., 2019). AMPs secreted by CoNS produce a range of structurally diverse inhibitors and certain studies showed their interactions and interference towards bacterial strains of the same or different species, highlighting their therapeutic potential (Paharik et al., 2017).

Figure 1. 1. Competition between CoNS and S. aureus on human skin: CoNS compete with S. aureus on human skin by producing small-molecule products such as antimicrobial peptides (AMPs), lantibiotics, and phenol-soluble modulins (PSMs) (Figure taken from Parlet *et al.*, 2019).



1.8 Threats from antibiotic resistance

Antibiotics, antimicrobial compounds with the capacity to either kill microorganisms or limit their growth, are commonly used to treat bacterial illnesses in both people and animals (Serwecińska, 2020). Historically, the early warning of antibiotic resistance was reported in 1945 by Sir Alexander Fleming (Ventola, 2015). However, antibiotic resistance remains more and more dangerous to human health and to treat clinical disease worldwide (Haddadin, 2022). Antibiotic resistance of bacteria can be a natural defence mechanism for them against threats to their survival, however, overuse, easy access to antibiotics, and poor diagnosis of pathogenic bacteria, have led to an increase in bacterial resistance to antibiotics (Ventola, 2015; Baquero, 2021; Tarrant and Krockow, 2022). Despite decades of study on bacterial drug resistance, antibiotic resistance remains a concern to public health. The Centres for Disease Control and Prevention (CDC) in the United States has reported 16 antimicrobial-resistant bacterial

infections that cause over 2.8 million illnesses and at least 35,000 deaths per year. The estimated cost of this infection ranges from $100 million to $30 billion each year (Antibiotic resistance threats in the United States, 2019). According to the World Health Organization (WHO), infections caused by multidrug-resistant (MDR) bacteria cause 700,000 deaths worldwide each year, of whom 200,000 are children (Romandini et al., 2021). By 2050, it is estimated that antibiotic resistance would cause 10 million yearly deaths (Serwecińska, 2020). With this challenge in mind, *S. aureus* is one of the first bacteria that selects successful evolutionary changes and become a multidrug-resistant bacterium that can rapidly acquire resistance genes (Liang et al., 2022).

1.9 Methicillin-resistant *S. aureus*

Methicillin-resistant *S. aureus* (MRSA) is among the most common antibiotic-resistant threats that can cause serious infections. Each year, MRSA is responsible for approximately 11,285 deaths in the United States (Ventola, 2015). These mortalities are more than those from HIV and tuberculosis combined, which has placed an additional burden on the health care system (MRSA infection in the US, 2008; Jarvis et al 2012; Koch et al, 2014). MRSA is a major nosocomial pathogen throughout the world and these strains are found in 29% to 35% of all clinical isolates (Haddadin, 2022). This means that they are common in hospitals all over the United States and Europe. *S. aureus* also contributes to SSTIs. In 11 US cities in 2004, MRSA was detected in 59% of SSTI patients (Hindy et al., 2021). SSTIs caused by *S. aureus* are known to be difficult to treat because the elements of antibiotic resistance are often developed (Ferreira et al., 2021). Even though some antibiotics, such as vancomycin, teicoplanin, linezolid, tigecycline, daptomycin, ceftaroline, and seftoperol, still have an effect against MRSA, MRSA is becoming more and more prevalent in hospitals, the public, and more lately animals. This spread complicates the challenge to health care and is of great concern because MRSA may become resistant to more antibiotics. Due to this challenge, the WHO and the CDC

have considered MRSA as serious risks (Mariger, 2018; Hardy et al., 2019). The higher incidence of these strains has already led to higher morbidity and mortality, posing a threat to public health.

1.10 Penicillin and methicillin resistance

Antibiotic treatment for *S. aureus* infections frequently leads to antibiotic resistance (Foster, 2017). *S. aureus* is a multi-drug-resistant bacterium that can rapidly acquire resistance genes. However, antibiotic resistance to *S. aureus* has been reported since the early 1940's after a strain of *S. aureus* developed resistance against penicillin (Musser and Kapur, 1992). The first cases of methicillin-resistant *S. aureus* (MRSA) were found in the United Kingdom in 1962 and the United States in 1968 (Ventola, 2015). Drug resistance mechanisms include complex methods such as efflux pumps, enzymatic inhibition, drug target site alteration, and target protein mutation (Ylmaz and Aslantaş, 2017). Penicillin resistance was discovered by penicillin drugs detected through penicillin-binding proteins (PBPs), which were found to have a role in how penicillin works. These transpeptidases participate in the construction of the peptidoglycan portion of the bacterial cell wall. Once penicillin enters the bacterial cell, it binds to PBPs and blocks the ability of PBPs to function normally. The resistance to penicillin is mediated by *blaZ*, the gene which codes for β-lactamase and is controlled by two adjacent regulatory genes known as *blaI a*nd *blaRI* (Bitrus et al., 2018). Penicillinase (β-lactamases), in the form of extracellular enzymes, are synthesised when staphylococci are exposed to β-lactam antibiotics resulting in the hydrolysis of the β-lactam ring which reduces the therapeutic effect of penicillin. Ampicillin, oxacillin, and methicillin were other modified-structure ß-lactam antibiotics that were developed by antimicrobial researchers to protect the β-lactam ring from attack by the β-lactamases. However, the bacteria acquired a new resistance called PBP2a or PBP2', which was also resistant to methicillin and most other β-lactam drugs. *mecA* is the gene that encodes the PBP2a protein. This gene is part of the staphylococcal cassette

chromosome *mec* (*SCCmec*) that includes two required components, the *mec* gene complex, and the ccr gene complex.

Based on the structure and combination of *mec* and *ccr* gene complexes present, *SCCmec is* classified into eight types (I-VIII) (Foster, 2017). Types I, IV, V, VI, and VII often do not carry other resistance genes, while types II, III, and VIII can include one or more additional resistance genes, such as *tetK* (tetracycline), *ermA* (erythromycin), and *aadD* (tobramycin). These genes are an important trait in hospital-associated MRSA (HA-MRSA) and community-associated MRSA. Most strains of HA-MRSA have SCCmec types I, II, III, VI, and VIII. Most CA-MRSA strains are type IV, but some are also typing V and VII (Malachowa and DeLeo, 2010). According to a study conducted in a Chinese hospital between 2012 and 2017 on 835 isolates of MRSA, most CA-MRSA strains showed susceptibility and sensitivity to ciprofloxacin, gentamicin, rifampicin, vancomycin, linezolid, tigecycline, quinupristin/dalfopristin, and nitrofurantoin. While CA-MRSA strains showed resistance to erythromycin and clindamycin. On the other hand, 100 % of the HA-MRSA isolates were sensitive to vancomycin, linezolid, tigecycline, and quinupristin/dalfopristin. The sensitivity of HA-MRSA isolates to clindamycin was less than 10 % (Tsouklidis et al., 2020). According to another study conducted in the USA in 2004, skin and soft tissue infections can be caused by both CA-MRSA and HA-MRSA strains. Since current antibiotic therapy shows limitations for treating MRSA, several approaches are being investigated to overcome or minimise this challenge. Such attempts include developing new antibiotics, developing quorum-sensing inhibitors, and gaining insights from microbial competition strategies between CoNS and *S. aureus*. More studies are, however, needed to evaluate the likely success of novel strategies (Rutherford and Bassler, 2012; Koch et al., 2014).

1.11 Antimicrobial resistance in *S. aureus*

S. aureus is a notorious bacterial species with an extraordinary ability to develop resistance to a wide range of antimicrobial compounds, including most available classes of antibiotics (Hiron et al., 2011). *S. aureus* develops resistance to antimicrobials in a variety of ways. Limiting drug uptake, changing the drug target, enzymatic inactivation, and active efflux are all examples of these strategies (Yılmaz and Aslantaş, 2017). In addition to the *mecA* gene encoding penicillin-binding proteins (PBPs) (Foster, 2017), there are other mechanisms known as efflux pumps that have a role in resistance of *S. aureus* (Rezende-Júnior et al., 2020). NorA is among these well-studied systems in *S. aureus*. Historically, the *norA* gene was first reported in Japan in 1986 (Ubukata., 1989). The gene was identified in *S. aureus* that developed resistance to fluoroquinolones. NorA is a 388-amino acid protein with 12 transmembrane segments that belongs to the major facilitator superfamily of secondary transporters. This efflux system acts by extruding fluoroquinolone and other antimicrobials from the cell using the proton motive force. However, other studies reported NorA association to fusaric acid and siderophore transport (Costa et al., 2019). This fact has motivated extensive efforts to understand the genetic basis for antimicrobial resistance through the mechanisms by which these determinants evolve over time within bacterial populations (Jensen and Lyon, 2009).

1.12 The role of two component systems (TCS) in *S. aureus* antimicrobial resistance

In addition to the antibiotic resistances described above, antimicrobial resistance is also regulated in *S. aureus* by two-component signal transduction systems (TCS) (Coates-Brown et al., 2018). Bacterial TCS were the first major mode of signal transduction identified in bacteria (Nixon et al., 1986). These systems are frequently used to regulate multiple genes (regulons) involved in activities like cell proliferation and pathogenicity (Utsumi and Igarashi, 2012).

Bacteria use TCS as a type of signalling to adjust their genetic response to changes in their environment. These systems code for histidine kinase (HK), a signal sensor that phosphorylates in response to environmental change, as well as a response regulator (RR), which interacts with phosphorylated HK (Mitrophanov and Groisman, 2008). It was suggested that *S. aureus* also uses TCS to aid its survival in difficult bacterial communities. According to the genome studies and in addition to *mecA*-linked TCS of MRSA, there are 16 distinct TCS (Foster, 2017; Hiron et al., 2011). Thus, understanding the evolution of antimicrobial resistance phenotypes and resistance mechanisms, particularly in relation to TCS regulation, helps in limiting global growth in bacterial antimicrobial resistance (Jensen et al., 2017). Early knowledge of antimicrobial resistance and the discovery of drug resistance mechanisms at the genetic level are critical and will greatly increase drug efficacy and safety. Although there is one TCS that has not been characterised in *S. aureus*, there are four HKs that have been classified as intramembrane sensing histidine kinases (IM-HKs), namely VraS, BraS, GraS and SaeS. WalK, along with other HKs, has only one possible ligand interaction site for the Per-ARNT-Sim (PAS) domain (Fig 1.2). While Agr is a quorum sensor that enables the bacteria to monitor cell density (Fig 1.3) (Bleul et al., 2021).

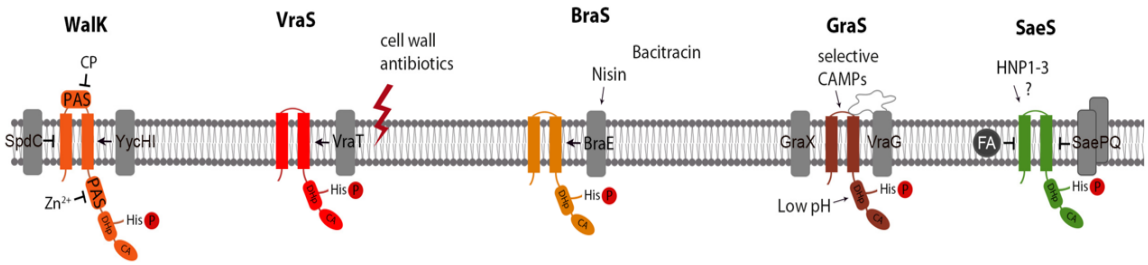


Figure 1. 2. Five HKs of *S. aureus*: Structure and signalling mechanisms. Peptidoglycan cleavage products (CP), cationic antimicrobial peptide (CAMP) and fatty acid (FA) (Bleul et al., 2021).

1.12.1 VraSR

Vancomycin-associated resistance (VraSR) is a TCS responsible for resistance to cell wall-damaging peptides such as β-lactam antibiotics and other AMPs through regulation of cell wall biosynthesis and antibiotic resistance genes (Ahmad et al., 2020). It was found that the higher expression of the *vraRS* genes in resistant *S. aureus* strain was related to a reduction in vancomycin sensitivity. However, other antibiotics such as ceftizoxime, teicoplanin, imipenem, cycloserine, and bacitracin can also induce expression levels of the *vraS* gene (Wu et al., 2020). VraS belongs to the IM-HK group and senses agents that inhibit the growth of cell walls (Mascher, 2006). The gene that codes VraS is part of an autoregulated four-gene operon (*vraUTSR*). Although the molecular mechanism involved in VraS sensing is yet unknown, the relationship between VraT and VraS appears to be important (Hu 2016 et al., Bleul, Francois and Wolz, 2021). This significance comes from the suggestion that constitutive *vra* regulon expression results from deletion of the putative membrane protein VraT. It was suggested that cell wall damage alters the VraT-VraS connection to affect the activity of the VraS kinase (Bleul et al., 2021).

1.12.2 GraRS

The GraRS TCS (sometimes termed ApsSR) is a resistance system to many cationic antimicrobial peptides (CAMPs) and vancomycin (Bleul et al., 2021). GraRS showed sensory activity to AMPs such as nisin, however, not to daptomycin and gentamycin antibiotics (Rapun-Araiz, Haag, Solano and Lasa, 2020). GraRS is an IM-HK and primarily regulates the expression of *vraFG*, *mprF*, and *dltABCD* genes (Yang et al*.,* 2012). Although the mechanism of GraRS activation still requires further investigation, the TCS controls the expression of the *dlt* and *mprF* genes that encode modifying enzymes that alter the negative potential on the

surface of the cell wall, which reduces the ability of positively charged antibiotics to adhere to bacteria and reduces antimicrobial impact (Wu et al., 2020).

1.12.3 BraRS

Bacitracin resistance associated system (BraRS or BceRS), sometimes also termed nisin susceptibility associated (NsaRS) is an important TCS that responds to bacteriocins, particularly bacitracin, nisin and nukacin ISK-1 (Kawada-Matsuo et al., 2013; Arii et al., 2019). BraS is a member of the IM-HKs, and skin-colonising staphylococci have of the BraRS TCS homologues (Coates-Brown et al., 2018). The BraRS resistance pathway in the case of bacitracin or nisin involves the ABC transporters of BraDE and VraDE. BraDE is essential for the detection of antibiotics while VraDE acts as an efflux pump to provide resistance (Ahmad, Majaz and Nouroz, 2020). Although single nucleotide polymorphisms (SNPs) associated with *braRS* can indicate nisin resistance, other bacteriocins can also activate the expression of BraR (Bleul et al., 2021). The BraRS system in *S. aureus* is also associated with resistance to nukacin ISK-1 antimicrobial that is produced by *S. warneri* (Kawada-Matsuo et al., 2013).

1.12.4 SaeRS

In *S. aureus*, the SaeRS TCS controls the expression of several virulence factors and pathogenicity (Haag and Bagnoli, 2015). HK SaeS belongs to the IM-HK class and similar other TCS, SaeRS is activated by phagocytosis-related signals among others (Mascher, 2006; Geiger et al., 2008). Human immune response effectors such as hydrogen peroxide, human neutrophil peptides (HNPs) and calprotectin activate SaeS (Wittekind et al., 2022). In the *S. aureus* USA300 strains, SaeS activation was verified with subinhibitory concentrations of HNP1-3. (Flack et al., 2014). However, other related *S. au*reus strains did not show any HNP1-3 sensing that could lead to SaeS activation (Geiger et al., 2008). Although SaeR interactions

with other regulators are mostly unclear, *agr* mutants may be able to reduce *sae* expression (Bleul et al*.,* 2021).

1.12.5 WalRK

The WalRK (sometimes termed YycGF or VicSR) system has been reported to be associated with cell wall metabolism (Liu et al., 2016). Accordingly, this system effectively regulates autolysis, virulence factors, and biofilm formation. Vancomycin-resistant *S. aureus* (VISA) was linked with the function of WalRK TCS. SNPs in WalRK genes are frequently found in clinical VISA as well as resistant strains selected in vitro (Machado et al., 2021). The HK WalRK gene was discovered in *Bacillus subtilis* and encodes four genetic factors, *walRKHI*, that seems not to be autoregulated. It has been reported that in *S. aureus*, WalRK contains an intracellular and extracellular PAS domain likely involved in sensing and associated with Zn^{2+} binding (Kim et al*.,* 2016; Bleul et al., 2021). Although the regulation mechanism of WalK remains unclear in *S. aureus*, disruption of two proteins called *yycH* and *yycI* that are transcribed with *walKR* caused downregulation of WalRK regulon. Another protein SpdC has also been described to interact with the regulation of WalK (Cameron et al*.,* 2016; Poupel et al*.,* 2018).

1.12.6 Agr

The *S. aureus* accessory gene regulator (Agr) system comprises two divergent transcriptional units, RNAII and RNAIII, driven by promoters P2 and P3. The P2 RNAII transcript contains the operon *agrBDCA.* P3 drives transcription of the riboregulator RNAIII. AgrA and AgrC comprise a two-component system that responds to the secreted autoinducing peptide (AIP). The AIP is processed from the *agrD* product by AgrB (Figure 1). Signalling via the Agr system is modulated in concert with other regulatory elements, such as SarA (Cheung, Bayer and

Heinrichs, 1997). AgrA and SarA increase transcription from both the P2 and P3 promoters, resulting in elevated intracellular concentrations of RNAIII that act to raise the expression of secreted virulence factors (Wang et al., 2007; Queck et al., 2008; Wang and Muir, 2016; Hardy et al., 2019). Since Agr regulates *S. aureus* virulence factor expression that plays a key role in the development of skin infections (Cheung et al., 2011), there have been many studies into Agr dysfunction to reduce the virulence of staphylococci. (Yarwood and Schlievert, 2003; rackman and Coenye, 2015; Le and Otto, 2015).

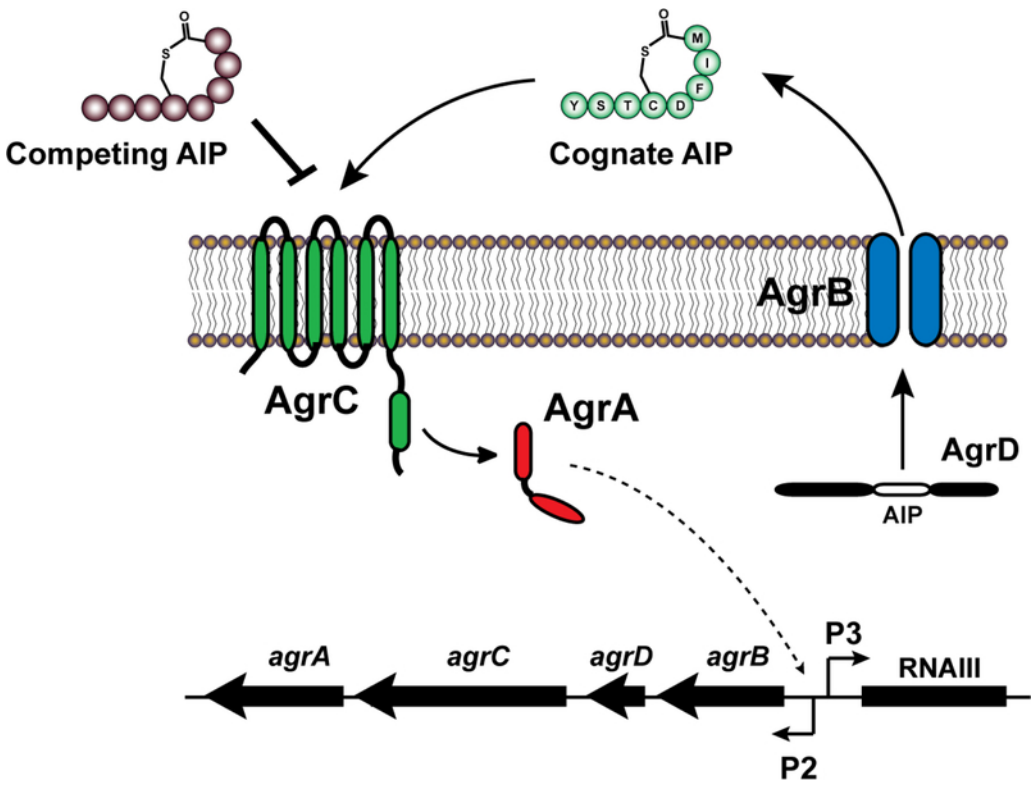


Figure 1. 3. *S. aureus* Agr QS TCS. Autoinducing peptide (AIP) is cleaved from AgrD and transported out of the cell by AgrB. AIPs are detected by sensor AgrC that autophosphorylates to phosphorylate the AgrA regulator, which activates the P2 and P3 promoters, encoding the Agr operon. RNAIII activates virulence factor production and represses expression of surface adhesins (Rutherford and Bassler, 2012; Figure taken from Parlet et al., 2019).

1.12.6 DesKR

DesKR is a relatively uncharacterised TCS in *S. aureus* and sometimes termed TCS-7. In *B. subtilis*, DesKR TCS plays a significant role in the response to temperature sensing and modifies membrane lipid through a regulated desaturase activity that is absent from *S. aureus*. Recent studies in *S. aureus* establish that DesKR is involved in temperature sensing and DesK likely functions similarly to that of *B. subtilis* but its associated roles with adaptation to lower temperature conditions is unclear. (Fernández et al., 2020; Bleul, Francois et al., 2021).

1.13 antiSMASH and secondary metabolites

Bacteria and fungi create many chemicals and other bioactive substances that they use in a variety of ways to defend themselves against other species (Newman and Cragg, 2020). The secondary metabolites released by microorganisms have emerged as an important source of antimicrobials (Takano et al., 2015). The genes that code for the biosynthetic pathway that produces such a secondary metabolite are frequently clustered together at a certain location on the chromosome, and this group of genes is known as a secondary metabolite biosynthetic gene cluster (BGC) (Blin et al., 2019). As the cost of sequencing bacteria and fungi has fallen dramatically in recent years and many more genome sequences have become available, a software tool called antiSMASH has begun to be used. This tool, which was established in 2011, can detect gene clusters that code for secondary metabolites in bacterial and fungal genomes (Medema et al., 2011; Introduction-antiSMASH Documentation, 2022). antiSMASH recognises a wide range of BGCs, counting those generating polyketide (PK) pathways, nonribosomal peptides (NRPs), terpenoids, ribosomal peptides, aminoglycosides, and non-NRP siderophores. PKs and NRPs are produced via megasynthase enzymes which include many protein domains, such as adenylation (A), condensation (C) and PCP-binding domains in nonribosomal peptide synthetases (NRPSs), acyltransferase (AT), and ketosynthase (KS),

and ACP-binding domains in polyketide synthases (PKSs). AntiSMASH has a library of pHMMs that can identify all these protein domains as well as discriminate between different subtypes. The antiSMASH output shows the domain structures of any NRPSs or PKSs that are encoded in a gene cluster. Each domain provides different analysis options. For example, some domains of NRPSs can show the antimicrobial similarity that found in the gene cluster (Medema et al., 2011; Blin et al., 2021).

1.14 Experimental evolution

Experimental evolution is a technique that can be used to investigate the evolutionary process. This includes measuring the fitness costs of the mutations that cause antibiotic resistance and determine the rate and probability of the evolution of resistance (Cooper, 2018; McDonald, 2019). As antibiotic resistance is the result of evolutionary processes, experimental evolution offers a powerful tool for researchers to study evolutionary processes in real time, including mutation as well as how bacteria develop (Kawecki et al., 2012; Barbosa et al., 2017). Although evolution can be established in a laboratory, it can also be observed when populations adapt to new environmental conditions through natural selection and adaptations. Among these natural selections, there can be a dynamic evolutionary process that helps organisms adapt to their environment, such as population increases during that process. Natural selections can also be a phenotypic feature that has a functional role in each individual organism (Bozdag et al., 2023; Chavhan et al., 2023; Guo et al.,2023; Santos et al.,2023). In experimental evolution, adaptation can occur in different ways. First, when an organism acquires a new beneficial mutation. Second, it can be from changes in allele frequency in genetic variation that have been present in an organism's population (Long et al., 2015). In addition to mutation and natural selection, genetic drift and gene flow are two additional evolutionary mechanisms that may contribute to or be included in experimental studies of evolution (Kawecki et al., 2012). Evolutionary changes in organismal characteristics can happen slowly or quickly (Barrick and

Lenski, 2013). Historically, experimental evolution was developed over many years and first used by Cleric Dallinger in the 1870s to continuous culture experiments at slowly rising temperatures (O'Malley et al., 2015). Nine FX174 isolates were evolved and sequenced in 1997 (Bull et al*.,* 1997). In 2007, the genome of an evolved *Myxococcus xanthus* was successfully sequenced (Velicer et al*.,* 2006). In 2009, whole genome sequencing of evolved *Escherichia coli* samples was prepared to investigate genetic polymorphisms (Barrick and Lenski, 2009). Recent studies of mutation rates and genome evolution have benefited from whole-genome sequencing, which has provided new clarity and insights. DNA sequencing technology has made it possible to detect genetic differences between ancestral and derived organisms on the entire genome scale of all species (Barrick and Lenski, 2013).

Although evolution in natural populations is difficult to understand due to the large number of elements that can impact the outcome of evolution, controlled experimental evolution in the laboratory usually begins with populations cloned from a model organism that obtains a well-characterized genetic starting point (Barrick and Lenski, 2013; McDonald, 2019). Evolution experiments between microorganisms can be prepared in different ways. Most experiments, however, begin by culturing microbes in a medium and incubating them until they reach a high population density. The microbes that transferred to the new culture medium to allow continued growth. This procedure can be repeated indefinitely, thus accumulating generations. Natural selection will prompt microbes to adapt to new laboratory conditions (McDonald, 2019). Interactions and competition through toxins between bacteria often lead to evolution (Kawecki et al., 2012; Gorter et al., 2021). Thus, coculture with other species is one technique of experimental evolution that, when compared to monoculture, might lead to the discovery of novel genetic functions (McDonald, 2019).

1.15 Illumina

Illumina is a technology that analyses genetic function and variation and provides a different way to generate DNA fragments. Studies that were not even possible a few years ago are now possible because of this tool (Petersen et al., 2019). With fluorescent-labeled reversible terminator technology, single base substitutions (SBS) are the foundation of the llumina next-generation sequencing (NGS) method (Mardis et al., 2013; Hu et al., 2021). Sequencing from both ends of a DNA fragment (paired-end sequencing) is possible with Illumina NGS platforms. This technique produces high-quality sequence data with wide coverage and high numbers of reads. With an error rate of just 0.1%, Illumina is one of the most accurate sequencing methods. The long run times of Illumina platforms are one of their disadvantages; for example, MiSeq utilising a reagent kit version 3 with a read length of 2 x 300 bp can take up to 56 h of total sequencing time. Modern models, such as MiniSeq, NextSeq 550, and NovaSeq 6000, can speed up data processing while retaining high quality and accuracy (Hu et al., 2021). As an advantage, NGS has been reported to be less expensive than other conventional approaches such as Sanger (Bahassi et al., 2014). The short read sequences that NGS can provide have some limitations, including longer running times and difficulties with *de novo* assembly. On the other hand, short-read sequencing has the benefit of having many computational tools created specifically for short read data analysis. Another benefit of short read sequences is that they are more accurate than long read sequences (van Dijk et al., 2018; Hu et al., 2021).

1.16 Aims of thesis

A key rationale for this study comes from the treatment challenges that *S. aureus* infections create for the medical community, notably skin infections, and their difficulty in being cured. There is an urgent need for new approaches that can overcome the mechanisms of *S.*

aureus resistance that could aid the development of new antibiotics. Equally important, investigation of microbial competition is also significant in this study. Our understanding of the ability to use and resist antimicrobials in the natural environment broadens our knowledge of both the mechanisms employed during competition and the potential avenues for treatments and understanding resistance mechanisms. The aims of this study were to investigate the genetic evolution and resistance mechanisms of *S. aureus* against secreted antimicrobial producers that are isolated from coagulase-negative staphylococcus (CoNS) species with novel biosynthetic gene clusters (BGS) using experimental evolution of interference competition and genome sequencing to identify single nucleotide polymorphisms (SNPs) associated with resistance genes. Understanding the genetic drivers of competition evolution in the *S. aureus* niche will help to limit global growth in bacterial antimicrobial resistance.

1.16.1 Specific research aims:

- To screen CoNS species for their ability to produce antimicrobial compounds that inhibit the growth of different staphylococci from the same niche to be used later for experimental evolution. Additionally, to study the experimental evolution of interference competition between antimicrobial producer *capitis* 47 and *S. aureus* strains184 and SH1000, separately.
- To study the experimental evolution of interference competition among additional CoNS species that included the interference competition between the antimicrobial producer *S. epidermidis* IVK83 and *S. aureus* USA300, and between the antimicrobial producer *S. lugdunensis* 1007 and *S. aureus* USA300.
- Develop a model for competition with resident and invading species of *S. aureus* in the presence and absence of co-resident species of bacteria from the same niche to determine the interference effect of co-resident *S. epidermidis* expressing specific

antimicrobial activity on the stability of resident *S. aureus* from invading strains of *S. aureus* expressing the same or different Agr group AIPs.

Chapter 2: Methods and Materials

2.1 Bacterial strains

Strains were obtained from the culture collection of the *Staphylococcus* Research Group, University of Liverpool. Strains were cultured on BHI agar plates then overnight cultures (O/C) were made by inoculating one colony in a 30 ml sterile universal tube containing 5 ml of BHI, incubated aerobically at 37^0C for 24 h with shaking at 150-200 rpm. Strain stocks were generated by mixing 700 µl of O/C with 300 µl of glycerol (G/0650/17) 50 % v/v and stored at -80 ^{0}C to be used later. Bacterial strains with publication references are listed in Table 2.1.

Table 2. 1. Bacterial strains used in this research study.

Species	Strain ID	Relevant characteristics	References
S. aureus	SH1000	Wild type	(Horsburgh et al., 2002)
S. aureus	BEN032	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	BEN34	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	BEN44	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	BEN130	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	BEN137	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	BEN184	Wild type; nasal isolate	(Libberton et al., 2014)
S. aureus	USA300	Wild type; clinical isolate	(Montgomery et al., 2009)
S. capitis	38	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	47	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	50	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	55	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	56	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	58	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	62	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	65	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	66	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	85	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	86	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	88	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	104	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	108	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	DSM6717	Wild type; scalp isolate	(Chong et al., 2022)
S. capitis	NCTC11045	Wild type; scalp isolate	(Chong et al., 2022)
S. epidermidis	BEN04	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	14	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	55	Wild type; nasal isolate	(Libberton et al., 2014)

S. epidermidis	50	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	155	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	180	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	273	Wild type; nasal isolate	(Libberton et al., 2014)
S. epidermidis	IVK83	Wild type; nasal isolate	(Torres et al., 2023)
S. epidermidis	IVK83 *ΔefiTP*	epifadin mutant	(Torres et al., 2023)
S. epidermidis	Tü3298	Wild type; nasal isolate	(Ebner et al., 2018)
S. epidermidis	039331N1	Wild type; ocular isolate	(Ghabban, 2019)
S. epidermidis	23936-048204N1	Wild type; ocular isolate	(Ghabban, 2019)
S. epidermidis	23954-039174N1	Wild type; ocular isolate	(Ghabban, 2019)
S. epidermidis	039331N1	Wild type; ocular isolate	(Ghabban, 2019)
S. lugdunensis	166	Wild type; nasal isolate	(Libberton et al., 2014)
S. lugdunensis	229	Wild type; nasal isolate	(Libberton et al., 2014)
S. lugdunensis	189	Wild type; nasal isolate	(Libberton et al., 2014)
S. lugdunensis	250	Wild type; nasal isolate	(Libberton et al., 2014)
S. lugdunensis	G310	Wild type; foot isolate	(Unilever, 2014)
S. lugdunensis	1003- N920143	Wild type; breast abscess isolate	(Mitchell et al., 2004)
S. lugdunensis	1004-N930432	Wild type; endocarditis isolate	(Mitchell et al., 2004)
S. lugdunensis	1005-N940025	Wild type; finger pulp infection isolate	(Mitchell et al., 2004)
S. lugdunensis	1006-N940084	Wild type; finger pulp infection isolate	(Mitchell et al., 2004)
S. lugdunensis	1007-N940113	Wild type; vertebral infection isolate	(Mitchell et al., 2004)
S. lugdunensis	1008-N940135	Wild type; perineal infection isolate	(Mitchell et al., 2004)
S. lugdunensis	1009-N940164		(Mitchell et al., 2004)
S. lugdunensis	1010-N950646		(Mitchell et al., 2004)
S. lugdunensis	211421	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211482	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211491	Wild type; ocular isolate	((Somerville, 2022)
S. lugdunensis	211493	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211551	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211589	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211633	Wild type; ocular isolate	((Somerville, 2022)
S. lugdunensis	211681	Wild type; ocular isolate	(Somerville, 2022)
S. lugdunensis	211704	Wild type; ocular isolate	(Somerville, 2022)

2.2 Preparation of media

Brain Heart Infusion Broth (BHI) medium (Neogen, NCM0016A) was prepared according to the manufacturer's instructions at 37g L^{-1}. LB broth medium (Millipore, VM782285 722) was prepared by dissolving 25g in 1 L of distilled water. BHI agar plates were produced with 15g L^{-1} of agar (Greiner A/1080/530). Trehalose dihydrate sugar-agar (TDSA) was prepared by mixing 2 g of peptone, 2 g of meat extract, 2 g of sodium chloride, 4 g of Trehalose, 0.025 g of phenol red and 15 g of agar in 1 L of distilled water. All the media were autoclaved at 121^0C for 20 min.

2.3 Preparation of stock solutions

100 ml of crystal violet (CV) (C0775-100G) stock solution (0.1% w/v) was made by dissolving 100 mg of CV in 100 ml of distilled water. Stocks of primers for amplification of 16S DNA and *Staphylococcus* Agr types were prepared by mixing 10 µl of each primer stock with 90 µl of filtered water (10 pmol μl^{-1}). PBS (Merforo, P32080-100T) was prepared by dissolving 1 tablet in 100 ml of distilled water. 1% w/v glucose stock was prepared by mixing 0.5 g of glucose (Fisher, 10/1826134) with 50 ml of distilled water.

2.4 Agarose gel electrophoresis

Agarose gels were prepared with 1 g of agarose (Geneflow, AGD1-500GM) in 100 ml of 1-x of TAE. TAE buffer was prepared as a 50-x stock solution containing 242 g of Tris base, 18.61 g of EDTA and 57.1 ml of acetic acid in 1 L of distilled water. The mixture was then heated in a microwave at full power for 3 min to melt the agarose, cooled to 50 ^{0}C and 2 µl of Midori green DNA Stain was added and mixed. The mixture was poured into a gel tray and cooled prior to electrophoresis.

2.5 DNA extraction

DNA was extracted by using DNeasy Tissue Kits (69506) according to its manufacturer. Bacterial strains of interest were inoculated into BHI medium for O/C. Next 700 µl of O/C was centrifuged in a sterilised Eppendorf tube for 10 min at 7500 rpm. Supernatant was discarded and 180 µl of enzymatic lysis buffer with 2 µl lysostaphin (5 1000 U ml $^{-1}$) and 5 µl mutanolysin (2.5 mg ml $^{-1}$) were added to the bacterial pellets, mixed gently, and incubated for 1 h at 37^{0}C. After that, 25 µl of proteinase K, 200 µl of buffer AL and 18 µl of RNase was added, vortexed and incubated for 1 h at 56^{0}C. Next, 200 µl of 99% (v/v) ethanol was added and mixed gently, the mixture after was then transferred onto the DNeasy Mini spin column and centrifuged for 1 min at 8000 rpm.

In the following step, 500 µl of AW1 buffer was added to the column, centrifuged for 1 min at 8000 rpm and the collection tube replaced. AW2 (500 µl) buffer was added to the column, centrifuged for 3 min at 14000 rpm, replacing the collection tube and the column was dried by centrifuging for 2 min. To elute the DNA, 100 µl of distilled water was added directly onto the spin column membrane and centrifuged for 1 min at 8000 rpm. The DNA concentration was measured by using a NanoDrop-1000 and high sensitivity Qubit-Fluorometer (Q32854) then DNA was stored at -20 ^{0}C to be used later in PCR (see below).

2.6 Genome Sequencing

One clone was selected from each separate biological replicate (three in total) to be sequenced. Cultures of each strain were grown in BHI for O/C. Cells were harvested to obtain 5x10^{9} cells and washed with PBS then resuspended in DNA shield (inactivation buffer provided by MicrobesNG added prior to cells being sent for sequencing. The Microbes NG protocols used to isolate the DNA are produced here from their literature for completeness: Five to forty microlitres of cell suspension were lysed with 120 µl of TE buffer containing lysozyme (final

concentration 0.1 mg mL^{-1}) and RNase A (ITW Reagents, Barcelona, Spain) (final concentration 0.1 mg mL^{-1}), incubated for 25 min at 37°C. Proteinase K (VWR Chemicals, Ohio, USA) (final concentration 0.1 mg mL^{-1}) and SDS (Sigma-Aldrich, Missouri, USA) (final concentration 0.5% v/v) were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer (Qiagen, Germany) (MicrobesNG., 2022). Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with modifications: input DNA was increased 2-fold, and PCR elongation time increased to 45 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries were sequenced using Illumina sequencers (HiSeq/NovaSeq) using a 250 bp paired end protocol (MicrobesNG, 2022).

2.7 PCR of extracted DNA

Primers for 16S rDNA targets were used to amplify the DNA of strains of interest for species identification. (Table 2.2). For amplification, 25 µl of Biomix red, 22 µl of filtered water, 1 µl of primer PA,1 µl of primer PH that designed by Alrajeh (2021) and 1 µl of DNA was mixed in one PCR Eppendorf tube and vortexed. In the next step, the samples were cycled with the following conditions: denaturation at 95^0C for 5 min, followed by 30 cycles 95^0C for 1 min, 55 ^{0}C for 1 min, 72^0C for 1.5 min and for the final extension at 72 ^{0}C for 7 min. PCR samples were stored at -20^0C.

Table 2. 2. Primers used to amplify 16S rDNA.

Primer	Primer Sequences
PA	5- 'AGA GTT TGA TCC TGG
PH	5- 'AAG GAG GTG ATC CAG

2.8 Purification of PCR product

PCR sample (20 µl) was loaded onto an agarose gel and electrophoresed at 135 V and 400 mA for 45 min. DNA bands of the correct size were excised using a UV transilluminator with a long wavelength (365 nm) to minimise the damage. The DNA bands were cut from the gel and weighed to determine the needed volume of PCR and Gel kit (Bio-52060). 200 ul of Binding Buffer Binding Buffer (CB) was needed for each100 mg gel. Gel and CB buffer was mixed and heated at 50 ^{0}C for 10 min to allow the gel to dissolve. Samples were transferred to a spin column membrane, centrifuged for 30 s at 11,000 rpm and the supernatant was discarded. Then 700 µl of wash buffer CW was added, centrifuged for 30 s at 11,000 rpm and the supernatant was discarded. After that, the membrane was centrifuged for 1 min at 11,000 rpm to dry. The DNA was then collected by adding 25 µl of distilled water onto the spin column membrane and left for 1 min at room temperature then centrifuged at 11.000 rpm for 1 min.

2.9 Agr Typing PCR

The Agr gene sequence of *S. aureus* type was determined by PCR (using the method above with modifications). Primer sequences used to identify each Agr type are listed in Table 2.3 (Peacock et al. 2002). PCR was performed using a thermal cycle of 94°C for 1min, followed by 35 cycles of 94°C for 1 min, 50°C for 1min, 72°C for 3 min, followed by a final elongation step of 72°C for 10 min. Agr typing was achieved by amplifying any type of Agr in the DNA of the interested strains.

Table 2. 3. Primers utilised in Agr typing PCRs

Locus	Primer	Primer Sequences (5'-3')
Allotype 1	AGR1-F	ATC GCA GCT TAT AGT ACT TGT
	AGR1-R	CTT GAT TAC GTT TAT ATT TCA TC
Allotype 2	AGR2-F	AAC GCT TGC AGC AGT TTA TTT
	AGR2-R	CGA CAT TAT AAG TAT TAC AAC A
Allotype 3	AGR3-F	TAT ATA AAT TGT GAT TTT TTA TTG

	AGR3-R	TTC TTT AAG AGT AAA TTG AGA A
Allotype 4	AGR4-F	GTT GCT TCT TAT AGT ACA TGT T
	AGR4-R	CTT AAA AAT ATA GTG ATT CCA ATA

2.10 Deferred growth inhibition assay

The method of Moran et al., 2016 was used to identify competitive antagonism from secreted antimicrobials. Briefly, 25 µl of overnight cultures (O/C) of each test strain was spotted in the middle of 15 ml of BHI agar plate and incubated at 37^0C for 24 h. The next day, the plate was sprayed in a sterilised chamber, using sterilised sprayer with a regular volume of indicator O/C strain that was diluted at 1:10 in BHI. The plate was then incubated at 37^0C for 24 h and the zone around the inhibitory strain was recorded, typically ranging in diameter from 0 to 8.5 mm.

2.11 Transwell biofilm assay for bacterial competition

Bacterial strains were cultured in a pre-sterilised 12-well (manufacturer, Greiner bio-one, LOT E17043R3) flat bottom plate in triplicate. Briefly, 50 µl of each O/C amended to OD = 0.5 in PBS was inoculated in the bottom of the well (strain B), which contained 1 ml total volume of BHI medium supplemented with 1% (w/v) glucose and 4% (w/v) NaCl (BHI-GN). In the next step, a pre-sterilised 0.4 µm transwell membrane insert (Greiner bio-one, LOT18380116) was added to each well by using sterilised tweezers. All strains were cultured with OD = 0.5 in PBS and different volumes of O/C were required to conduct each type of competition. For competition between two strains, 50 µl of O/C was inoculated up on the membrane of strain A and 50 µl of O/C was inoculated at bottom reservoir of strain B. For competition among three strains, 100 µl of O/C, which contains mixed two strains, (50 µl from each strain) was inoculated up on the membrane (strain A and C) and 50 µl was inoculated at bottom reservoir (strain B). Competition control of two strains was performed by inoculating 100 µl of O/C

divided into 50 µl on the membrane and 50 µl in the bottom reservoir of the same strain. Competition control among three strains was made by inoculating 150 µl of O/C divided into 100 µl on the membrane and 50 µl in the bottom reservoir of the same strain. Strains were inoculated in separate wells with a membrane that contained 1 ml total volume of the BHI-GN (table 2.4). After incubating transwell plates at 37^0C for 24 h to allow biofilm growth of the strain (B), biofilm production in the bottom well of the plate was determined by a crystal violet assay (CV) assay as described below.

Table 2. 4. Bacterial culture volume utilised in 12-well transwell biofilm assay

Competition	Top reservoir (above the membrane)	Bottom reservoir
Competition between two strains (Total 100 µl)	50 µl from strain A	50 µl from strain B
Control competition between two strains (Total 100 µl)	50 µl from strain B	50 µl from strain B
Competition among three strains (Total 150 µl)	50 µl from strain A + 50 µl from strain C	50 µl from strain B
Control competition among three strains (Total 150 µl)	100 µl from strain B	50 µl from strain B

2.12 Crystal violet (CV) assay for bacterial competition

After incubating cultured microwell plates at 37^0C for 24 h, the transwell membrane insert and growth medium were removed from each well. The wells were then washed twice with 1 ml of PBS by shaking at 100 rpm for 5 min. Next, 500 µl of CV solution (0.1% w/v) was added to each well, including the control wells. After 1 h at room temperature, the dye was removed, and the wells were washed twice with 1 ml of PBS, then dried and 200 µl of ethanol was added to each well with shaking at 100 rpm for 2 min. In the penultimate step, 200 µl of the ethanol/dye was transferred from each well to a 96-well microtiter plate and diluted 1:1 with ethanol. Finally, biofilm density was quantified by determining CV absorbance, at an OD_{550}, using a 96- well plate reader (Fluostar Omega).

2.13 Viable counting from transwell biofilms

Briefly, bacterial strains were cultured in 12-well transwell biofilm assay with 0.4 µm membrane (see above) for 24 h, then the cultured strains in the bottom of the well were removed and the adherent biofilm here transferred and mixed into sterile tubes containing 9 ml of PBS. After that, 100µl of cell mixture was serially diluted with an additional 900 µl of PBS in Eppendorf. Next, BHI-agar plates were divided into 4 sections with a pen and 10 µl of each diluted biofilm was pipetted in triplicate (3x10 µl) onto the surface of the agar in each section. In the penultimate step, the plates were dried and incubated at 37^0C overnight. Finally, an appropriate dilution was found, and the mean viable count recorded as colony-forming unit/millilitre (CFU ml^{-1}).

2.14 Experimental evolution on BHI agar

Bacterial strains were cultured on BHI agar and incubated at 37^0C for 24 h. The next day, the cultured cells were scraped off and suspended in 10 ml PBS by vortexing. After that, the suspended culture was diluted to OD = 0.5 in PBS and two chosen strains were mixed at ratio of 1:1 as a coculture. In next step, 50 µl of the coculture mixture was spotted on 25 ml of BHI agar. Control of each strain was created as monoculture and incubated with the test at 37^0C for 24 h. On the next day, the cell-spots were scraped using a sterile loop and resuspended in 10 ml PBS and transferred to a new BHI agar plate. This process was repeated every 24 h for a period of 8 d. Viable counts of each species in the competition were calculated daily and colonies were differentiated by morphology and pigmentation with confirmation using media that could discriminate species by carbohydrate fermentation. Finally, the deferred growth inhibition assay as previously described was used to test the selected clones for their resistance to the inhibitor producer.

2.15 Sequence analysis of selected clones from experimental evolution

The DNA sequences of clones picked from selection experiments and their parental wild type (WT), or matching day of experimental selection were received from the Centre for Genome Research (CGR), University of Liverpool or Microbes NG, Birmingham and analysed through several steps. The sequences were trimmed by the companies using Trimmomatic tool to remove the adapters from the reads. First, reads were download from the CGR website: http://cgr.liv.ac.uk/illum/LIMS21613_cd7706e1e12862e7/#download or Birmingham website https://microbesng.com/portal/projects/ to the computer then uploaded directly to Terminal by signing in FilZilla app. Sequence read files used included *_R0.fastq.gz, *_R1.fastq.gz and *_R2.fastq.gz files. R0 is a single read file that contains sequences whose pair has been removed due to poor sequence quality while R1 and R2 contain the corresponding paired-end sequences of length 15 to 151 base pair (bp). FastQC tool was used to examine the quality of the reads, which provides a modular set of analysis and gives an impression of whether the data has any quality problems. The main object of this step was to import data from FastQ files to detect any variant sequence. After examining FastQC quality files, the reads were assembled using Unicycler tool (version, 0.4.8) (Wick et al., 2017) which take Illumina paired end reads from the bacterial isolate output and assembled them. Next, Prokka (version 1.14.6) (Seemann, 2014) tool was used to annotate the genes of the assemblies and identify coding sequences. Burrows-Wheeler aligner (BWA) software (version 0.7.12) (Abuín et al.; 2015) was used to map the reads to WT and show confirmed similarity. Matching to WT within less than ~ 90 % was considered as contamination. The percentage mapping at this point ended at ~ 99 % for all samples compared to their WT. Finally, snippy program (version 4.6.0) (Seemann, 2015) was used to find the single nucleotide polymorphism (SNP) and the short indels variants between the WT and the experimentally evolved isolate reads.

2.16 Data presentation and statistical analysis

Graph Pad Prism 9.4.1 software, 2022 U.S.A was used, and based on the appearance of the graphs, a t-test analysis was applied to detect a significant difference. Probability (p) values < 0.05 were considered as statistically significant for biofilm formation ability after culture. While (p) values < 0.001 were considered as statistically significant for competition experiments. A star (*) was placed next to each p-value that was less than 0.05, two stars (**) flag was displayed for less than 0.01, and less than 0.001 was flagged with three stars (***).

Chapter 3: Antimicrobial activity of coagulase-negative *Staphylococcus* (CoNS) species and selection for resistance in *S. aureus*

3.1 Introduction

Human skin can be populated by a highly diverse community of bacteria that interact and compete for the purpose of colonisation and survival (Brown and Horswill, 2020). Competition between bacterial species can be experienced when they compete for a common nutrient or interfere by releasing specialised metabolites that may kill or inhibit related microbes, such as antimicrobial peptides (AMPs) (Wang, Gu, Breukink, 2020). Coagulase-negative staphylococci (CoNS), including *S. epidermidis, S. capitis, S. hominis, S. haemolyicus and S. lugdunensis,* are among the most prevalent species on healthy skin. CoNS can inhibit the expression of *S. aureus* virulence factors, thus limiting its pathological colonisation (Parlet, Brown and Horswill, 2019). The production of AMPs can strongly impact the ability of one species to inhibit another. The deferred growth inhibition assay is one method to identify production of antimicrobial compounds by in a test strain. This method was used to show a positive correlation between the presence of inhibitor-producing species and the absence of *S. aureus* in a nasal community (Libberton et al., 2014). The assay can be used to create quantitative (zone of clearing size) and or qualitative (visual pattern of inhibition) data, both of which can be overlaid onto the presence or absence of a species within a community to find correlations between species (Moran et al., 2016). Although the inhibitor strain requires overnight growth period to produce inhibitory compounds in this assay (Moran et al., 2016), other methods do not allow that period for production with the inhibitor strain (Krismer et al., 2014; Zipperer et al., 2016). Studies of microbial competition between *S. aureus* and those CoNS that produce antimicrobials can increase our understanding of species dynamics and how species interactions might contribute to evolution of antibiotic resistance with impacts on human health (Götz et al. 2014; Lynch et al., 2019). AMPs that are produced by CoNS target

various components and pathways in the bacterial cell through a variety of mechanisms. *S. aureus* can use two component systems (TCS) to adjust their genetic response to changes in their environment including AMPs, which helps them survive in bacterial communities (Bleul, Francois and Wolz, 2021). Although the human skin microbiome is becoming more common as a source of competitive strains and novel antimicrobials (O'Sullivan et al., 2018), little research has been done to study the genetic drivers of human skin microbiota in the evolution of competition and resistance to AMPs. Agar-based investigations and genomics analyses have shown that *S. capitis* strains can produce antimicrobials such as J Nisin or capidermicin (O'Sullivan et al., 2020; Lynch et al., 2021; Chong et al., 2022). However, these are still not well investigated regarding their mode of action in the *S. aureus* niche. Understanding the evolution of antimicrobial resistance (AMR) phenotypes and resistance mechanisms, particularly *S. aureus* resistance mechanisms, might help in limiting global growth in bacterial antimicrobial resistance (Jensen and Lyon, 2009; Yılmaz and Aslantaş, 2017). Whole genome sequencing (WGS) has evolved into a powerful technique for tracking antimicrobial resistance (AMR) genes and mobile genomic elements and providing critical information (Ellington et al*.,* 2017; Oniciuc et al*.,* 2022).

3.2 Aims

The aim of this chapter was to screen antimicrobial-producing CoNS and use whole-genome sequencing to identify the mutations responsible for resistance to antimicrobial products. In addition, specific focus was placed on competition between *S. capitis* 47 that produces an undescribed antimicrobial and *S. aureus*. Recent study of *S. capitis* genomes identified antimicrobial gene clusters that have not yet been investigated (personal communication Chong, 2022). The hypothesis proposed is that *S. aureus* will develop resistance to the antimicrobial produced by *S. capitis* 47.

3.3 Results

3.3.1 Screening for antimicrobial activity

Coagulase-negative *Staphylococcus* (CoNS) isolates were screened for their ability to produce antimicrobial activity that targeted *S. aureus* and *S. epidermidis*. The antimicrobial activity was identified in this study using a deferred growth inhibition assay (Moran et al., 2016). In the assay, a test strain was cultured as a spot of overnight culture on a BHI agar plate and incubated at 37^0C for 24 h. The next day, the plate was sprayed with an indicator *Staphylococcus* strain (approx. 10^8 cfu ml^{-1}) to identify inhibitor production by the test strain after overnight incubation (Section 2). For this analysis, 48 isolates from nose, scalp, or eye (Table 2.1) including *S. epidermidis*, *S. lugdunensis* and *S. capitis* were tested for their antimicrobial production against eight *S. aureus* strains. Known and unknown antimicrobial producers were included in the test strain panel with *S. epidermidis* strains: Tü3298 epidermin producer (Ebner et al., 2018), B155 (Ghabban, 2019) and IVK83 (Torres Salazar et al., 2023) epifadin producers plus B14, 273 and 039331N1 as unknown antimicrobial producers (Alrajeh, 2021). Antimicrobial activities of the test strains were quantitatively recorded by measuring zones of clearing of varying sizes in millimetres (Fig 3.1).

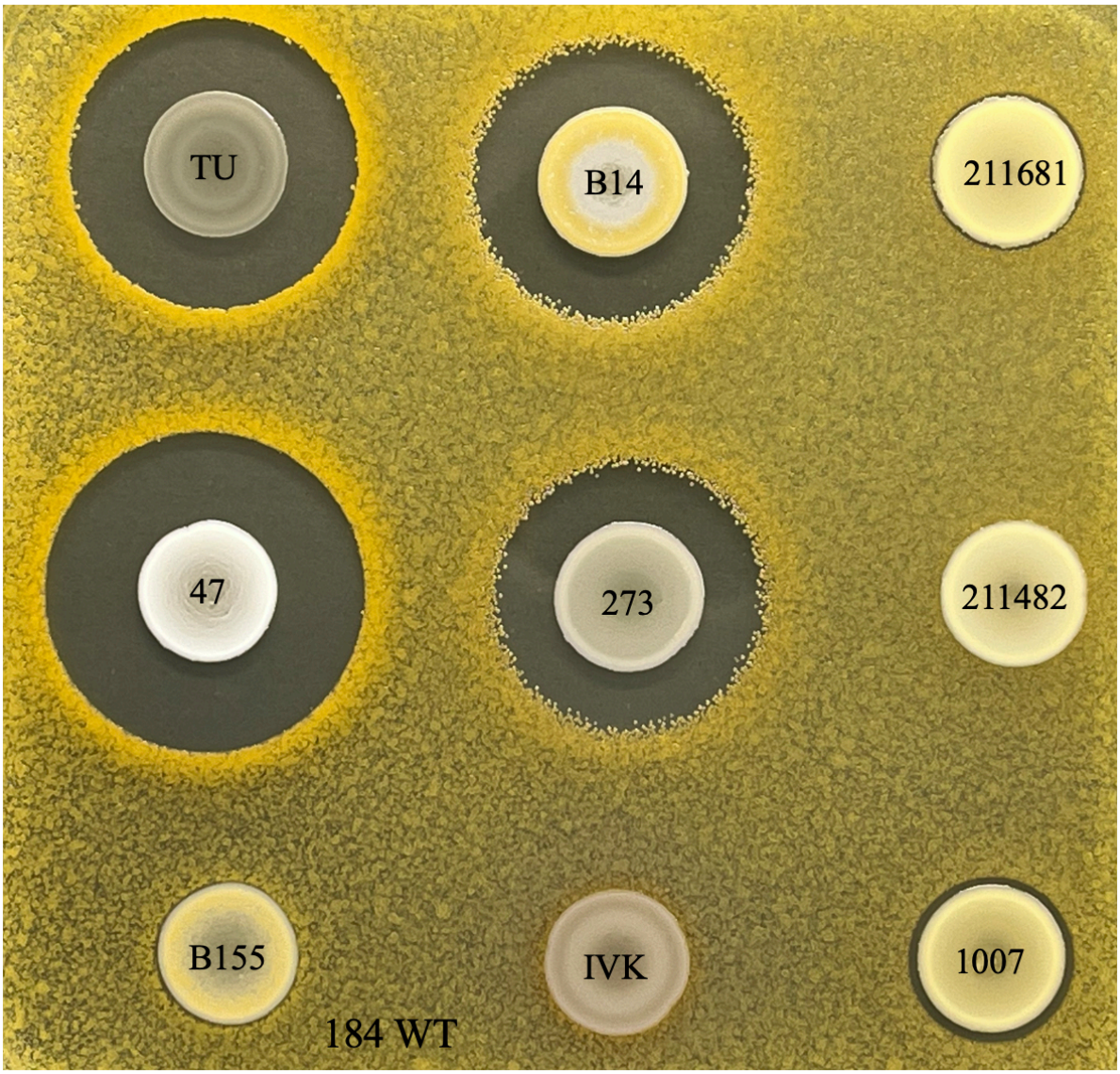


Figure 3. 1. Inhibitory activity of *S. epidermidis* and *S. lugdunensis* and *S. capitis,* against *S. aureus* 184 WT on BHI agar. Deferred growth inhibition assay was used to determine antimicrobial activities. The test strains: *S. lugdunensis* 211681, 211482 and 1007 and *S. capitis* 47, *S. epidermidis* B14, 273, epidermin producer Tü3298, and epifadin producers B155 and IVK83 were cultured as a spot of overnight culture on BHI agar and incubated at 37°C for 24 h. The next day, the plates were sprayed with the indicator strain, *S. aureus* 184 WT (approx. 10^8 cfu ml^{-1}).

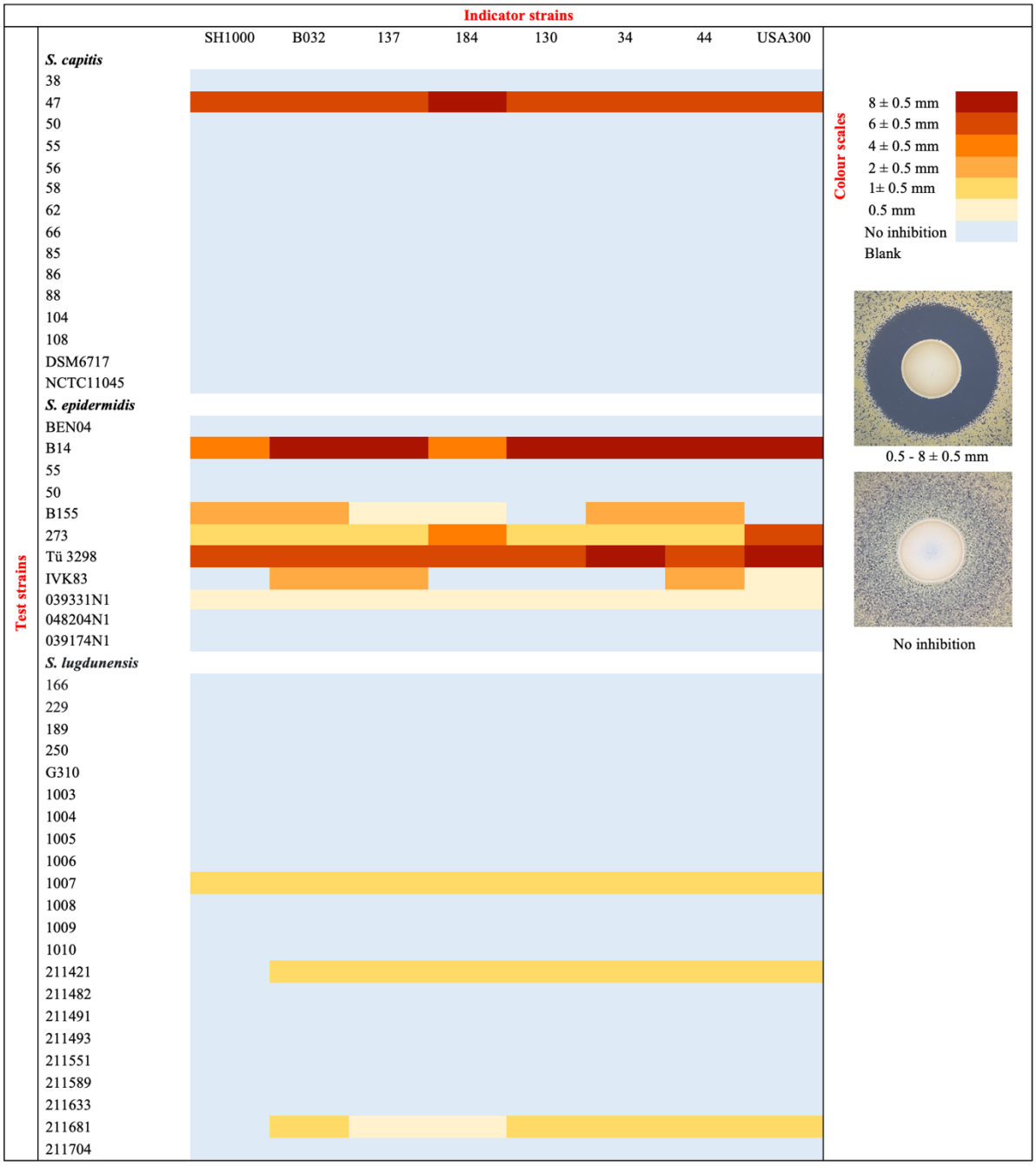


Figure 3. 2. Antimicrobial activity of 48 strains of CoNS, including *S. capitis, S. epidermidis, S. lugdunensis* against *S. aureus*. Inhibition zone sizes (mm) were measured and represented incrementally as a colour scale in the key. Images are indicative of inhibition or its absence. Data are the mean of 4 biological replicates.

3.3.2 Testing reciprocal inhibition between identified antimicrobial producing CoNS

As a means of gaining insight into species expressing either functionally similar activities or resistance mechanisms, the antimicrobial-producing strains with inhibitory activity were tested against each other. Most of the antimicrobial producers identified (Figure 3.1), were included in the reciprocal assay: *S. epidermidis* strains Tü3298, B155 IVK83, B14, 273, 039331N1; *S. capitis* 47; and *S. lugdunensis* strains 1007, 211421 and 211681. The ability to produce antimicrobial compounds again used the deferred growth inhibition assay. Examples of the extent of inhibition on agar plates is shown (Fig 3.3) with the quantified zone diameter (Figure 3.4).

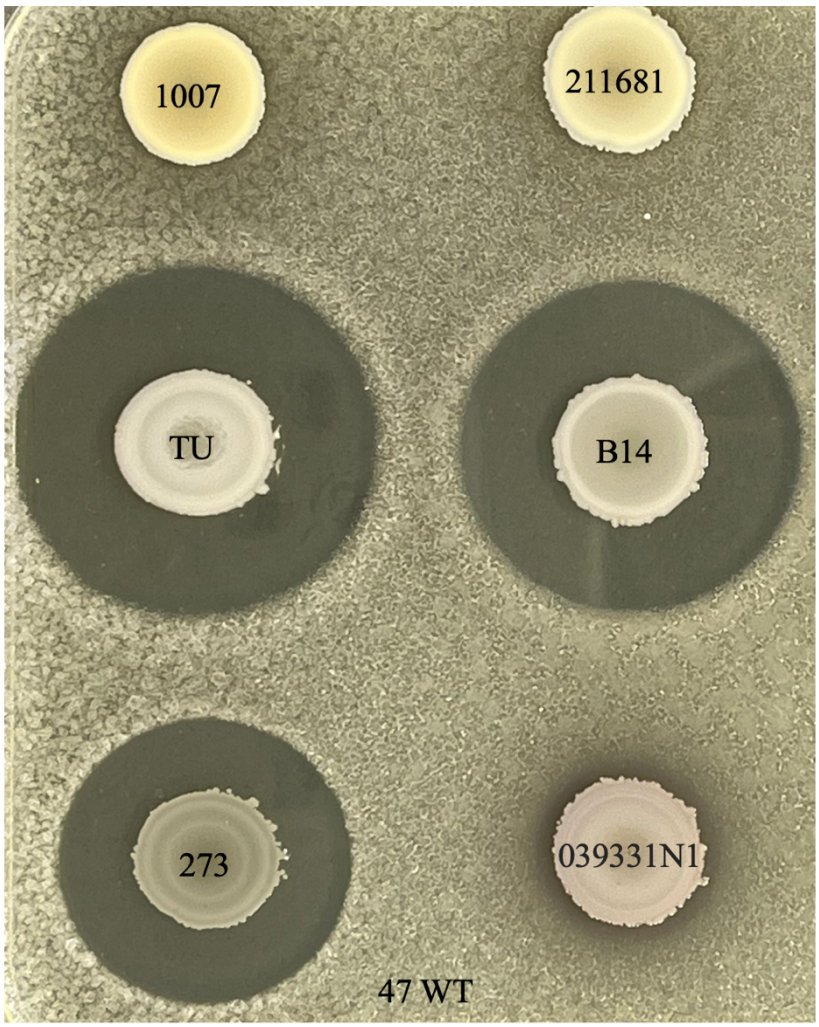


Figure 3. 3. Inhibitory activity of *S. epidermidis* and *S. lugdunensis* against *S. capitis*. Deferred growth inhibition assay was used to determine the antimicrobial activities. Strains: *S. lugdunensis* 1007 and 211681 and *S. epidermidis* Tü3298, B14, 273 and 039331N1 were cultured as a spot of overnight culture on BHI agar plate and incubated at 37°C for 24 h. The next day, the plates were sprayed with *S. capitis* 47 WT (approx. 10^8 cfu ml^{-1}).

From the patterns of inhibitory activities or resistance mechanisms several insights could be determined. Therefore, the deferred growth inhibition assay was used to test 10 antimicrobial producers including the controls against each other to investigate their activities (Fig 3.4). The *S. epidermidis* B155 epifadin producer did not inhibit any of *S. epidermidis* strains B14, IVK8, 273, Tü3298, 039331N1 and *S. capitis* 47 and *S. lugdunesis* 1007, 211681 and 211421. All the 10 antimicrobial producer strains could be inhibited by *S. epidermidis* 14, but not *S. epidermidis* Tü3298. On the other hand, the producer strain of Tü3298 epidermin (Ebner et al., 2018) was able to inhibit all the 10 antimicrobial producer strains. Finally, only *S. lugdunesis* 1007 could not be inhibited by the antimicrobial producer *S. capitis* 47. *S. lugdunensis* did not show activities against other *S. lugdunensis*, but also showed low range of activity generally, which could be reflective of the assay format or conditions.

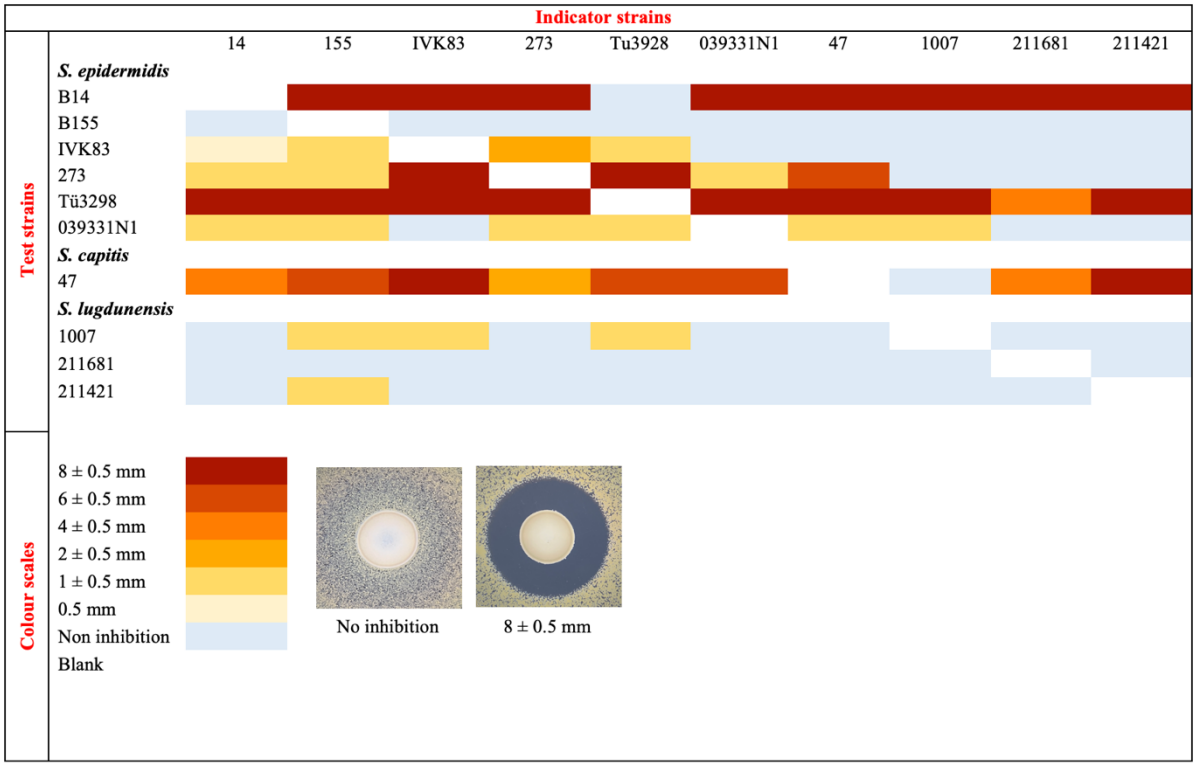


Figure 3. 4. Antimicrobial activity of 10 antimicrobial producing strains against each other, including *S. epidermidis, S. capitis* and *S. lugdunensis*. The key indicates relative inhibition zone sizes (mm) presented incrementally as a colour scale Images are indicative of inhibition or its absence. Data are the mean of 4 biological replicates.

3.3.3 Using antiSMASH of genomes to interrogate antimicrobial production

The antiSMASH web-based application identifies, annotates, and compares gene clusters encoding the production of secondary metabolites in bacterial and fungal genomes. Secondary metabolites which are encoded by Biosynthetic Gene Clusters (BGC) are an important source of antimicrobials. The antibiotics and secondary metabolite analysis shell of antiSMASH was used to detect and characterise the BGCs present in the genomes of strains used in the study (Medema et al., 2011; Introduction-antiSMASH Documentation, 2022). Genome sequences (GenBank) of S. *epidermidis* B14, B155, 273, Tü3298, 039331N and *S. capitis* 47 and *S. lugdunensis* 1007, 211421 and 21148 were used and their GenBank file was submitted in

bacterial antiSMASH version 6.0 website http://antismash.secondarymetabolites.org. Secondary metabolite classes were recorded (Table 3.1) showing antiSMASH detected a wide range of BGC at varying levels of identity. With respect to antimicrobial-encoding clusters, those BGC of *S. epidermidis* B14 had a 37 % similarity to Epidermin in region one and an 81 % similarity to Gallidermin in region six supporting that the strain synthesises one or other of these similar antimicrobials. In region one, *S. epidermidis* B155 clusters was 27 % similar Nukacin ISK-1. *S. epidermidis* Tü3298 clusters showed 36 % similarity with Gallidermin in region three while antiSMASH did not detected any BGC for *S. epidermidis* 039331N and 237. Clusters revealed 27 % similarity to Gallidermin in region one for *S. capitis* 47. The clusters for *S. lugdunensis* 1007 in region one was a 28% similarity to Equibactin, 93 % similarity to Lugdunin in region two, 50 % similarity to Listeriolysin in region three and 4 % similarity to Kijanimicin in region four. In region one, *S. lugdunensis* 211421clusters were 93 % similar Lugdunin and 50 % similar Listeriolysin, in region two was 28 % similarity to Equibctin, and in region fifteen was 4 % similarity to Kijanimicin. *S. lugdunensis* 2111681 clusters showed 50 % similarity to Listeriolysin, 93 % similarity to Lugdunin in region one, 28 % similarity to Equibctin in region three and 4 % similarity to Kijanimicin in region eleven (Table 3.1).

Table 3. 1. BGC identification in genomes using antiSMASH.

Genome sequence files (nucleotides) were interrogated from each of *S. epidermidis* B14, B155, 273, Tü3298 and 039331N, *S. capitis* 47 and *S. lugdunensis* 1007, 211421 and 211481 using antiSMASH to identify matches to secondary metabolites class sequences.

Strain Species/ ID	Detected types of BGC	Location in contig no	Most similar known cluster	Similarity	Region in /contig no
S. capitis					
47	1anthipeptide-class-I	785,429-809,647	Gallidermin	27%	1/1
	Terpene, T3PKS	1-48,241	None		2/3
S. epidermidis					
B14	Lanthipeptide-class-I	1-16,309	Epidermin	37 %	1/1
	Lanthipeptide-class-I	1-11,591	Gallidermin	81%	1/6
	NRPS	1-4,002	None		1/132
IVK83	NRPS	110,603-157,766	None		1/4
	Cyclic-lactone-autoinducer	49,182-69,891	None		1/9
	NRPS, TransAT-PKS	1-54,550	Nukacin ISK-1	27%	1/17
	T3PKS	1-34,601	None		1/23
	Ripp-like	16,441-26,833	None		1/25
	CDPS	1-11,086	None		1/32
B155	Cyclic-lactone-autoinducer	547,975-568,684	None		3/1
	NRPS	2,306,195-2,353,358	None		4/1
	TransAT-PKS, NRPS	1-59,998	Nukacin ISK-1	27%	1/2
273	NRPS	571,748-618,915	Aureusimine	100%	1/1
	Cyclic-lactone-autoinducer	387,945-408,653	None		1/2
	T3PKS	59,430-100,038	None		1/3
Tü3298	Lanthipeptide-class-I	1-5,082	Gallidermin	36%	
	T3PKS	1-24,830			1/18
039331N1	NRPS	1-36,284	None		1/12
	T3PKS	1-24,739	None		1/38
	Cyclic-lactone-autoinducer	1-10,797	None		1/61
S. lugdunensis					
1007	NRPS	346,716-392,737	Equibactin	28%	1/1
	NRPS	9,915-75,834	Lugdunin	93%	1/2
	T3PKS	404,174-445,343	None		2/2
	Terpene	163,485-184,348	None		1/3
	NRPS	282,488-338.593	Listeriolysin	50%	3/2
	Cyclic-lactone-autoinducer	18,971-39,683	Kijanimicin	4%	1/4
211421	NRPS	10,172-75,892	Lugdunin	93%	1/1
	T3PKS	392,298-433,467	None		2/1
	CDPS	620,057-640,761	None		3/1
	Terpene	680,785-701,648	None		4/1
	NRPS	791,994-839,175	Listeriolysin	50%	5/1
	NRPS	297,068-343,091	Equibactin	28%	2/1
	NRPS	19,007-39,719	Kijanimicin	4%	1/12
211681	LAP, NRPS	98,839-145,453	Listeriolysin	50%	1/1
	Terpene	236,312-257,175	None		2/1
	CDPS	297,199-317,903	None		3/1
	T3PKS	513,600-554,769	None		4/1
	NRPS	870,363-936,285	Lugdunin	93%	5/1
	NRPS	263,148-303,171	Equibactin	28%	1/3
	Cyclic-latone-autoinducer	4,964-25,676	Kijanimicin	4%	1/11

3.3.4 Experimental evolution of interference competition between antimicrobial producer *S. capitis* 47 and *S. aureus* strains SH1000 and 184.

On the basis that *S. capitis* 47 expressed antimicrobial activity that was potentially a novel molecule, based on bioinformatic analysis of genome sequences (Chong, 2022, Chong et al., 2022), an experimental evolution approach was used to gain insights on resistance. Interference competition was based on the methods of Ghabban, (2019) with serial coculture *S. capitis* 47 (test/antimicrobial producer) with either *S. aureus* strains SH1000 or 184 (indicator). *S. aureus* SH1000 and 184 were the most inhibited strains (Fig 3.2) of those tested with *S. capitis* 47 (zone size 6 ± 0.5 mm and 8 ± 0.5 mm respectively). To interrogate the interference competition of the antimicrobial producer *S. capitis* 47, two *S. aureus* strains SH1000 and 184 were examined to identify species and strain-specific effects on evolutionary outcomes. The competition experiment was started by mixing approx. 10^8 cfu ml^{-1} of *S. capitis* 47 with either *S. aureus* strain SH1000 or 184 with sequential day transfers to fresh agar BHI plates (Fig 3.5). As comparators, monoculture controls of test/indicator strains were sequentially transferred in parallel. Viable counts were determined daily to calculate the yield as colony forming unit/mL (CFU ml^{-1}) and evaluate growth dynamics of each species/strains over nine days.

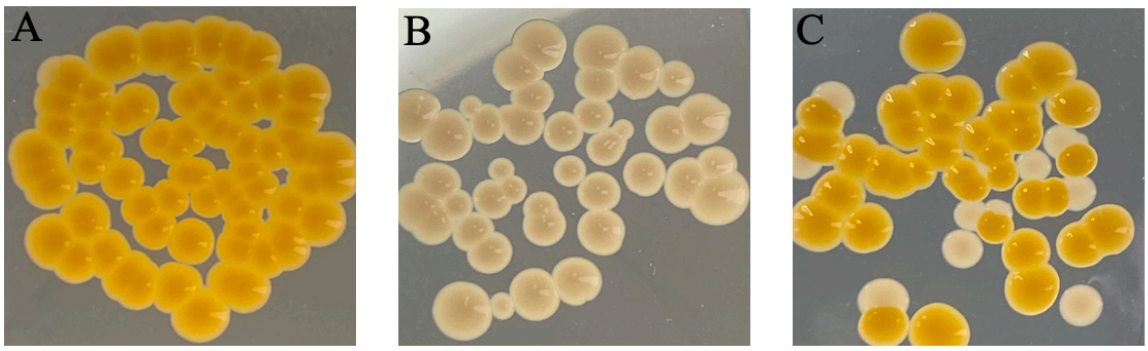
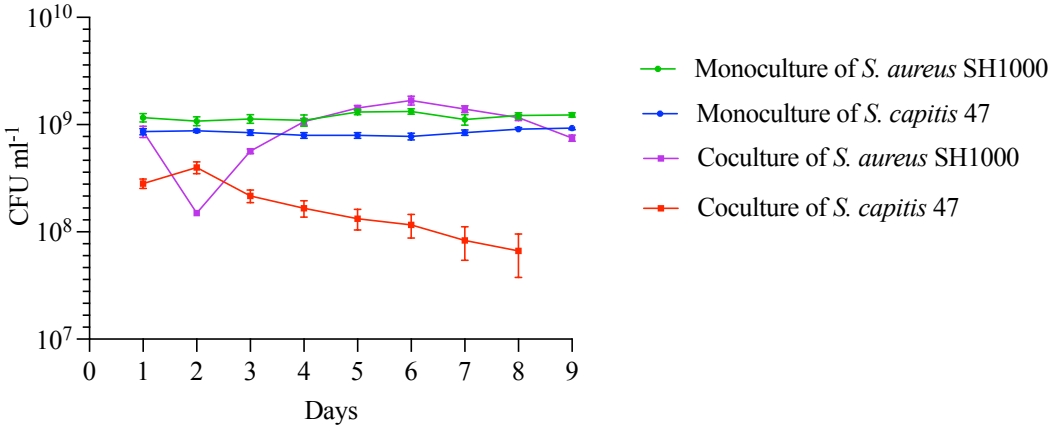


Figure 3. 5. Interference competition between *S. capitis* 47 and *S. aureus* SH1000. Experimental evolution on BHI agar was used perform the competition. Strains were culture in either monoculture or coculture and incubated over consecutive days at 37°C for 24 h with dilution and culture daily. (A) (gold colonies) monoculture of *S. aureus* SH1000 WT, (B)

(white colonies) monoculture of *S. capitis* 47 WT, (C) coculture (gold and white colonies) of *S. capitis* 47 and *S. aureus* SH1000. Images were taken from day one at dilution 10^5.

As monoculture, *S. capitis* 47 grew with similar yield for nine days at ~ 8.1 X 10^8 CFU ml^{-1}, while *S. aureus* SH1000 and 184 yields were both enumerated at ~ 1.4 x 10^9 CFU ml^{-1} on BHI agar (Fig 3.6 A, B). A different growth dynamic was detected when the species were cocultured. For competition between *S. capitis* 47 and *S. aureus* SH1000 (Fig 3.6 A), after the first day, *S. aureus* SH1000 showed a significantly higher yield (~ 8.6 x 10^8 CFU ml^{-1}) than *S. capitis* 47 (~ 2.8 x 10^8 CFU ml^{-1}) (P value < 0.001). After the second day, *S. aureus* SH1000 numbers increased in the coculture as *S. capitis* 47 yield halved to ~ 1.5 x 10^8 CFU ml^{-1}. Strain SH1000 yields increased from days three to six with peak ~ 1.7 x 10^9 CFU ml^{-1} then reducing to ~ 7.5 x 10^8 CFU ml^{-1} by day nine. In contrast, *S. capitis* 47 yields gradually reduced over days three to eight, reaching its lowest yield of ~ 6 x 10^7 CFU ml^{-1} on the eighth day and was not detected by the ninth day, at the dilutions tested. For competition between *S. capitis* 47 and *S. aureus* 184 a quite similar yield profile was observed for the two species with a broadly comparable output in terms of relative viable counts (Fig 3.6 B). *S. aureus* 184 had a similar growth yield after one day (~ 4 x 10^8 CFU ml^{-1}) as *S. capitis* 47 (~ 3.1 x 10^8 CFU ml^{-1}) and followed the similar change in yield on day two as strain SH1000. From the third to sixth day, *S. aureus* 184 increased its population size threefold to reach the highest yield of ~ 1.8 x 10^9 CFU ml^{-1} that was significantly greater than *S. capitis* 47 (P value < 0.001) before *S. aureus* 184 declined over days seven to nine reaching ~ 3.5 x 10^8 CFU ml^{-1}. *S. capitis* 47 increased over the first four days, then declined steadily to a minimum yield at ~ 5 10^7 CFU ml^{-1} on the eighth day that was significantly less than *S. aureus* (P value < 0.001), and undetectable by the ninth day.

A



B

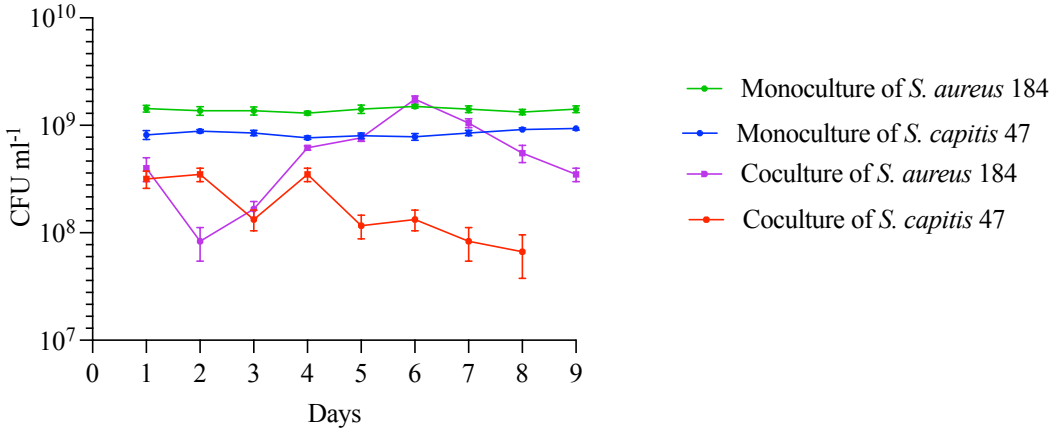


Figure 3. 6. Competition dynamics of antimicrobial producer *S. capitis* 47 and *S. aureus* SH1000 or 184. Strains were cultured alone or as coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar. Viable counts were used to determine population changes. *S. aureus* SH1000 (A) or 184 (B) monoculture control (green circles). *S. aureus* SH1000 (A) or 184 (B) coculture (purple squares). *S. capitis* 47 monoculture control (A, B) (blue circles). *S. capitis* 47 coculture (A, B) (red squares). Values are the mean of three biological replicates counted in triplicate. Error bars represent mean +/- STDEV across the three replicates with t-test analysis for statistical significance. Y-axis was presented in log 10 scale.

3.3.5 *S. aureus* SH1000 and 184 evolved resistances to antimicrobial producer *S. capitis* 47

Ten colonies of *S. aureus* SH1000 were selected from different days of competition cocultures and tested for their inhibition by *S. capitis* 47 using the deferred growth inhibition assay (Fig 3.7). From the selected colonies of day one, *S. aureus* SH1000 showed an inhibition zone matching its parental strain, which was 6 ± 0.5 mm. By day two, *S. aureus* SH1000 exhibited reduced inhibition (SH1000 D-2) by the antimicrobial producer *S. capitis* 47 (Fig 3.7 B), and by day eight *S. aureus* SH1000 (SH1000 D-8) displayed apparent resistance with no inhibition zone compared to the WT (Fig 3.7 C).

To investigate whether resistance phenomena occur with similar timescale in *S. aureus* 184, competition isolates were tested by growth inhibition assay with *S. capitis* 47. The results revealed *S. aureus* 184 was sensitive to *S. capitis* 47 with zone matching WT at 8 ± 0.5 mm on day one. The partial resistance phenomenon occurred again on day 2 and from day three to eight, *S. aureus* 184 was resistant to *S. capitis* 47 based on the absence of an inhibition zone.

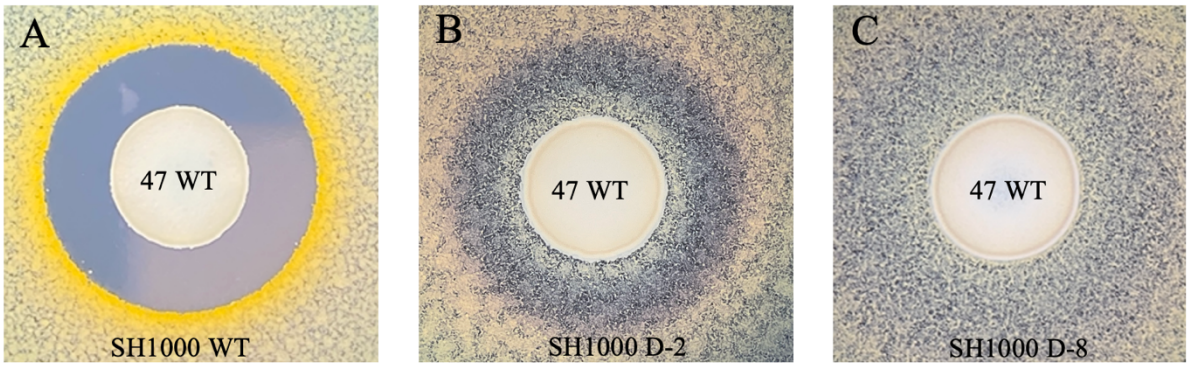


Figure 3. 7. *S. aureus* SH1000 developed resistance to antimicrobial producer *S. capitis* 47. Deferred growth inhibition assay with *S. capitis* 47 WT (inhibitor producer) cultured as a spot of overnight culture on BHI agar plate and after 24 h of incubation at 37°C, the plate was sprayed with either WT or evolved *S. aureus* SH1000 (indicator). (A) *S. capitis* 47 WT zone

of inhibition (6 ± 0.5 mm) against *S. aureus* SH1000 WT (B) *S. aureus* SH1000 from day two of competition showed incomplete resistance to *S. capitis* 47 WT while (C) *S. aureus* SH1000 from day eight presented complete resistance to *S. capitis* 47 WT.

3.3.6 Different phenotypes of *S. aureus* emerged during evolution of resistance

Golden colour imparted by carotenoid pigments is the eponymous feature of the human pathogen *S. aureus.* During the competition over eight days, both SH1000 and 184 showed colonies with changes in pigmentation. This phenomenon appeared between the third to eighth day in both WT and competition experiments and was observed as two-colour phenotypes. In addition to the WT gold pigment (G-Pig) there were colonies with either less gold pigment (L-G-Pig) or white non-pigmented (W-N-Pig) (Fig 3.8). Since pigment variants emerged in experimental and control experiments it was unclear how these might impact on the concomitant resistance that was selected with the antimicrobial producer.

Figure 3. 8. Loss of *S. aureus* pigmentation during competition. Experimental evolution of *S. aureus* SH1000 and *S. capitis* 47 in repeated coculture on BHI agar over eight days. The

image is from day eight and shows three distinct colour phenotypes of *S. aureus* pigmentation. (A) white (B) gold (C) less-gold.

3.3.7 Diverse resistance or no resistance phenotypes of *S. aureus* SH1000

Colonies with different pigmentation phenotypes from both the competition and monoculture control of *S. aureus* SH1000 were tested for their resistance to antimicrobial producer *S. capitis* 47. With focus on day eight, clones were picked to include day eight gold pigment (D-8-G-Pig), day eight less gold-pigment (D-8-L-G-Pig) and day eight white-non-pigment (D-8-W-N-Pig). Each of the colour phenotypes were tested by using deferred growth inhibition assay and revealed that complete resistance to the antimicrobial producer *S. capitis* 47 was only found within the isolated colonies from the competition but not within the monoculture/control of *S. aureus* SH1000 (Fig 3.9). The day eight white-non-pigment (D-8-W-N-Pig) from the WT control culture showed reduced growth inhibition revealing a complex picture of resistance.

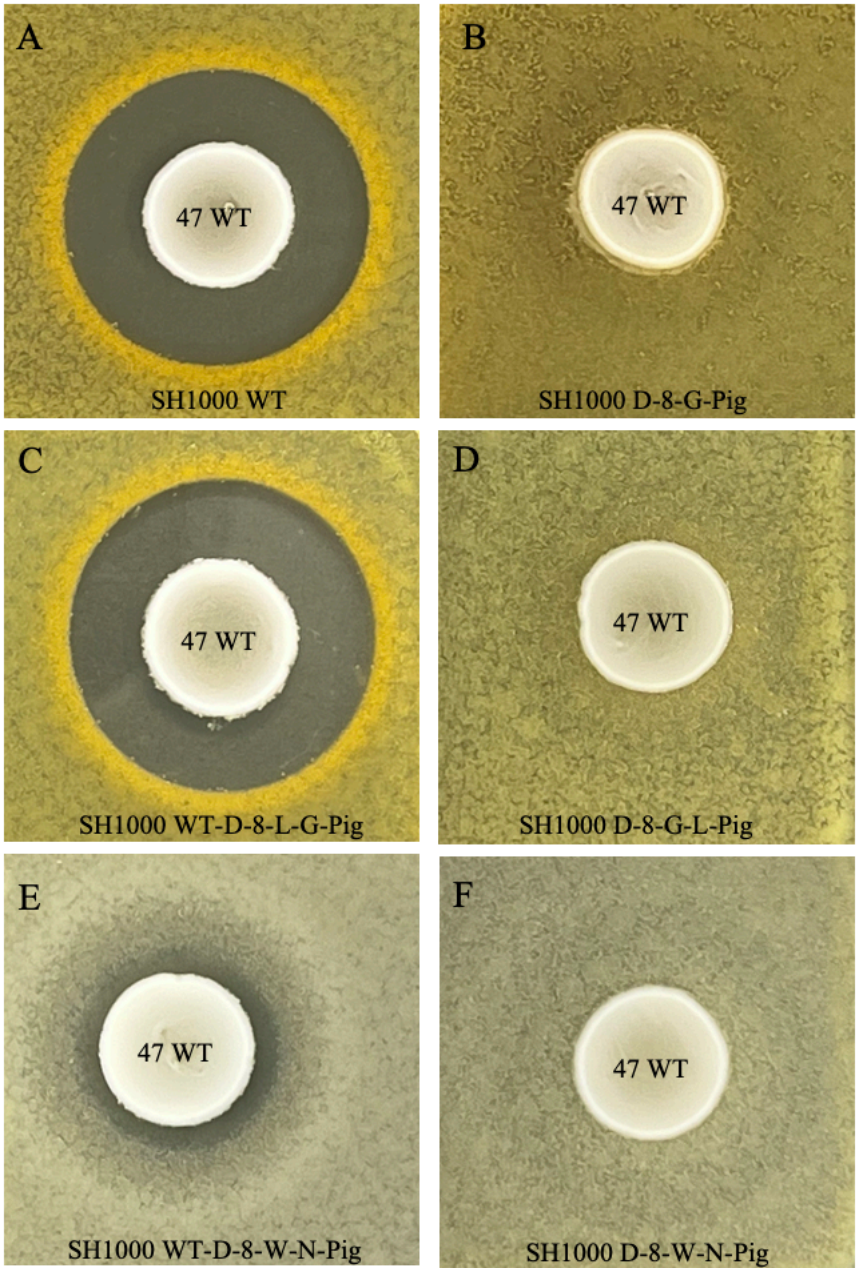


Figure 3. 9. Resistance phenotypes of *S. aureus* SH1000 with different pigmentation taken from day eight of both competition and control cultures. Deferred growth inhibition assay was used to determine the antimicrobial activities. Antimicrobial producer S. capitis 47 WT (test, inhibitor producer) was cultured as a spot of overnight culture on BHI agar and after 24 h of incubation at 37°C, the plate was sprayed with either different pigment phenotypes of indicator *S. aureus* SH1000 that were isolated from day 8 control or competition cultures. *S.*

capitis 47 assay with: (A) *S. aureus* SH1000 WT-day 0, zone of inhibition (6 ± 0.5 mm) (B) *S. aureus* SH1000 with gold pigment (D-8-G-Pig). (C) *S. aureus* SH1000 WT with less gold pigment (WT D-8-L-G-Pig) zone of inhibition (6 ± 0.5 mm). (D) *S. aureus* SH1000 with less gold pigment (D-8-L-G-Pig). (E) *S. aureus* SH1000 WT white-non-pigment zone of inhibition (2 ± 0.5 mm). (F) *S. aureus* SH1000 white-non-pigment (D-8-W-N-Pig).

3.3.8 Species discrimination using sugar fermentation agar

Due to the difficulty of discriminating *S. aureus* from *S. capitis* during the competition experiment due to pigment loss in *S. aureus*, trehalose dihydrate sugar-agar media (TDSA) (Alrajeh, 2021) was used to distinguish the strains from each other. Cultured on TDSA, *S. aureus* can ferment trehalose to produce acid that causes yellow colour change from the pH indicator in the media, while *S. capitis* cannot ferment trehalose (Fig 3.10).

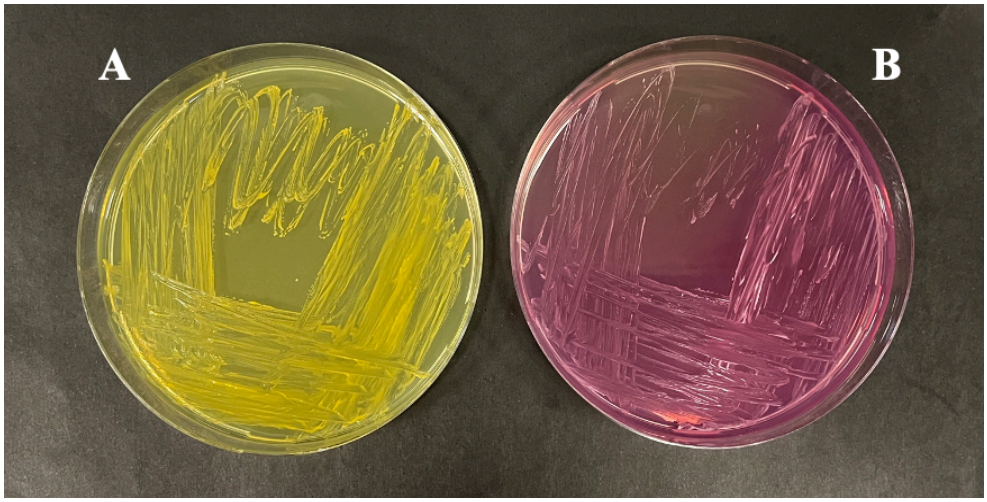


Figure 3. 10. Trehalose dihydrate sugar-agar media (TDSA) test to discriminate *S. aureus* and *S. capitis.* Strains were cultured on TDSA at 37°C for 24 h. (A) *S. aureus* ferments trehalose to produce acid that causes a yellow colour change (B) *S. capitis* cannot ferment trehalose.

3.3.9 Effect of experimentally evolved resistance to *S. capitis* 47 on resistance to other antimicrobial producers.

To assess whether the *S. aureus* with resistance to *S. capitis* 47 that were selected in the competition experiment showed altered inhibition from other antimicrobial producers, the species in Fig 3.4 were tested again. Both *S. aureus* SH1000 and 184 clones, which included D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig and D-8-W-N-Pig were assayed. No differences with inhibition profiles relative to their parent strains were detected, except for the resistance to *S. capitis* 47 that was selected in the competition experiment. Antimicrobial activities were recorded and measured as clear or unclear zones of varying sizes in millimetres (Fig 3.11).

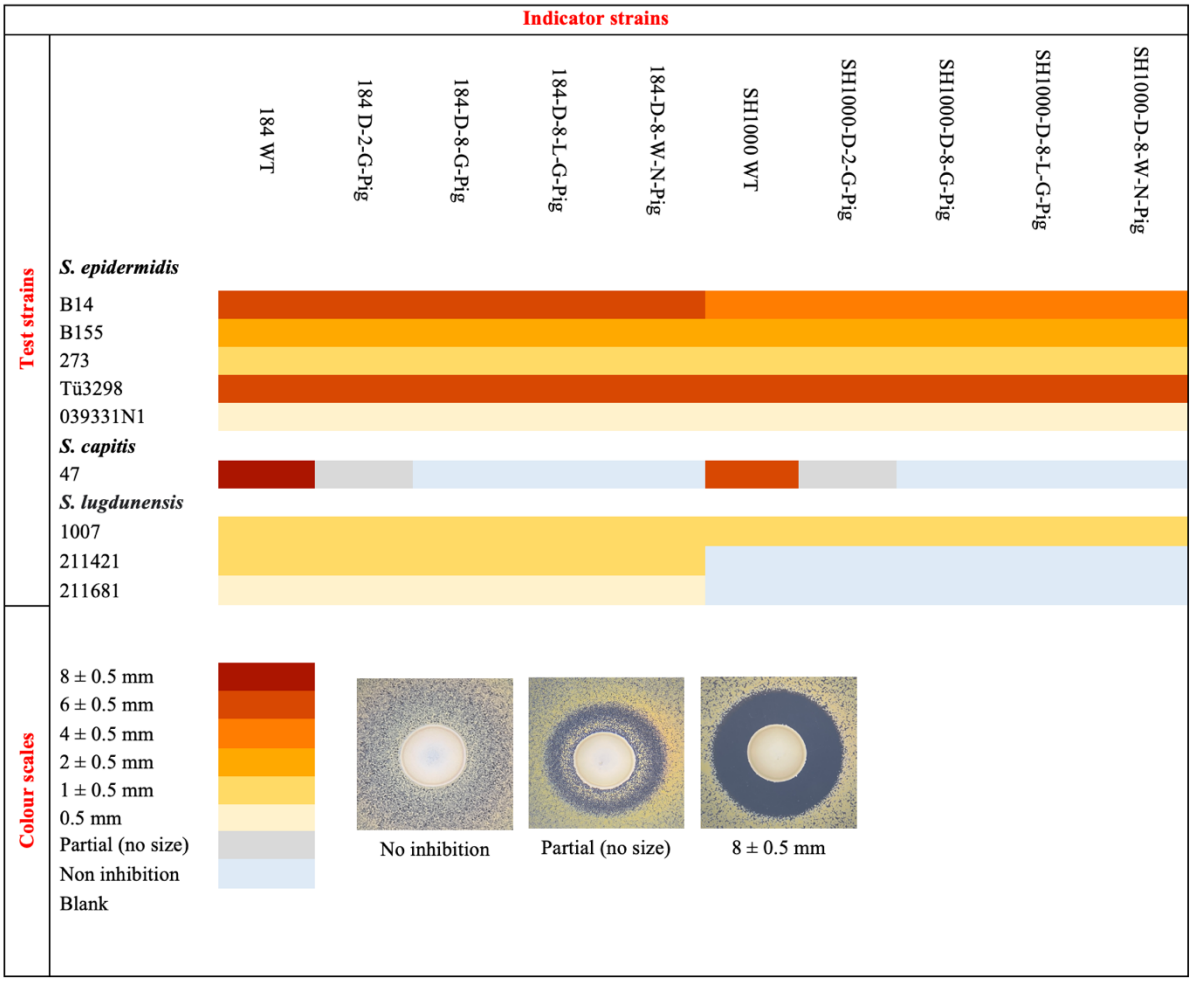


Figure 3. 11. Antimicrobial producer activity against-evolved resistance of both *S. aureus* SH1000 and 184 strains on BHI agar. The zone sizes of inhibition were measured and

presented as shown in the colour key. Images as indicators of inhibition and no inhibition zone were provided into the table. These data are the mean of 4 biological replicates.

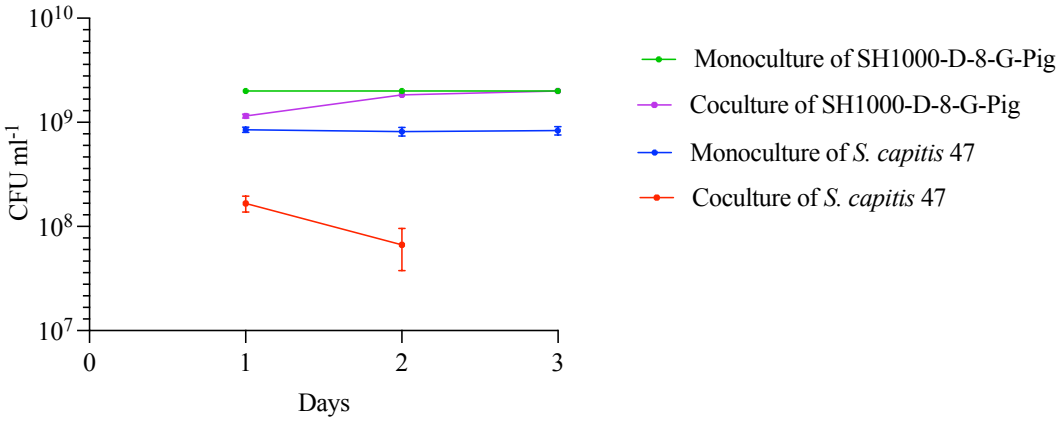
3.3.10 Resistant clones of *S. aureus* SH1000 and 184 from experimental evolution have increased competition with ancestral *S. capitis* 47

Competition experiments were performed to determine the ability of resistant *S. aureus clones* to compete with the ancestral strain *S. capitis* 47. The growth yields of both *S. aureus* SH1000 and 184 (D-8-G-Pig clone No 1) strains resistant to *S. capitis* 47 WT antimicrobial, were grown as cocultures with monoculture controls. In coculture, both *S. aureus* SH1000-D-8-G-Pig (fig 3.12A) and 184-D-8-G-Pig (Fig 3.12B) clones were more competitive in culture with ancestral *S. aureus* 47, producing greater yields as compared to *S. aureus* SH1000 and 184 WT strains (compare with Fig 3.6).

Specifically, in coculture of *S. aureus* SH1000-D-8-G-Pig had increased growth yields of ~1.1 x 10^9 CFU ml^{-1} on day one and ~1.8 x 10^9 CFU ml^{-1} on day two and three (Fig 3.12A) compared with coculture of *S. aureus* SH1000 WT and *S. capitis* 47 WT (Fig 3.6A), which had lower yields of *S. aureus* with ~8.6 x 10^8 CFU ml^{-1} on day one, ~ 1.5 x 10^8 CFU ml^{-1} on day two and 5.6 x 10^8 CFU ml^{-1} on day three. The yield of the ancestor *S. capitis* 47 in competition with *S. aureus* SH1000-D-8-G-Pig was decreased (Fig 3.12A) with significantly lower number of cells of the former on day one at ~1.5 x 10^8 CFU ml^{-1} and 5 x 10^7 CFU ml^{-1} on day two (Fig 3.12A) compared to coculture of *S. capitis* 47 WT in competition with SH1000 WT (Fig 3.6A), which were ~2.8 x 10^8 CFU ml^{-1} on day one and ~4 x 10^8 CFU ml^{-1} on day two. *S. capitis* 47 was undetectable and disappeared from culture on the third day in competition with *S. aureus* SH1000-D-8-G-Pig (Fig 3.12 A) at the dilutions plated, in contrast to its yield in the competition with *S. aureus* SH1000 WT that remained at ~2.1 x 10^8 CFU ml^{-1} (Fig 3.6 A).

A similar trend was identified with *S. aureus* 184 (184-D-8-G-Pig) resistance-selected pigmented strain having increased growth yields in competition with *S. capitis* 47 also becoming undetectable after 3 days. Specifically, *S. aureus* 184-D-8-G-Pig in coculture had a higher yield of cells at ~2 x 10^9 CFU ml^{-1} on day one and two, and ~1.1 x 10^9 CFU ml^{-1} on day three (Fig 3.12 B), compared to WT yields that were ~ 4 x 10^8 CFU ml^{-1}on day one, ~ 2.3 x 10^8 CFU ml^{-1} on day two and ~1.3 x 10^8 CFU ml^{-1}on day three in competition with *S. capitis* 47 (Fig 3.6 B). Ancestral *S. capitis* 47 was outcompeted by *S. aureus* 184-D-8-G-Pig (Fig 3.12 B). *S. capitis* 47 WT had a lower number of colonies on day one at ~ 1.8 x 10^8 day two at ~ 1.3 x 10^8 and 5 x 10^7 cells on day three and disappeared from coculture on day 4 (Fig 3.12 B)compared to the coculture of *S. capitis* 47 WT in competition with 184 WT which were ~ 3.3 x 10^8 cells on day one, day two at ~ 1.5 x 10^8 and ~ 1.3 x 10^8 cells on the day three and persisted to day 8 (Fig 3.6 B)

A



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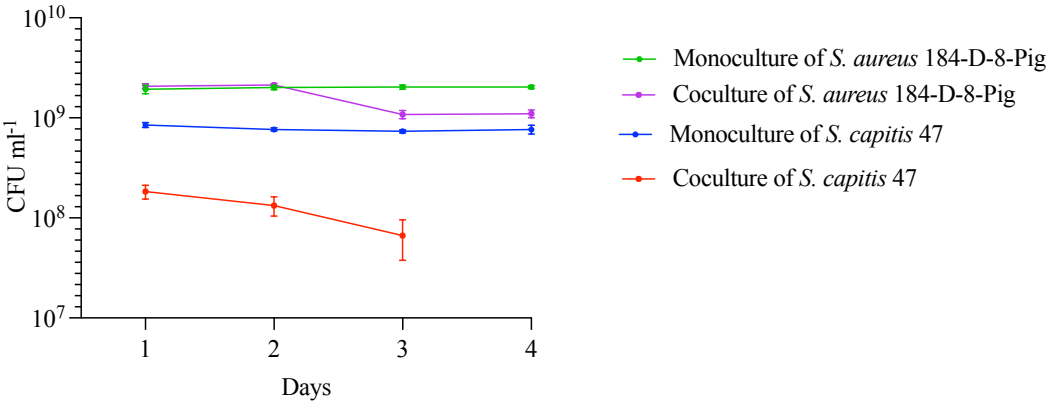

Figure 3. 12. Competition between antimicrobial producer *S. capitis* 47 and resistant *S. aureus* clones SH1000-D-8-G-Pig or 184-D-8-G-Pig. Strains were cultured in monoculture and coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar as part of an experimental evolution procedure. Viable counting was used to determine growth yields. Monoculture controls of *S. aureus* SH1000-D-8-G-Pig (A) or 184-D-8-G-Pig (B) (green circles). Coculture of *S. aureus* SH1000-D-8-G-Pig (A) or 184-D-8-G-Pig (B) (purple squares). Monoculture control of *S. capitis* 47 (A, B) (blue circles). Coculture of *S. capitis* 47 (A, B) (red squares). Values are the mean of three biological replicates counted in triplicate. Error bars represent mean +/- STDEV across the three replicates with t-test analysis for statistical significance. Y-axis was presented in log 10 scale.

3.3.11 Whole Genome Sequencing

To identify the genetic basis for altered *S. aureus* growth inhibition by *S. capitis* 47 in the previous findings (Fig 3.7 and 3.9), DNA was extracted from three biological triplicates (24 resistance clones and 15 WT) was extracted using DNeasy Blood and Tissue Kit-Qiagen and submitted to the Centre for Genomic Research (CGR), University of Liverpool. Whole genome sequencing was performed using the Illumina platform to enable comparison of

evolved *S. aureus* compared to the WT across the selection experiment, including colour phenotype variants. The specific selected clones for sequencing are shown in Table 3.2.

Table 3. 2. The selected colonies and days.

Strains	Selected day	Sequence number of clones	Sample ID
S. aureus SH1000	0	3	WT D-0
	8	3	WT D-8-L-G-Pig
	8	3	WT D-8-W-N-Pig
	2	3	Partial resistance D-2-G-Pig
	8	3	Complete resistance D-8-G-Pig
	8	3	Complete resistance D-8-L-G-Pig
	8	3	Complete resistance D-8-W-N-Pig
	8	3	WT D-8-L-G-Pig
	8	3	WT D-8-W-N-Pig
S. aureus 184	2	3	Partial resistance D-2-G-Pig
	8	3	Complete resistance D-8-G-Pig
	8	3	Complete resistance D-8-L-G-Pig
	8	3	Complete resistance D-8-W-N-Pig

3.3.12 Genomic Changes

After isolates were sequenced, raw reads were assembled using the Unicycler tool (version, 0.4.8) and annotated by Prokka (version 1.14.6). The Snippy tool (version 4.6.0) was used to detect genetic variants by comparing sequences. Snippy aligns raw reads with WT-day 0, which acted as a reference genome (the genes ID presented in the tables below was from the self-annotated reference genome). The detected single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were considered potential variants that could contribute to *S. aureus* resistance, colour change, and increased competitiveness. Changes in genes (SNPs or

INDELS) that were present in both the competition control and the evolved clones were identified from the three clones that were sequenced for each timepoint/phenotype (clones 1, 2, 3). The genomes of the WT (colour change) and resistant *S. aureus* SH1000 and 184 clones from day 2 and day 8 were aligned to reference genomes from day 0. Overall, sequencing revealed a total of 38 observed mutations across all clones (tables 3.3, 3.4,3.5 and 3.6).

3.3.13 SNPs associated with resistance in *S. aureus* SH1000

Fifteen non-synonymous SNPs were discovered in resistant *S. aureus* SH1000. Among these, seven SNPs were found in each of the resistant phenotype clones: D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig, and D-8-W-N-Pig (Table 3.3). The seven sequence variants included first, the *saeS* gene, which encodes the sensor kinase of the SaeSR TCS that controls the expression of multiple virulence factors contributing to *S. aureus* pathogenicity (Haag and Bagnoli, 2015). Second, a SNP was detected in a gene encoding a helix-turn-helix (HTH)-type transcriptional regulator, which is a DNA-binding protein used to regulate gene expression (Aravind et al., 2005). A further SNP was found in *ecfA1*, which encodes a transporter channel protein associated with the capacity of cells to adjust to their environment by importing nutrients and metals, as well as responding to osmotic stress, which exports antibiotics and toxins (Alhareth et al., 2022). In *S. aureus* USA 300, *ecfA1* was suggested to be related to the transport of cobalt (Machado et al., 2019). A fourth SNP was detected in the gene for a transposase, involved in mechanisms of DNA transposition. A further SNP was identified in a gene for a putative TrmH family tRNA/rRNA methyltransferase, which is responsible for transfer RNA (tRNA, also named G18) recognition described as a methyltransferase of *Escherichia (E) coli* (Ochi et al., 2010). Another SNP was found in the *gyrB* gene which is responsible for catalysing the breakage and formation of double-stranded DNA as well as the removal of supercoiled DNA (Hull et al., 2022). Mutations in active locations of DNA gyrase, have been associated with changes in permeability of the outer membrane and to altered

antimicrobial resistance (Chen et al., 2021). The final common SNP was detected in the *pbuG* gene (also named *yebB* or *stgP*), encoding a guanine-xanthine permease.

A further three mutations were found in resistant *S. aureus* SH1000 clones but not in each single clone meaning they could contribute to the resistance phenotype at the clone level only. In D-8-W-N-Pig clones 2 and 3, SNPs of y*heS* gene encoding an ATP-binding protein were observed. While D-8-W-N-Pig clones 2 and 3 both had a mutation in *yhfS,* encoding a biotin transporter component. Lastly, a SNP in a gene for a putative ABC transporter ATP-binding protein was detected in clones 2 and 3. The ABC transporters are a broad group of transmembrane proteins (Orelle et al., 2019).

3.3.14 SNPs associated with resistance in *S. aureus* 184

Fifteen non-synonymous SNPs and one INDEL were discovered across the resistant clones of *S. aureus* 184 (table 3.5). Of these mutations, four SNPs were shared by more than one clone/phenotype. The most frequent mutation that was detected was in a gene encoding an HTH-type transcriptional regulator and was detected in all the clone numbers/phenotypes except clone three of D-2-G-Pig. Of those found in more than one clone, a SNP in a putative ABC transporter ATP-binding protein was detected in the resistant clones 2 and 3 of D-8-W-N-Pig. Furthermore, these resistant clones 2 and 3 of D-8-W-N-Pig also had a SNP in a putative ABC transporter ATP-binding protein. A SNP in y*heS* was seen in clone No 1 of D-8-W-N-Pig. Another SNP of *yhfS* was detected in clones 2 and 3 of D-8-L-G-Pig and in clone No 1 of D-8-W-N-Pig.

3.3.15 Common SNPs between resistant in *S. aureus* SH1000 and 184

Examining common sequence changes between the two *S. aureus* strains, SH1000 and 184 provided a means to look for common sequence changes from their experimental evolution.

There was not a single SNP common to all clones of both strains indicating multiple routes to antimicrobial resistance from coculture experimental evolution with *S. capitis* 47. However, six SNPs were shared between individual clones of resistant *S. aureus* SH1000 and 148.

Among all the SNPs, those in the HTH-type transcriptional regulator gene was found in all clones of resistant *S. aureus* SH1000 and 8 out of 12 resistant clones of resistant *S. aureus* 184. In total, this mutation was observed in 20 out of 24 resistant clones of both strains making the mutation the most common of all clones. Hence, it was suspected that mutations in the gene encoding the HTH-type transcriptional regulator could play an important role in resistance to the unknown antimicrobial produced by *S. capitis* 47. Of note, the mutations in the gene for the HTH repressor protein differed across the clones with three variants identified.

HTH repressors have been reported to regulate gene expression involved in antibiotic resistance. One example is the HTH-type transcriptional regulator NorR, a positive regulator of NorA expression and a repressor of NorB that functions in the efflux pump system associated with fluoroquinolone resistance of *S. aureus* (Aravind et al., 2005; Costa et al., 2019; Vall-Jääskeläinen., 2021; Leal et al., 2021). According to the public NCBI database, the gene encoding HTH-type transcriptional regulator identified in this study is associated with gene ID *SAUSA_300 0350*, encoding a Cro/CI transcriptional regulator-like protein. In all the resistance clones of *S. aureus* SH1000, mutations were observed in the gene for the Cro/CI repressor with a stop codon introduced at amino acid 7. In the resistance clones of *S. aureus* 148, the Cro/CI gene mutations result in substitutions of amino acids at different positions, amino acid Glu34Asp and Arg27Ile. Although there are different mutations of the gene between the resistance clones of *S. aureus* SH1000 and 148, the finding of multiple mutations in the same gene of Cro/CI can be a resistance mechanism to the *S. capitis* 47 antimicrobial.

The next widespread mutation affected the *pbuG* gene and was found in all the *S. aureus* SH1000 resistant clones as well as in two resistant clone of *S. aureus* 184. In total, the *pbuG*

mutant was detected in 14 clones out of 24, indicate a possible alternative resistance mechanism in *S. aureus* SH1000 and 184. The *pbuG* gene also named as *yebB*/stgP (NE283, guanine/hypoxanthine), encodes a hypoxanthine-guanine transporter that was shown to encode a purine nucleoside transporter and renamed as NupG (NE1419, guanine/guanosine) (Johansen et al., 2003; Nolan et al., 2022). Mutations found in the *pbuG*-related purine synthesis was reported for resistance to azaguanine, vancomycin and daptomycin (Saxild et al., 2001*;* Salazar et al., 2002; Nolan et al., 2022). Therefore, mutations in *pbuG* gene can be alternative resistance mechanism to the *S. capitis* 47 antimicrobial.

A SNP in a putative ABC transporter ATP-binding protein was detected in clones 2 and 3 of both resistant *S. aureus* SH1000 and 184 D-8-W-N-Pig. As a relevant resistance function in bacteria, ABC transporters are a broad group of transmembrane proteins with a variety of purposes that participate in the efflux pump of a wide range of molecules such as lipids, multiple drugs, natural products, and peptides (Orelle et al., 2019). It is possible that the ABC transporter found in this study serves as efflux pump for antimicrobial produced by *S. capitis* 47 to maintain bacterial viability. However, mutations of the ABC transporter were only found in 4 clones out of 24, indicating a low contribution, if any.

An y*heS* mutation was seen in both resistance *S. aureus* SH1000 D-8-W-N-Pig clones 2 and 3 and in clone No 1 of resistance *S. aureus* 184 D-8-W-N-Pig. The YheS ATP-binding protein has been suggested to play a role of this gene in antimicrobial resistance among *E. coli* isolates but required for further investigations (Lu et al., 2010). Thus, the function of mutation of *yheS* in resistance of *S. aureus* SH1000 and 148 is not clear. Finally, the SNP of *yhfS* was shared by clones 2 and 3 of *S. aureus* SH1000 D-8-W-N-Pig as well as by clones 2 and 3 of the resistant *S. aureus* 184 D-8-L-G-Pig and by clone No 1 D-8-W-N-Pig of the 184. In all, the *yhfS* mutant was shared by 5 out of 24 resistant clones of both strains. YhfS is a biotin transporter component with the name BioY and may have a role in maintaining the homeostasis of fatty

acids. BioY was suggested to help signal to *S. aureus* for effective scavenging in low-biotin environments (Satiaputra et al., 2018).

3.3. 16 Other SNPs associated with resistance in *S. aureus* SH1000 and 184

As a means of discriminating the day 2 and day 8 phenotypes of *S. aureus* SH1000, two common SNPs in the *thiD* and *rhaS* genes for metabolism were detected in all three clones isolated from D-2-G-Pig in addition to the five common SNPs that observed in all *S. aureus* SH1000 clones (table 3.3). RhaS is required for L-rhamnose uptake in *E. coli* (Fricke et al., 2022). However, none of these SNPs were found in the resistant *S. aureus* 184 D-2-G-Pig.

Eight SNPs were detected in *S. aureus* 184 D-2-G-Pig but not in each single clone. First, a SNP was detected in the gene for a transposase of clone1 and 2. An additional SNP occurred in the *bioD* gene, the first gene in the biotin biosynthesis operon in clone 2 of the resistant *S. aureus* 184 D-2-G-Pig. This gene could contribute to increased amounts of biotin synthesis (Machado et al., 2019). A mutation of *pdhC_2* was identified in the resistant clone *S. aureus* 184 D-2-G-Pig. This component was reported to be highly expressed in heterogeneous vancomycin-intermediate *S. aureus* (hVISA) Liu et al., 2020). Likewise, a SNP of HutH was also found in resistant *S. aureus* 184 D-2-G-Pig. HutH is an amino acid catabolic system reported to be associated with zinc in *Pseudomonas aeruginosa*, (Wang et al., 2021). Also, a mutation in a gene encoding a transcriptional regulator HTH-type was detected in clones 1 and 2. A *ktrB 2* SNP mutation was found in resistant *S. aureus* 184 D-2-G-Pig clone 3. In *B. subtilis* Ktr has a role in potassium uptake (Do and Gries 2021). Lastly, further SNPs were detected in clone 1 and 3 of resistance *S. aureus* 184 D-2-G-Pig in a hypothetical protein (PILBBNHO_01805) alpha/beta hydrolase.

Accordingly, there is no clear explanation from the SNPs described above for the visually determined incomplete resistance phenotype to *S. capitis* 47 associated with both D-2-G-Pig

of *S. aureus* SH1000 and 184. However, in a disc diffusion assay, a high concentration of the drug was proposed to activate efflux pumps in bacteria that lead to two rings (Shrestha et al., 2022). Because mutations of a transcriptional regulator were observed in all the resistant *S. aureus* SH1000 D-2-G-Pig clones and in 2 out of 3 of the 184 D-2-G-Pig, it is suggested that the transcriptional regulator mutant contributed to the incomplete resistance in day 2 of both strains and contributions of other mutations producing the different visual phenomena.

A range of additional mutations were present across clones without a clear relationship to resistance. Six different SNPs were detected across the clones of the resistant *S. aureus* phenotypes of SH1000 and 184. A SNP of *ydjZ* gene belongs to the DedA family was detected in clone 2 and 3 of D-8-W-N-Pig resistant *S. aureus* SH1000. This protein family of highly conserved transmembrane proteins is present in most organisms but have no recognised biological roles. However, defects in membrane potential and cell division, the production of additional cytoplasmic stress responses, sensitivity to alkaline pH, temperature sensitivity were discovered in an *E. coli*-DedA family mutant (Boughner et al*.,* 2013, Panta et al*.,* 2021). Another SNP was found in SarA, a transcriptional regulator of *S. aureus* in clone 2 and 3 of D-8-W-N-Pig. This regulator controls the expression of target genes important for the pathogenicity of the organism (Oriol et al., 2021). Next, a SNP mutation with RpsN2 zinc-dependent ribosomal protein gene was detected in clone No 1 resistant *S. aureus* SH1000 D-8-W-N-Pig. In *S. aureus* culture, *rpsN2* transcription can be activated during zinc limitation (Grim et al., 2020).

SNPs of *fusA* and *atpD* genes were identified in all the clones of resistance *S. aureus* 184 D-8-L-G-Pig (table 3.5). The *fusA* gene has been shown to encode a fusaric acid target in *S. aureus* SH1000 in addition to mutations in a putative phage gene with an unidentified function (Gupta et al., 2022). Finally, a mutation as deletion SNP in *clpB* was detected in clone No 1 of resistant *S. aureus* 184 D-8-L-G-Pig in clone No 1. The ClpB is a protein belonging to the AAA+ family

that functions in ATPases involved in different cellular processes. It has been suggested that one of these essential roles of ClpB is to regulate environmental stress responses (Alam et al., 2021).

3.3.17 Characterization of less pigmented and non-pigmented phenotypes

3.3.17.1 Less pigmented phenotype

During the experimental evolution, WT of both *S. aureus* SH1000 and 148 showed colonies with changes in pigmentation. Therefore, it was decided to sequence these clones to understand this phenomenon. Different *agrA* mutations were found in the *S. aureus* SH1000 and 148 clones that include the WT/resistance D-8-L-Pig and resistant D-8-L-Pig (Tables 3.4). The Agr TCS regulates toxin and adhesin virulence factors, biofilm formation and mutations are associated with a reduction in pigmentation in *S. aureus* (Tan et al., 2018; Ghabban 2019). Here *agrA* mutations were detected in 9 out of 12 lesser gold clones linking the TCS with pigment phenotype.

3.3.17.2 Non-pigmented phenotype

In *S. aureus,* stress-activated alternative sigma factor SigB is involved in adaptation to different environments and loss of pigmentation (Pannu et al., 2018; Zhang et al., 2019). A sigma factor B termination codon mutation was found in clone No 1 of the resistant *S. aureus* SH1000 D-8-W-N-Pig. Additionally, it was detected in clones 2 and 3 of the resistant D-8-W-N-Pig and in all clones of WT D-8 -W-N of *S. aureus* 184. In *S. aureus* SH1000, a *rsbU* mutation was associated with all the clones of both WT D-8-L-Pig and resistance D-8-W-N-Pig. In clone 1 of *S. aureus* 184 a mutation in *rsbU* was seen with D-8-W-N-Pig (Table 3.6). RsbU is a major activator of SigB during stress (Jenul and Horswill, 2019) and both *rsbU* and *sigB* mutations are reported for non-pigmented phenotypes in *S. aureus*.

Table 3. 3. SNPs and INDELs identified in *S. aureus* SH1000 resistance of D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig, and D-8-W-N-Pig.

SNPs and INDELs were detected in three clones (1,2,3) after either two or eight days of interference competition with antimicrobial producer *S. capitis* 47. NCBI protein BLAST was used to provide more information on the hypothetical proteins and the number in bracket indicates the percentage of protein identity. Asterisk corresponds with a termination codon. Shading colour indicates shared SNPs that were found in all clones of the resistant phenotype.

Non-synonymous					
Gene ID (Prokka) SH1000	Gene product description	Genome position	Codon change	Base change	Day of change/ detected in clones No
JEJILOIK _00102	Histidine protein kinase *saeS*	107481	Lys161Glu	T to C	D-2-G-Pig/1,2,3 D-8-G-Pig/1,2,3 D-8-L-G-Pig/1,2,3 D-8-W-N-Pig/1,2,3
JEJILOIK _00284	Putative TrmH family tRNA/rRNA methyltransferase	60361	Phe90Ile	A to T	
JEJILOIK _00379	Hypothetical protein- (100 %) helix-turn-helix (HTH) transcriptional regulator	49726	Glu7*	G to T	
JEJILOIK _00642	Energy-coupling factor transporter ATP-binding protein *ecfA1*	19243	His201Gln	A to T	
JEJILOIK _00673	Guanine/hypoxanthine permease *pbuG*	35475	Gly366Cys	C to A	
JEJILOIK _01315	DNA gyrase subunit B *gyrB*	35185	Leu531Phe	T to A	
JEJILOIK_00226	Hypothetical protein-transposase (100 %)	314	Tyr53fs	CT to C	
JEJILOIK _00716	HTH-type transcriptional activator *rhaS*	75620	Ser639Arg	G to T	D-2-G-Pig/1,2,3
JEJILOIK _01086	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase *thiD*	35562	Cys215Tyr	G to T	
JEJILOIK_00217	Putative ABC transporter ATP-binding protein y*heS*	101293	Glu403Lys	G to A	D-8-W-N-Pig/2,3
JEJILOIK_00477	Putative acetyl-CoA C-acetyltransferase *yhfS*	36253	Glu25Lys	C to T	
JEJILOIK_00551	TVP38/TMEM64 family inner membrane protein *ydjZ*	18977	Gly59*	G to T	
JEJILOIK_00620	Putative ABC transporter ATP-binding protein	98310	Glu137Val	A to T	

JEJILOIK_00715	Hypothetical protein-SarA family transcriptional regulator (100 %)	75551	Ser2Tyr	G to T	
JEJILOIK_02302	Alternate 30S ribosomal protein S14 *rpsN2*	4101	Ile81Asn	A to T	

Table 3. 4. Shared SNPs and INDELs identified in resistance and passaged *S. aureus* SH1000 of D-8-L-G-Pig, and D-8-W-N-Pig.

SNPs and INDELs were detected in three clones (1,2,3) after either competition with antimicrobial producer *S. capitis* 47 or eight days of passaging on BHI Agar. NCBI protein BLAST was used to provide more information on the hypothetical proteins and the number in brackets indicates the percentage of protein identity.

Non-synonymous variant					
Gene ID (Prokka) SH1000	Gene product description	Genome position	Codon change	Base change	Day of change/ Detected in clones No
JEJILOIK_00209	Accessory gene regulator protein A *agrA*	91287	Glu7Lys	G to A	Resistance D-8-L-Pig/1,2,3 WT D-8-L-Pig/1,2,3
JEJILOIK_01111	Phosphoserine phosphatase *rsbU*	62673	Asp264Tyr	G to T	Resistance D-8-W-N-Pig/2,3 WT-D-8-W-N-Pig/1,2,3
JEJILOIK_02402	Hypothetical protein-transposase (100 %)	124	Ter34Glnext*	T to G	WT-D-8-W-N-Pig/3

Table 3. 5. SNPs and INDELs identified in *S. aureus* 184 resistance of D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig, and D-8-W-N-Pig.

SNPs and INDELs were detected in three clones (1,2,3) after either two or eight days of interference competition with antimicrobial producer *S. capitis* 47. NCBI protein BLAST was used to provide mor information on the hypothetical proteins and the number in bracket indicates the percentage of protein identity. Shading colour indicates shared SNPs that were found in 8 out of 12 clones of the resistant phenotype.

Non-synonymous variant					
Gene ID (Prokka) 184	Gene product description	Genome position	Codon change	Base change	Day of change/ Detected in clones No
PILBBNHO_01621	Hypothetical protein-helix-turn-helix (HTH) transcriptional regulator (100 %)	56254	Glu34Asp	C to A	D-8-G-Pig/1,2,3 D-8-W-N-Pig/1,2,3
PILBBNHO_01439	Hypothetical protein-transposase (100 %)	144843	Arg61His	C to T	D-2-G-Pig/1,2 D-8-L-G-Pig/1
PILBBNHO_01370	ATP-dependent dethiobiotin synthetase *bioD*	72758	Asp11Ala	A to C	D-2-G-Pig/3
PILBBNHO_01009	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex *pdhC_2*	114357	Ile38Ser	ATA to CTC	
PILBBNHO_00852	Histidine ammonia-lyase *hutH*	260183	Leu452	T to A	
PILBBNHO_01621	Hypothetical protein-helix-turn-helix transcriptional regulator (100 %)	56276	Arg27Ile	C to A	D-2-G-Pig/1,2
PILBBNHO_01805	Hypothetical protein (100 %)	52693	Ile225Thr	A to G	D-2-G-Pig/1,3
PILBBNHO_01446	Hypothetical protein-alpha/beta hydrolase (100 %)	6512	Ile105Asn	C to T	
PILBBNHO_01990	Ktr system potassium uptake protein B *ktrB_2*	20991	Val203Leu	G to T	
PILBBNHO_01697	Elongation factor G *fusA*	33826	Arg354Cys	C to T	D-8-L-G-Pig/1,2,3
PILBBNHO_01871	ATP synthase subunit beta *atpD*	40359	Ser291Tyr	G to T	
PILBBNHO_00247	Chaperone protein *clpB*	259301	Ala746Val	C to T	D-8-L-G-Pig/1
PILBBNHO_00305	Putative ABC transporter ATP-binding protein	320452	Val137Glu	T to A	D-8-W-N-Pig/2,3
PILBBNHO_01724	Putative acetyl-CoA C-acetyltransferase *yhfS*	69257	Glu25Lys	G to A	D-8-L-G-Pig/2,3

					D-8-W-N-Pig/1
PILBBNHO_02011	Putative ABC transporter ATP-binding protein *yhes*	44530	Lys403Glu	A to G	D-8-W-N-Pig/2
PILBBNHO_01507	Guanine/hypoxanthine permease *pbuG*	53251	Gly366Cys	C to A	D-8-W-N-Pig/2,3

Table 3. 6. Shared SNPs and INDELs identified in resistance and passaged *S. aureus* 184 of D-8-L-G-Pig, and D-8-W-N-Pig.

SNPs and INDELs were detected in three clones (1,2,3) after either competition with antimicrobial producer *S. capitis* 47 or eight days of passaging on BHI Agar. NCBI protein BLAST was used to provide more information on the hypothetical proteins and the number in brackets indicates the percentage of protein identity. Asterisk corresponds with a termination codon.

Non-synonymous variant					
Gene ID (Prokka) 184	Gene product description	Genome position	Codon change	Base change	Day of change/ Detected in clones No
PILBBNHO_00663	Hypothetical protein (100 %)	43921	Asp38Asn	G to A	
PILBBNHO_01439	Hypothetical protein-transposase (100 %)	144843	Arg61His	C to T	WT D-8-L-G-Pig/1,2,3 WT D-8-W-N-Pig/1,2,3
PILBBNHO_01834	RNA polymerase sigma-B factor *sigB*	4198	Glu149*	C to A	WT D-8-W-N-Pig/1,2,3 Resistance D-8-W-N-Pig/2,3
PILBBNHO_02003	Accessory gene regulator protein A *agrA*	34805	Tyr100*	C to A	WT D-8-L-G-Pig/1,2,3
PILBBNHO_02450	Hypothetical protein (100 %)	124	Ter34Glnext*	T to C	WT D-8-L-G-Pig/1,2,3 WT D-8-W-N-Pig/3

3.3.18 Assessing relative growth rates as a measure of fitness of evolved clones

Growth rates of the sequenced *S. aureus* SH1000 clone No 1 of each different phenotypes with respect to inhibition and pigmentation were measured using a growth profiler (960 by Enzyscreen) (Fig **3.13)**. Selected mutations can give a fitness advantage by increasing growth rate and/or resistance and could explain why multiple mutations were observed in the genome sequencing results.

Doubling time was calculated from hours 3 to 7 to compare the exponential growth rates of *S. aureus* SH1000 clones with different phenotypes versus WT. A lower doubling time indicates a higher growth rate. All phenotypes had the lower doubling time and a higher growth rate than WT-day-0 except the resistance day-8-white-non-pigmented (3.13 and table 3.7). Resistance day-8-less-gold-pigment showed the lowest doubling time at 1.120 h with a highest growth rate compared to WT-day-0 which had a doubling time of 1.333 h. According to the doubling time of 1.224 h, WT-day-8-less-gold pigment has the second highest growth rate. Moreover, the resistance day-8-gold-pigment, WT-day-8-white-non-pigmented and resistance day-2-gold-pigment showed a similar doubling time to each other from 1.302 h to 1.312 h and a higher growth rate compared to the WT-day-0. However, resistance day-8-white-non-pigmented has the lowest growth rate with doubling time at 1.718 h.

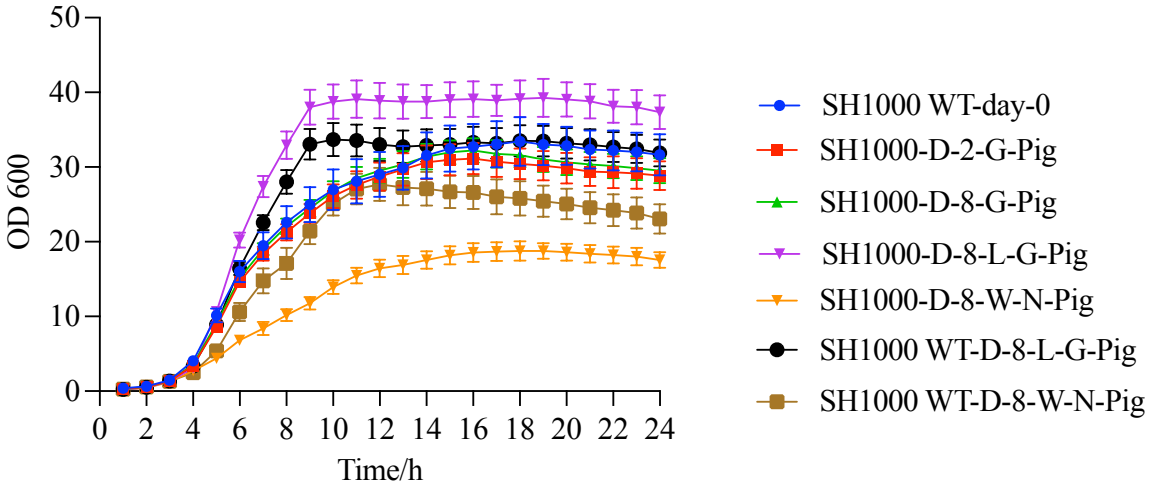

Figure 3. 13. Growth rates of *S. aureus* SH1000 clones with different pigment and resistance phenotypes. Strains were cultured in BHI medium at 37°C inoculated to 0.05 OD600 that was measured hourly for 24 h. *S. aureus* SH1000 of either WT-day-0 (blue circles), *S. aureus* SH1000-WT-L-D-8-G-Pig (black circles), *S. aureus* SH1000 WT-D-8-W-N-Pig (gold square), incomplete resistance *S. aureus* SH1000-D-2 (red square), complete resistance *S. aureus* SH1000-D-8-G-Pig (green triangle), complete resistance *S. aureus* SH1000-D-8-L-G-Pig (purple triangle), complete resistance *S. aureus* SH1000-D-8-W-N-Pig (orange triangle). Data are presented as mean of 6 biological replicates +/- STDEV.

Table 3. 7. Doubling time of *S. aureus* SH1000 with different phenotypes.

The doubling time for the growth rates (Fig. 3.13) calculated from hours 3 to 7 (5 h) representing the mean values from 6 biological replicates.

Strain	Duration of growth/h	Doubling time/h
Resistance SH1000-D-8-L-G-Pig	5	1.120
WT-D-8-L-G-Pig		1.224
Resistance D-8-L-G-Pig		1.302
WT-D-8-W-N-Pig		1.308
Resistance D-2-G-Pig		1.312
WT-day-0		1.333
Resistance D-8-W-N-Pig		1.718

3.4 Discussion

The aim of this chapter was to investigate 42 CoNS for their ability to produce antimicrobial activity that inhibited growth of *S. aureus*. The major focus was on undescribed antimicrobial gene clusters of *S. capitis* from a comparative genomics study (Chong et al., 2022). Antimicrobial activity of CoNS was determined only by the absence and presence of an inhibition zone surrounding the antimicrobial producer strains in a deferred growth inhibition assay used successfully previously (Moran et al., 2016). To study the resistance mechanism, experimental evolution of interference competition was used to select resistance in *S. aureus* to the antimicrobial producer. Whole genome sequencing of *S. aureus* was used to identify mutations responsible for variations in growth inhibition by the antimicrobial producer.

3.4.1 Antimicrobial activity

Based on the assay screening, 4 CoNS strains were found to have antimicrobial activities with different inhibition profiles against 8 isolates of *S. aureus*. Although 38 CoNS strains did not show any activities towards *S. aureus* (Fig 3.2), they may target CoNS, but this was not tested further here. The antimicrobial producers identified here included *S. capitis* 47, *S. lugdunensis* 1007, 211421 and 211681, and these were investigated in the context that CoNS can be a source of antimicrobials and compete with *S. aureus* on human skin (Paharik et al., 2017).

Previous studies have demonstrated that inhibitor producer strains show self-resistance to their own or related antimicrobials (Krauss et al., 2020). The antimicrobial producer strains *S. epidermidis,* B14, IVK8, 273, Tü3298, 039331N1 and *S. capitis* 47 and *S. lugdunensis* 1007*,* 211681 and 211421 were not inhibited by epifadin producer *S. epidermidis* B155, but several of these strains were inhibited by a second epifadin producer *S. epidermidis* IVK83 (Fig 3.4), suggesting some other antimicrobial related difference between these epifadin producers. A second antimicrobial activity could be present in *S. epidermidis* IVK83. *S. epidermidis* B14

was able to inhibit all strains but not the epidermin producer *S. epidermidis* Tü3298, suggesting the strain B14 antimicrobial is either epidermin or a similar molecule exported by the epidermin biosynthesis-resistance module of strain Tü3298; alternatively, Tü3298 has an efflux system distinct from its epidermin resistance. Otherwise, the *S. epidermidis* strains appeared to be producing different antimicrobial activities exemplified with *S. epidermidis* 273 antimicrobials not inhibiting *S. lugdunensis*. The *S. lugdunesis* were also not inhibited by either epifadin producers *S. epidermidis* IVK83 or B155 but were inhibited by Tü3298 epidermin producer and B14. *S. capitis* 47 was found to inhibit all species strains except *S. lugdunesis* 1007 indicating a potential similar resistance mechanism in the latter. *S. lugdunensis* 1007*,* 211681 and 211421 did not show any inhibitory activities against each other indicating that all *S. lugdunensis* may produce the same antimicrobial, such as the described lugdunin. Although these results suggest that antimicrobial-producing strains may share similar antimicrobials or resistance mechanism this relationship remains to be determined.

3.4.2 The effect of antimicrobial producer *S. capitis* 47 upon *S. aureus* SH1000 and 184

Human skin can be populated by a highly diverse community of bacteria that interact and compete for the purpose of colonisation and survival (Brown and Horswill, 2020). CoNS are reported to compete with *S. aureus* on the skin by producing a group of small-molecule products, such as AIPs and AMPs (Horswill, 2019; Severn et al., 2022). To increase knowledge about *S. capitis* strains that compete via AMPs on the skin, antimicrobial producer *S. capitis* 47 was selected for further investigation. Although *S. capitis* strains can produce antimicrobials such as J Nisin or capidermicin (O'Sullivan et al., 2020; Lynch et al., 2021, Chong et al., 2022), the antimicrobials encoded by BGCs observed in genome comparisons of *S. capitis* are still not well understood. Therefore, to provide more information about the effect of antimicrobial

produced by *S. capitis* 47, an experimental evolution of interference competition between antimicrobial producer *S. capitis* 47 and *S. aureus* strains SH1000 and 184 was performed.
In the experimental evolution experiment, numbers of cells in the monoculture control cultures were greater than when grown in cocultures, suggesting that the species were competing, and growth yield was limited by resources of BHI medium and potentially antimicrobial activity of *S. capitis* 47 against *S. aureus* (Fig 3.6). In the cocultures, antimicrobial producer *S. capitis* 47 likely limited growth of *S. aureus* SH1000 and 184 in the first two days of the competition based on reduced population sizes. Subsequently, the populations of *S. aureus* SH1000 and 184 broadly doubled in number. Following continued exposure to antimicrobial produced by *S. capitis* 47 on BHI agar there was evolution of resistance either incompletely by day two or completely by day eight. The phenotype at day two was determined visually.

3.4.3 Evaluation of *S. aureus* to antimicrobial produced by *S. capitis* 47

AMPs produced by CoNS against pathogenic bacteria that causes soft-tissue infection (SSTI) such as *S. aureus* have potential for beneficial use (Parlet, Brown and Horswill, 2019). However, the use of bacteriocins as therapeutically active molecules is limited mainly due to difficulties in production, purification, delivery systems and regulatory approvals. Therefore, development of resistance must be addressed and can be used to increase our knowledge about these antimicrobials (Schofs et al., 2020). Through the study presented here, *S. aureus* SH1000 and 184 were experimentally evolved to resistance (Fig 3.7). *S. aureus* can become resistant to antimicrobials in various ways. These mechanisms include reducing drug absorption, altering the drug target, enzymatic inactivation, and active efflux (Yılmaz and Aslantaş, 2017). Although no mutations were found in TCS genes encoding known antimicrobial peptide defence regulators, such as BraRS that mediates resistance to nisin A and nukacin ISK-1 (Bleul, Francois and Wolz, 2021), other mutations were detected. There were common non-

synonymous SNPs detected in both *S. aureus* SH1000 and 184 clones with resistance to the antimicrobial producer *S. capitis* 47 (table 3.3 and 3.5). Notably, in all the clones of resistant *S. aureus* SH1000 and 8 out of 12 resistant clones of resistant *S. aureus* 184, the standout genetic change selected that could account for the resistance phenotype was observed in a gene encoding a Cro/CI-like repressor. Comparing *S. aureus* and SH1000 and 184 resistance clones, mutation differences were found with a termination codon at amino acid position 7 in the Cro/CI repressor gene in all the resistant clones of *S. aureus* SH1000 while two non-synonymous SNPs were identified with strain 184.

In *S. aureus*, a Cro/CI homolog repressor controls the expression of an operon required for resistance to an inhibitor compound of type I signal peptidase SpsB (Morisaki et al., 2016). This novel arylomycin antibiotic inhibits the SpsB membrane-bound protein and has an MIC ~1 ug/ml but has low potency in *vivo*. It was reported that the Cro/CI encoding operon consists of four genes, one of which is *cro/cI* itself (Morisaki et al., 2016), plus, genes *SAUSA300_0351*, *SAUSA300_0352* and *SAUSA300_0353* that encode putative ABC transport proteins. In their study, a wide range of *cro/cI* gene mutations including stop codon mutations were identified to be associated with arylomycin compound resistance, including the Glu34Asp mutation identified here. The ABC transporters are a large class of transmembrane proteins that serve a variety of functions in the efflux of diverse compounds, including antimicrobials (Orelle et al., 2019).

In the study here as with the Morisaki et al study, the stop codon mutation of the Cro/CI repressor homologue gene in the *S. aureus* SH1000 resistant clones and the Glu34Asp mutation in the resistance clone of *S. aureus* 148 would derepress the operon enabling expression of the ABC transport proteins. The finding of multiple mutations in the same gene in the study here is strong evidence that the loss of repression by the Cro/CI repressor could be a key resistance mechanism to *S. capitis* 47 antimicrobial. Future research should involve allelic replacement

of the *cro/cI* gene in both strains to confirm its functionality in competition with *S. capitis* 47 and screen for other antimicrobials that this might functionally relate to mechanistically. Furthermore, inactivation of the ABC operon in the *cro/cI* mutant should restore sensitivity to the *S. capitis* 47 antimicrobial.

In addition to mutations of *cro/cI*, a mutation in *pbuG* was identified in 14 out of 24 clones, with different observed substitutions. In previous studies, a *pbuG* inactivation mutation was reported for resistance to azaguanine and recently to oxacillin in *B. subtilis* (Saxild et al., 2001; Salazar et al., 2002; Nolan et al., 2022). There is evidence that purine biosynthesis plays a part in the development of resistance to vancomycin and daptomycin, two antibiotics with cell wall and membrane targets (Salazar et al., 2002). MRSA can become resistant to oxacillin and other B-lactam antibiotics if it develops gene mutations that affect purine biosynthesis (*pur* operon) or transport (*nupG/pbuG*). Mechanistically, levels of small cyclic molecules called c-di-AMP dinucleotides have been reported to be significantly reduced when MRSA is exposed to purine nucleosides, such as guanosine (Nolan et al., 2022). The c-di-AMP was recently discovered to play a role in maintaining osmotic pressure, responding to DNA damage, regulating central metabolism, forming biofilms, and avoiding acid stress, all of which are required for B-lactam resistance (Zarrella et al., 2020; Nolan et al., 2022). Although it is not yet known whether specific c-di-AMP transporters belong to the family of efflux pumps (Nolan et al., 2022), mutations found in the *pbuG*-related purine synthesis in *S. aureus* may have occurred to increase c-di-AMP for a resistance-related function via its transport. However, it remains to be determined that resistance to the antimicrobial produced by *S. capitis* 47 is affected directly by NupG/PbuG gene function or the Gly366C amino acid change in the study here.

Further mutations in all the SH1000 clones were found in transporter gene *ecfA1* and in *gyrB* gene encoding DNA gyrase. The EcfA1 transporter is associated with import of nutrients and

metals demonstrating a fundamental role in the ability of cells to adapt to surrounding condition (Alhareth et al., 2022). Bacterial DNA gyrase is a key target of antibiotics ciprofloxacin and norfloxacin, play an important role in DNA replication. The *ecfA1* and *gyrB* gene variants were not observed in resistant clones of *S. aureus* 184, therefore, additional studies are needed to verify these gene functions in either resistance or nutrient acquisition to determine their contribution.

Other mutations seen in the *yhfS* and *bioD* genes are involved in the biotin biosynthetic pathway, which is an essential nutrient for bacteria. These mutations happened multiple times but not across all the replicates of resistant *S. aureus* SH1000 and 184. One explanation for these mutations is bacterial competition for biotin due to importance in several essential metabolic processes during adaptation to surrounding environment (Machado et al., 2019).

For some resistant clones of *S. aureus* SH1000 and 184, mutations were found in the genes encoding the proteins, RpsN2 and KtrB2 that control metals such as zinc and potassium (Grim et al., 2020; Do and Gries 2021). A crucial element, zinc has a role in key enzymes of cell metabolism. However, zinc is also toxic and must be efflux when in excess to maintain cell viability (Capdevila et al., 2016). Moreover, in bacterial growth and division, potassium is required to maintain the pH, membrane potential, and osmotic pressure (Sweet et al., 2021). Thus, it is suggested that these mutations occurred due to the central roles of potassium and zinc in the survival of *S. aureus* during competition. Many AMPs such as nisin, epidermin or gallidermin act mostly by creating pores in bacterial membranes that cause small molecules to flow into and eventually damage the cell (Wang et al., 2020). Although the activation of these proteins can be related to the antimicrobial produced by *S. capitis* 47 against *S. aureus* SH1000 or 184, the function of these proteins in resistance needs further investigation.

3.4.4 Antimicrobial produced by *S. capitis* 47

According to recent studies, *S. aureus* can use TCS for antimicrobial resistance. For instance, *S. aureus* regulates resistance to bacitracin, nisin A and nukacin ISK-1 by the TCS BraSR. Vancomycin-resistance in *S. aureus* was found to be caused by mutations of TCS, BraSR, GraRS, and WalK. (Bleu, Francois, and Wolz, 2021). According to Ghabban (2019), epifadin antimicrobial has been linked to DesKR TCS, which was confirmed in this study (Chapter 4). Moreover, Gallidermin resistance reported mutation in three-component system VraH (Popella et al., 2016). In this study, none of the above mutations were found in resistant *S. aureus* SH1000 or 184 that occurred due to the antimicrobial producer *S. capiti*s 47. *S. aureus* SH1000 or 184 mutations to the antimicrobial producer *S. capitis* 47 was tested against other known or unknown antimicrobial producers. All the mutations of *S. aureus* SH1000 and 184 were tested against 10 additional antimicrobial producers, including *S. epidermidis* B155 producing novel inhibitor epifadin (Ghabban 2019), *S. epidermidis* 039331N1 producing a novel inhibitor (Alrajeh, 2022), Tü3298 epidermin producer (Moran and Horsburgh, 2016; Ebner et al., 2018), and *S. epidermidis* B14 and 273 with undescribed inhibitors. The mutations selected were specific to resistance towards antimicrobial produced by *S. capitis* but none of the other tested antimicrobials. Since the identified mutations in *S. aureus* SH1000 and 184 associated with resistance to *S. capitis* 47 antimicrobial are not those known mechanisms (including the TCS GraSR, BraSR, VraSR, WalKR, DesKR or VraH), it is possible that the antimicrobial produced by *S. capitis* 47 differs among the antimicrobials secreted by staphylococci. The identified *S. capitis* 47 antiSMASH clusters showed 27 % similarity to Gallidermin (table 3.1). *S. capitis* APC 2923 is reported to be a producer of a novel antimicrobial, J Nisin (O'Sullivan et al., 2020). More recently, *S. capitis* CIT060 was shown to produce capidermicin with inhibitory activity against a variety of Gram-positive bacteria, including *Lactococcus*, *Streptococcus* and *Staphylococcus* (Lynch et al., 2021). According to antiSMASH version 6.0, *S. capitis* APC

2923 and *S. capitis* 47 contain lanthipeptides that may lead to the same antimicrobial activity. Further studies could determine whether *S. capitis* producing antimicrobial J Nisin or capidermicin select for the same mutations in *S. aureus* SH1000 and 184 as those identified here with antimicrobial produced by *S. capitis* 47.

3.4.5 Altered growth and loss of pigmentation

3.4.5.1 Agr

In *S. aureus*, staphyloxanthin is golden yellow colour carotenoid pigment located in the plasma membrane and serves as an antioxidant from host mediated immune responses and other environmental stressors. Staphyloxanthin provides structural integrity to the membrane and is associated with survival of bacteria under harsh environments (Valliammai et al., 2021). During the selection experiment, in addition to the WT gold pigmented strain there were colonies that were less gold pigmented or white non-pigmented (Fig 3.8) at the eighth day of competition, in both the control and coculture populations. Reduced staphyloxanthin by thymol treatment was reported to increase the sensitivity of MRSA membranes to polymyxin B (Valliammai et al., 2021). However, in this study, the reduction of pigmentation was seen in repeated passage of *S. aureus*. This less gold pigmentation was associated with *agrA* mutants in 9 out of 12 lesser gold clones of *S. aureus* (tables 3.4 and 3.6). The *agrA* mutation was also reported by Ghabban (2019) in less gold pigmented *S. aureus* clones. The *agr* mutation was not seen in the presence of the *sigB* mutant supporting hypothesis that SigB could suppress the Agr system (Ersoy, et al., 2021).

3.4.5.2 SigB

A *sigB* mutation was detected in *S. aureus* 184 white clones that were isolated from the eighth day of both WT and resistance phenotypes (tables 3.6). In *S. aureus*, SigB is the global regulator of the expression of stress responsive genes. It is known to regulate as many as 251

genes, including genes with functions in virulence, biofilm formation, persistence, cell internalisation, membrane transport, and antibiotic resistance (Guldimann et al., 2016). SigB recruit's RNA polymerase to promoters of the staphyloxanthin pathway operon and as a result, the *S. aureus sigB* mutants are deficient in carotenoid pigment. It was recently reported that the glutamine-225 in SigB is critical for the regulation of staphyloxanthin production as its substitution with proline-led to non-pigmented colonies of *S. aureus* (Pannu et al., 2018). In *S. aureus,* stress-activated SigB has been reported to be involved in adaptation to different environments and resistance to active cell wall antibiotics, such as beta-lactamase and vancomycin (Zhang et al., 2019). The SigB mutant located in three clones of 184 WT-day-8-less-gold-pigment (tables 3.6). Although it is unclear why SigB was activated, it is suggested this mutant may occurred as an adaptation to daily passing on BHI agar.

The *rsbU* gene has been reported to be a major activator of SigB during stress (Jenul and Horswill, 2019). A mutation in the *rsbU* gene was seen within 5 out of 6 clones of white non-pigmented *S. aureus* SH1000 WT/resistance (table 3.4) which supports that *rsbU* gene is necessary for the activation of SigB (Horsburgh et al., 2002). Notably, *S. aureus* SH1000 WT-day-8-white-non-pigmented which is associated with *sigB* mutation had a significant reduction in the growth rate compared to WT **(**Fig 3.13). The SH1000 WT-day-8-white-non-pigmented, which was not exposed to the antimicrobial produced by *S. capitis* 47 did not show resistance. However, a shrink by ~ 5 mm in the zone size was realised and linked with increase in the growth rate compared to the WT (Fig 3.9 and 3.13), indicating that self-activation of *sigB* mutation allowed *S. aureus* SH1000 to be more competitive. To understand the *sigB* gene contributions, more studies are required.

3.5 Conclusion

The key rationale for this study comes from the treatment challenges that *S. aureus* infections create for the medical community, notably skin infections, and their difficulty in being cured. This chapter was started by screening CoNS isolates for their antimicrobial activities using a deferred growth inhibition assay. Four inhibitory strains, including *S. capitis* 47 and *S. lugdunensis* 1007, 211681, and 211421, were found to be active against eight isolates of *S. aureus*.

Experimental evolution of interference competition was used to study unidentified antimicrobial produced by *S. capitis* 47 in competition with *S. aureus* SH1000 and 184. Whole-genome sequencing was used to investigate selected mutations. The repressor gene *cro/cI* was identified in evolved mutations of both *S. aureus* SH1000 and 184. An efflux transporter's regulation by the Cro/CI repressor has previously been shown, supporting the idea that *S. aureus* could take advantage of this efflux pump system to survive this antimicrobial activity in competition with other producing species. Finally, further mutations that decreased pigment production were selected in the *agr* and *sigB* genes via serial passages of the *S. aureus* SH1000 and 184 strains.

Chapter 4: Experimental evolution with *Staphylococcus* (CoNS) species as a tool to interrogate interference competition

4.1 Introduction

Several species of *Staphylococcus* are significant opportunistic pathogens and are found in the microbiomes of animals and humans. Antimicrobial resistance (AMR) has emerged as a worldwide public health concern, posing a substantial threat since it severely restricts treatment choices. Methicillin-resistant *Staphylococcus aureus* (MRSA) is among the most common antibiotic-resistant threats that can cause serious infections (Belhout et al., 2022). *S. epidermidis* is the most frequently isolated species of the CoNS and has been extensively studied for its inhibitory products targeting *S. aureus* (Paharik et al., 2017; Otto, 2009; Kollef et al., 2021). The study of competition between *S. aureus* and CoNS is less pronounced than that of *S. epidermidis*. CoNS were found to be a rich source of antimicrobial peptides (AMPs) that have not yet been well studied and represent a potential wealth of mechanistic information about interactions between microorganisms and the genetic basis of successful colonisation of human skin, which correlates with the development of resistance to antimicrobial. The effect of AMPs acting against closely related bacteria greatly aid in understanding the global rise in antimicrobial resistance of bacteria (Brown and Horswill, 2020; Wang, et al, 2020).

Lugdunin is an antimicrobial produced by *S. lugdunensis* IVK28 that was proposed as a novel compound to prevent staphylococcal infections (Zipperer et al., 2016). *S. lugdunensis* IVK28 was tested in *vivo* by co-inoculation into rat noses with *S. aureus*. The outcome of these experiments showed that significantly fewer *S. aureus* cells were regained from animals co-colonised by IVK28 wild type (WT) compared to those co-colonised with IVK28 which had the genes deleted for lugdunin antimicrobial activity. Lugdunin was shown to have no risk of resistance in a mouse skin infection model. Due to less *S. aureus* cells being retrieved from

animals co-colonised by IVK28 WT compared to those co-colonised with epifadin mutant antimicrobial producer *S. epidermidis* IVK83 Δ*efiTP* over the 5 days, it has been proposed that it may be particularly difficult for *S. aureus* to develop resistance to lugdunin (Zipperer et al., 2016).

Methicillin-resistant *S. aureus* (MRSA) USA300, a designated strain by the Centers for Disease Control and Prevention (CDC), has been identified as the primary type that causes skin and soft tissue infections (SSTIs) in most countries around the world the world (King et al., 2006; Malachowa et al., 2022). The USA300 isolates were methicillin-resistant, however, resistance to mupirocin, tetracyclines, vancomycin, macrolides, and fluoroquinolones, as well as daptomycin, has developed over time, as has cross-resistance to gentamicin and trimethoprim-sulfamethoxazole. Due to these diverse antibiotic resistances, whole genome sequencing (WGS) of USA300 isolates was used to assess antimicrobial resistance phenomena. Amongst the USA300 isolates, 73.5% were community-associated, 21.3% healthcare-associated, and 5.2% with unknown acquisition. Erythromycin had the highest non-lactam resistance rate (86%), followed by fluoroquinolones (68-69%). 57% of the isolates were resistant to fluoroquinolone and erythromycin (Enström et al., 2018). Investigating the resistance mechanisms of AMPs produced by bacteria from the same primary niche will increase our knowledge of the relationship between the strains and their evolution over time (Belhout et al., 2022).

4.2 Aims

Given the outcomes of the previous chapter identifying resistance mutations from competition with *S. capitis* 47, the aim of this chapter was to examine alternative antimicrobial producer CoNS and to use whole genome sequencing to identify mutations responsible for variations in these strains' resistance to the antimicrobial products. This framework would allow for

exploration of the types of mutations that are selected during competition, both common mutations from competition events and those determined by alternative selection due to different antimicrobial producers. Previously competition between *S. epidermidis* B155 epifadin producer and *S. aureus* SH1000 was done to identify resistance mutations (Ghabban, 2019). Here, firstly, competition was established between the epifadin antimicrobial producer *S. epidermidis* IVK83 (Torres Salazar et al., 2023) and *S. aureus* USA300 to look for similarities in resistance outcomes. Second, competition between antimicrobial producers *S. lungdunensis* 1007 and *S. aureus* USA300 was performed as a comparator due to the report that resistance mutations do not emerge (Zipperer et al., 2016) and this would provide a useful comparator dataset of mutations relating to increased competition fitness.

4.3 Results

4.3.1 Experimental evolution of interference competition between antimicrobial producer *S. epidermidis* IVK83 and *S. aureus* USA300

An interference competition experiment between the epifadin producer *S. epidermidis* IVK83 and *S. aureus* USA300 was set up to select for resistance. *S. epidermidis* IVK83 and *S. aureus* USA300 were cultured together on BHI agar with controls of monoculture to distinguish the effects of repeated daily culturing.

Growth yield changes of both the monoculture and coculture were calculated from viable counts using the Miles and Misra technique. Pigmentation and morphology were used to distinguish between the species. The competition experiment occurred over five days to identify outcomes. According to viable counts (CFU), the monoculture of each species showed a similar dynamic of growth in contrast to coculture (Fig 4.1). Compared with *S. epidermidis* IVK83, *S. aureus* USA300 grew stably in monoculture for five days with a significantly higher population (~3.5 x 10^9 cfu ml^{-1}) than *S. epidermidis* IVK83 (~1.8 x 10^9) on BHI medium (P value < 0.001). During coculture after the first day, *S. epidermidis* IVK83 produced a significantly greater population (~ 6.6 x 10^8 cfu ml^{-1}) than *S. aureus* USA300 (~ 5 x 10^7 cfu ml^{-1}) (P value < 0.001) however, from the second day the *S. epidermidis* IVK83 population started to decline until the fourth-day conclusion of competition at ~ 5 x 10^7 cfu ml^{-1} when it had a significantly lower yield compared to the first day (P value < 0.001) followed by its absence at day five. In contrast, the *S. aureus* USA300 population increased from ~ 1 x 10^8 cfu ml^{-1} on day one and further through day two to three to reach ~ 2.1 x 10^9 cfu ml^{-1} on day four and was significantly greater on day five compared to the day 1 population (P value < 0.001) (Fig 4.1).

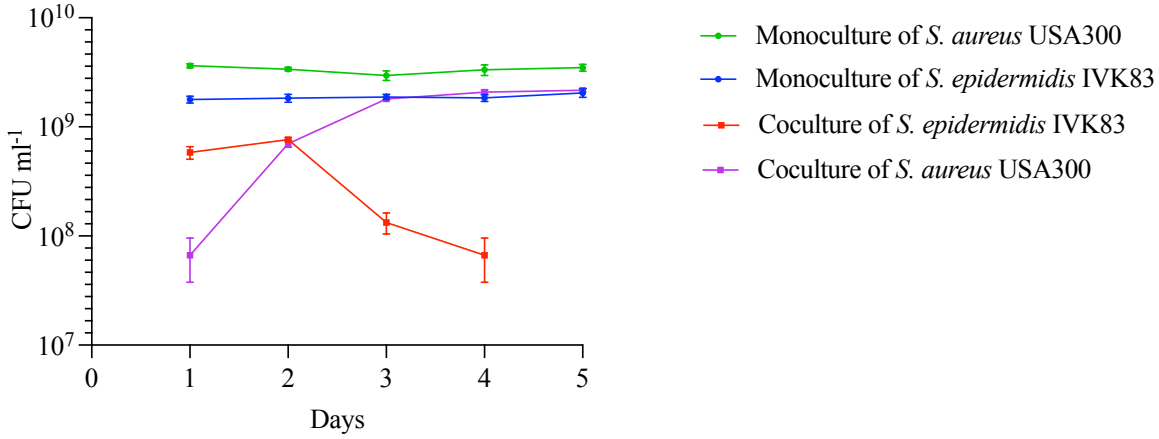

Figure 4. 1. Competition dynamic of antimicrobial producer *S. epidermidis* IVK83 and *S. aureus* USA300. Strains were cultured in monoculture and coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar. Viable counts were used to determine population changes. Monoculture of *S. aureus* USA300 control (green circle). *S. aureus* USA300 when cocultured with *S. epidermidis* IVK83 (purple square). Monoculture of *S. epidermidis* IVK83 control (blue circle). *S. epidermidis* IVK83 when cocultured with *S. aureus* USA300 (red square). Values are the mean of three biological replicates counted in triplicate. Error bars represent mean +/- STDEV across the three replicates with t-test analysis for statistical significance. Y-axis was presented in log 10 scale.

4.3.2 *S. aureus* USA300 evolved resistance to antimicrobial producer *S. epidermidis* IVK83

Ten colonies of *S. aureus* USA300 were selected from daily competition and tested for their resistance using the deferred growth inhibition assay. Comparing the antimicrobial producer strains (WT/control), the presence and absence of an inhibitory zone around the antimicrobial producer strains was examined. *S. aureus* USA300 clones from the chosen colonies of days one and two of the competition were inhibited by *S. epidermidis* IVK83 and displayed no change in inhibition zone (0.5 mm) compared to the WT (Fig 4.2). Conversely, colonies of *S.*

aureus USA300 that were selected from day three of the competition showed resistance (USA300-D-3) to the antimicrobial epifadin producer *S. epidermidis* IVK83 with no inhibition zone in comparison to the WT (Fig 4.2).

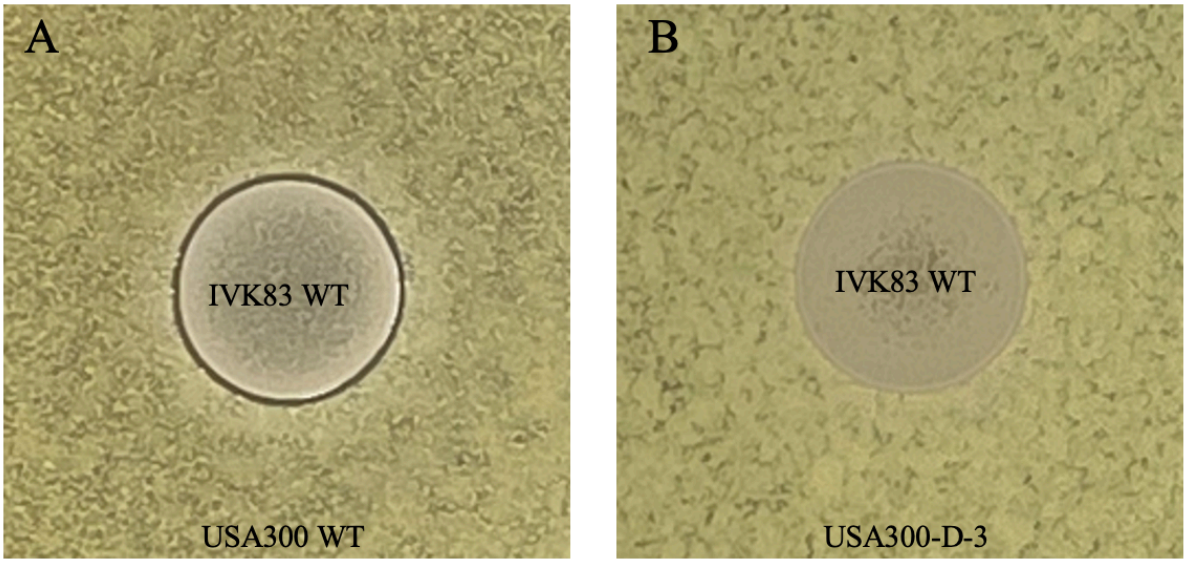

Figure 4. 2. *S. aureus* USA300 evolved resistance to antimicrobial p*roducer S. epidermidis* IVK83. Deferred growth inhibition assay was used to determine the antimicrobial activities. *S. epidermidis* IVK83 WT (epifadin producer) was cultured as a spot of overnight culture on BHI agar plate and after 24 h of incubation at 37ºC, the plate was sprayed with either WT or *S. aureus* from day 3 of competition (USA300-D-3). (A) *S. epidermidis* IVK83 WT zone of inhibition (0.5 mm) against USA300 WT (B) *S. aureus* USA300-D-3 from day three of competition showed resistance to *S. epidermidis* IVK83 WT.

4.3.3 Experimental evolution of interference competition between epifadin mutant *S. epidermidis* IVK83 Δ*efiTP* and *S. aureus* USA300

As a measure of comparison for the effect of the epifadin-derived selection from *S. epidermidis* IVK83, a mutant strain with a mutation of genes responsible for its biosynthesis was used in the competition experiment. *S. epidermidis* Δ*efiTP* produced no zone of inhibition against *S.*

aureus USA300 on BHI agar (Fig 4.3). Coculture of *S. epidermidis* IVK83 Δ*efiTP* and *S. aureus* USA300 was done on BHI agar as described above in chapter 2. Controls of monoculture were included as comparators.

According to viable counts (CFU), the monocultures of each species showed a dissimilar growth yield during competition compared with the coculture. Both species grew for three days with consistent population yields on each day in monoculture on BHI medium (*S. aureus* USA300, ~ 3.8 x 10^9 cfu ml^{-1}) (*S. epidermidis* IVK83 Δ*efiTP* (~ 2.7 x 10^9 cfu ml^{-1}). For coculture, *S. aureus* USA300 had a higher growth yield (~ 3 x 10^9 cfu ml^{-1}) than *S. epidermidis* IVK83 Δ*efiTP* (~ 1.3 x 10^8 cfu ml^{-1}) on both the first and second day (P value < 0.001). By the third day *S. epidermidis* IVK83 Δ*efiTP* disappeared from the competition while the *S. aureus* USA300 population number remained similar to previous days yields (Fig 4.3).

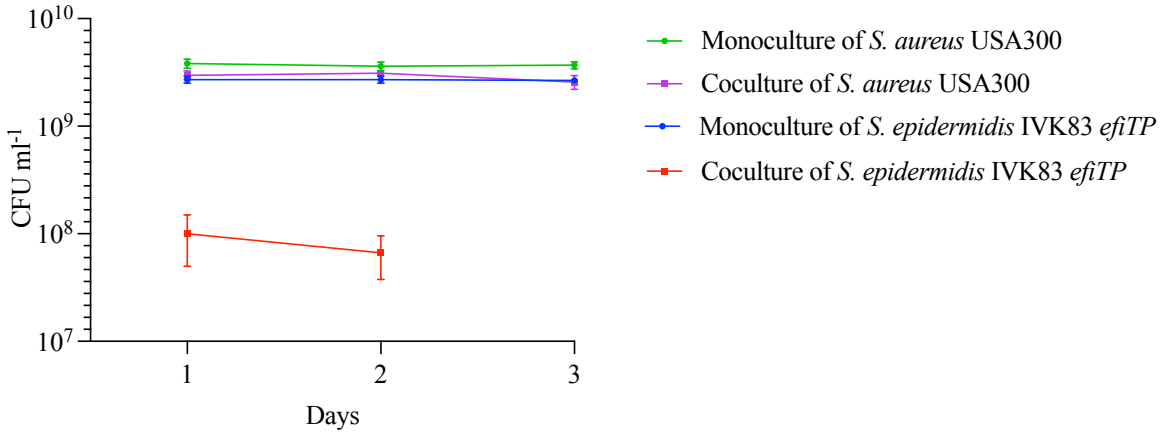

Figure 4. 3. Competition dynamic between epifadin mutant *S. epidermidis* IVK83 *ΔefiTP* and *S. aureus* USA300. Strains were grown in monoculture or coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar as part of the experimental evolution procedure. Viable counts were used to determine growth dynamics. Monoculture of *S. aureus* USA300/control (green circle). *S. aureus* USA300 when cocultured with *S. epidermidis* Δ*efiTP* (purple square). Monoculture of *S. epidermidis* Δ*efiTP*/control (blue circle). *S. epidermidis*

IVK83 Δ*efiTP* when cocultured with *S. aureus* USA300 (red square). Values are the mean of three biological replicates counted in triplicate. Error bars represent mean +/- STDEV across the three replicates with t-test analysis for statistical significance. Y-axis was presented in log 10 scale.

4.3.4 *S. epidermidis* IVK83 Δ*efiTP* does not select for epifadin resistance in *S. aureus* USA300

Several colonies of *S. aureus* USA300 selected daily from the competition were examined using the deferred growth inhibition assay. Comparing the antimicrobial producer strains (Wild type/control), the presence and absence of an inhibitory zone around the antimicrobial producer strains served as indicators of resistance and sensitivity. *S. aureus* USA300 from days one, two and three of the competition was sensitive (USA300-no resistance D-3) to *S. epidermidis* IVK83 WT and displayed no zone change in comparison to the 0.5 mm inhibition zone of the WT (Fig 4.4).

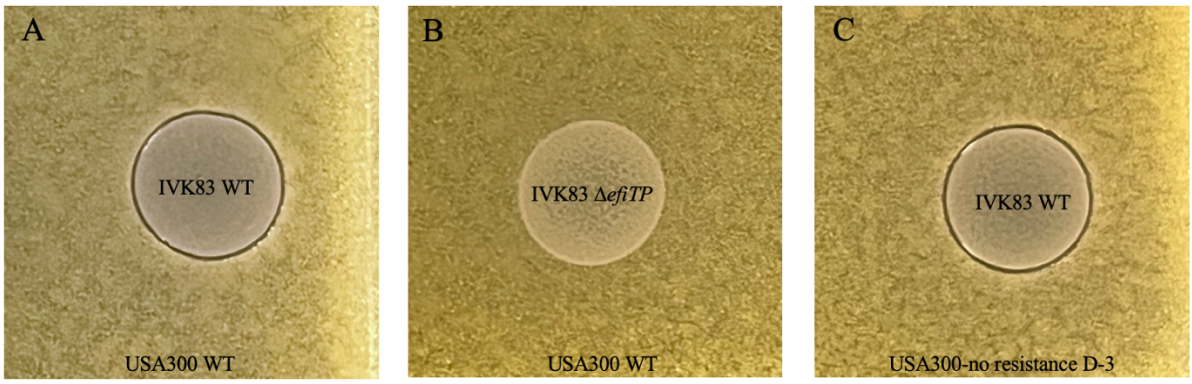

Figure 4. 4. Epifadin mutant *S. epidermidis* IVK83 *ΔefiTP,* does not select for resistance in *S. aureus* USA300. Deferred growth inhibition assay was used to determine the antimicrobial activities. Epifadin producer *S. epidermidis* IVK83 WT or epifadin mutant *S. epidermidis* IVK83 Δ*efiTP*, were cultured as a spot of overnight culture on BHI agar and after 24 h of incubation at 37°C, the plate was sprayed with either WT or the *S. aureus* USA300-no

resistance day-3 clone from day 3 of competition. (A) *S. epidermidis* IVK83 WT showed a zone of inhibition (0.5 mm) against *S. aureus* USA300-WT. (B) *S. epidermidis* IVK83 Δ*efiTP* showed no zone of inhibition against *S. aureus* USA300 WT. (C) *S. epidermidis* IVK83 WT showed a zone of inhibition (0.5 mm) against *S. aureus* USA300-no resistance D-3.

4.3.5 Whole Genome Sequencing

To evaluate the resistance selection phenomenon and its absence with the epifadin mutant co-coculture clones, colonies of *S. aureus* USA300 either WT, evolved or non-evolved isolated from the competition experiment were sequenced to identify its genetic basis. Three clones selected from three biological triplicates were sequenced by Microbes NG, Birmingham and whole genome sequencing was done using the Illumina platform methods (section 2). Three clones were sequenced from each selected day (Table 4.1).

Table 4. 1. The selected colonies and days.

S. aureus Strain /Sample ID	Selection	Sequence number of clones	Day
USA300 WT-day-0	None	3	0
USA300 WT-day-3	None	3	3
USA300-day-3-epifadin resistance	IVK83 WT and USA300 WT	3	3
USA300-no resistance day-3	IVK83 Δ*efiTP* and USA300 WT	3	3

4.3.6 Genome Sequence Changes

The Snippy tool was used to identify genomic variations arising from the selection experiment. Sequence reads were aligned with the USA300 WT-day-0 which was used as a reference genome (the genes ID presented in the tables below was from the self-annotated reference genome). The identified single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were curated to investigate selection that had occurred in *S. aureu*s USA300. Changes in genes that were present in both competition control and evolved clones are listed in tables 4.2, 4.3 and 4.4.

4.3.6 .1 Mutations identified in epifadin resistant *S. aureus* USA300

Five non-synonymous SNPs were identified in USA300-day-3-epifadin resistance and detected in all the sequenced clones (1,2 and 3). A SNP was identified in the *yezG_1* gene (also named *yeeF/yezG*), encoding a member of the polymorphic toxin family in *Bacillus* (*B*) *subtilis* that has been hypothesised to play a role in biofilm competition between *B. subtilis* strains. However, the biological function of YezG in *B. subtilis* remains unclear (Kobayashi et al*.,* 2021). A second SNP was present in *yhdG_1*, encoding an amino acid permease. In *Streptococcus mutans*, a nonsynonymous mutation in the gene encoding the permease YhdG has been linked to metabolism. (López et al*.,* 2020).

Notably, a further polymorphism was identified in the *desK* gene encoding a poorly characterised TCS. Relatedly, a SNP in this putative sensor kinase of the DesKR TCS was reported by Ghabban, (2019) and confirmed to be involved in resistance to epifadin antimicrobial. Therefore, as *S. epidermidis* IVK83 is another epifadin producer strain (Torres Salazar et al., 2023 unpublished), it is suggested that the *desK* mutation is responsible for causing resistance in the sequenced *S. aureus* USA300-day-3 clones. Another mutation located

in the *apt* gene was identified in each of the sequenced clones (1,2,3). The *apt* gene encodes a protein for uptake of exogenous adenine for growth of *S. aureus*.

4.3.6.2 Other SNPs of *S. aureus* USA300

Within the experimental evolution experiment selecting for epifadin resistance, both *S. aureus* USA300 WT-day-3 which was passaged alone on BHI agar for three days and *S. aureus* USA300-no resistance day-3 that was isolated from the coculture with *S. epidermidis* IVK83 Δ*efiTP* (epifadin mutant) for three days were sequenced as controls. This fundamental step was prepared to discount any common mutations unrelated to the resistance of epifadin.

In *S. aureus* USA300 WT-day-3, four non-synonymous variants were identified. Of these, SNPs were found in genes *yezG_1* gene and *yhdG_1*for all clones enabling their contribution to the epifadin resistance phenotype to be discounted. The two other mutations were found in the gene encoding a hypothetical protein in clone 4 and in *purR,* an operon repressor in clone 2 (Table 4.3).

The genome of the USA300-no resistance day-3 clone, had two non-synonymous SNPs identified: a mutation in *treA* and another in *yhdG_1* (table 4.4). The SNP of *yhdG_1* was shared by all the clones, while the mutant in *treA* gene was only found in clone 4. TreA is a member of the glycoside hydrolase family 13 (GH13) family that is responsible for catalysing the hydrolysis of trehalose 6-phosphate to create glucose and glucose-6-phosphate (Lin et al., 2016). As a means of discerning the cause of resistance, SNPs shared with the *S. aureus* USA300-day-3 clone or present in both controls indicate that they play no role in resistance to epifadin.

Table 4. 2. SNPs and INDELs identified in *S. aureus* USA300-day-3-epifadin resistance.

SNPs and INDELs identified in three clones (1,2,3) after interference competition of *S. aureus* with antimicrobial producer *S. epidermidis* IVK83. Shading colour indicates common SNPs that were found in all the clones.

Non-synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Detected in clones
NMEDEPAD_02274	Putative antitoxin *yezG_1*	144	Arg39Ser	A to C	1,2,3
NMEDEPAD_00066	Putative amino acid permease *yhdG_1*	79518	Ser379Gly	A to G	1,2,3
NMEDEPAD_00749	Sensor histidine kinase *desK*	785553	Ala363Val	C to T	1,2,3
NMEDEPAD_01120	Adenine phosphoribosyltransferase *apt*	1177816	Phe60Leu	A to G	1,2,3

Table 4. 3. SNPs and INDELs were found in the *S. aureus* USA300 WT-day-3.

SNPs and INDELs identified in three clones of *S. aureus* USA300 after three days of passage on BHI agar compared with day 0. NCBI protein BLAST was used to provide more information on the hypothetical proteins and the number in brackets indicates percentage identity. Shading colour indicates shared SNPs that were found in all the clones.

Non-synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Detected in clones No
NMEDEPAD_02274	Putative antitoxin *yezG_1*	144	Arg39Ser	A to C	1,2,3
NMEDEPAD_00066	Putative amino acid permease *yhdG_1*	79518	Ser379Gly	A to G	1,2,3
NMEDEPAD_02700	hypothetical protein (100 %)	110	Asp29Gly	A to T	4
NMEDEPAD_02598	Pur operon repressor *purR*	25058	Arg39Ser	G to T	2

Table 4. 4. SNPs and INDELs of *S. aureus* USA300-no resistance day-3.

SNPs and INDELs were detected in three colonies after interference competition of knockout antimicrobial producer *S. epidermidis* IVK83 Δ*efiTP*.

Shading colour indicates shared SNPs that were found in all the clones.

Non-synonymous					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Detected in clones
NMEDEPAD_00066	Putative amino acid permease *yhdG_1*	79518	Ser379Gly	A to G	1,2,3
NMEDEPAD_02525	Trehalose-6-phosphate hydrolase *treA*	6565	Leu160Leu	A to G	1

4.3.7 An epifadin resistant mutant of *S. aureus* USA300 has altered growth rate

Growth rate of a *S. aureus* USA300 epifadin resistant clone No 1 selected from day 3 of the competition was measured using Growth Profiler (960 by Enzyscreen) to investigate whether there might be changes in comparison with its parent strain. This mutation would provide a fitness advantage if there was an actual fitness increase due to their resistance to antimicrobial producer *S. epidermidis* IVK83. According to the cell density changes and compared to the WT/control, *S. aureus* USA300 epifadin resistant strain showed an apparent higher growth rate during exponential growth phase but a lower OD in stationary phase (Fig 4.5).

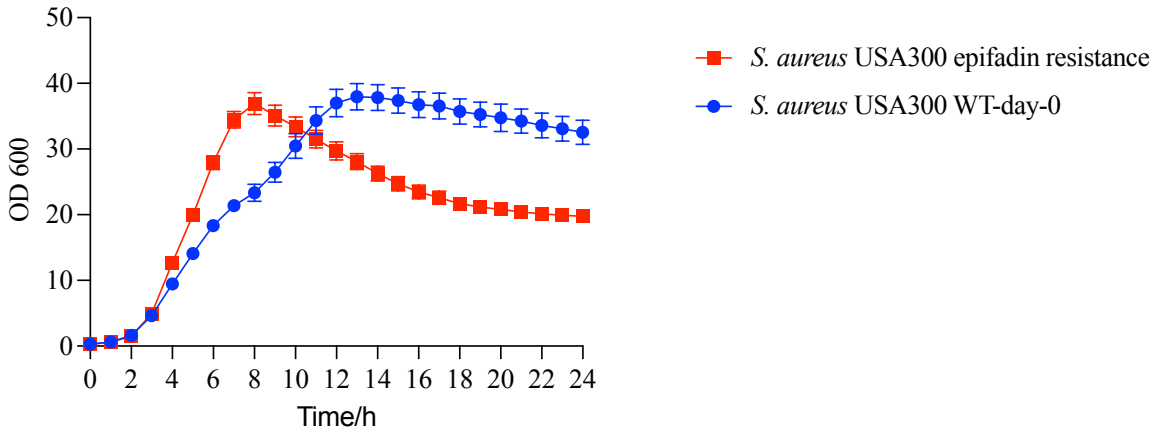

Figure 4. 5. Growth of *S. aureus* USA300 WT-day-0 and its epifadin resistance that selected from day 3 of the competition. Strains were inoculated in BHI medium at 37°C with OD600 0.05 and then measured hourly for 24 h. *S. aureus* USA300/control (WT-day-0) (blue circles) *S. aureus* USA300 epifadin resistance (red square). Data are presented as mean of 6 biological replicates +/- STDEV.

Doubling time was calculated from hours 3 to 7 (5 h) to compare the exponential growth phase rates of one chosen *S. aureus* USA300 epifadin resistant mutant versus WT. The mutant showed a lower doubling time (1.836/h) that matched with its significantly greater growth rate (P value < 0.001) compared to WT (doubling time 2.306/h) (Table 4.5).

Table 4. 5. The growth rates of *S. aureus* USA300 strains during active growth.

Strain	Duration of growth/h	Doubling time/h
S. aureus USA300 WT-day-0	5	2.306
S. aureus USA300 epifadin resistance day 3	5	1.836

4.3.8 Epifadin resistant *S. aureus* shows increased fitness compared with ancestral *S. epidermidis* IVK83

A coculture experiment was conducted to determine the ability of resistant *S. aureus* USA300 isolated from the third day of competition (*S. aureus* USA300 epifadin resistance day 3) to compete with the ancestral strain *S. epidermidis* IVK83. Monoculture controls served as comparators. The chosen clone (clone 1) of *S. aureus* USA300 resistance day 3 was identified with SNPs in genes *yezG_1, yhdG_1, desK* and *apt* and was cocultured with *S. epidermidis* IVK83 WT on BHI agar with viability determined daily to evaluate growth dynamics of mono- or cocultures. The monoculture of *S. aureus* USA300 epifadin resistant strain showed no difference in yield to the monoculture growth of *S. aureus* USA300 WT (Fig 4.6 and 4.1). However, in coculture, *S. aureus* USA300 epifadin resistance had a greater yield in the presence of *S. epidermidis* IVK83 compared to *S. aureus* USA300/control (Fig 4.6). Numbers of the *S. aureus* USA300 epifadin resistant strain were greater on days one to three ~ 1.2 x 10^9 ~ 1.6 x 10^9 and 2.3 x 10^9 cfu ml^{-1}, respectively (Fig 4.6), compared to *S. aureus* USA300/control ~ 5 x 10^8, ~ 6.6 x 10^8 and ~ 1.7 x 10^9 cfu ml^{-1}on days one to three, respectively (Fig 4.1). Moreover, the ability of ancestral *S. epidermidis* IVK83 to compete with the *S. aureus* USA300

epifadin resistant strain was approximately twofold reduced, where in coculture a decreased population of *S. epidermidis* IVK83 was seen, with ~ 1.3 x 10^8 cfu ml^{-1} on the first and second day and 5 x 10^7 cfu ml^{-1}on the third day (Fig 4.6). These numbers compared to the *S. aureus* USA300/control with a population of ~ 6.6 x 10^8 cfu ml^{-1} on the first and second and ~ 1.1 x 10^8 cfu ml^{-1}on the third day (Fig 4.1). Ultimately, *S. epidermidis* IVK83 was extinguished in competition with the *S. aureus* USA300 epifadin resistance day 3 clones by the fourth day of competition (Fig 4.6) compared to the ~ 5 x 10^7 cfu ml^{-1} determined in the competition with *S. aureus* USA300/control (Fig 4.1).

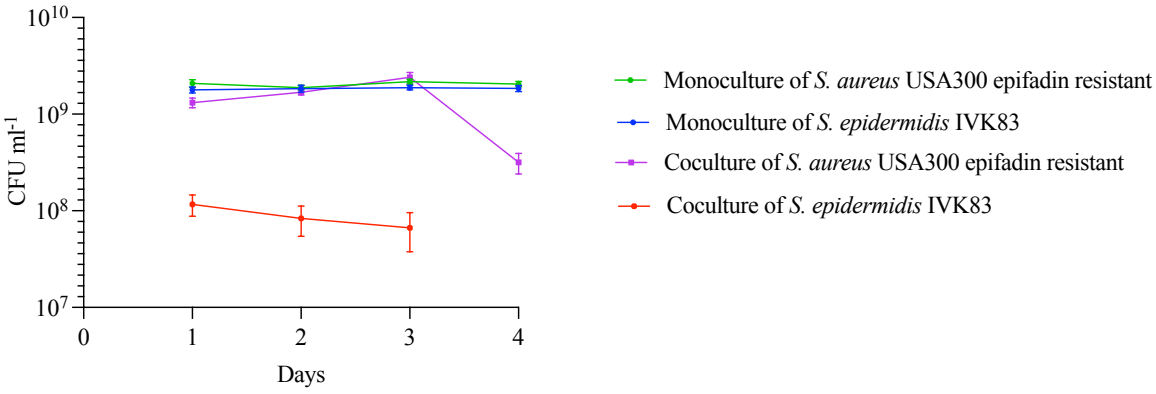

Figure 4. 6.Competition dynamic between ancestral *S. epidermidis* IVK83 and *S. aureus* USA300 epifadin resistance day 3 strain. Strains were cultured in monoculture and coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar as part of an experimental evolution procedure. Viable counting was used to determine growth dynamics. Monoculture of *S. aureus* USA300 epifadin resistant strain/control (green circle). *S. aureus* USA300 epifadin resistant strain when cocultured with *S. epidermidis* IVK83 (purple square). Monoculture of *S. epidermidis* IVK83/control (blue circle). *S. epidermidis* IVK83 when cocultured with *S. aureus* USA300 epifadin resistant strain (red square). Values are the mean of three biological replicates counted in triplicate. Error bars represent triple mean +/- STDEV across the three replicates with t-test analysis for statistical significance effect. Y-axis was presented in log 10 scale.

4.3.9 Experimental evolution to study interference competition between antimicrobial producer *S. lugdunensis* 1007 and *S. aureus* USA300

Previous studies propose that *S. aureus* cannot develop resistance to purified lugdunin compound or lugdunin-producing commensal bacteria (Zipperer et al., 2016). However, the processes governing bacterial fitness, competition and dissemination in microbiota remain poorly understood. Human skin is considered nutritionally poor, suggesting that colonising bacteria are possibly in strong competition with each other and may use a variety of strategies to overcome competitors (Krismer et al., 2014). Increasing fitness such as growth rate due to modified resource use over a competitor can be one such strategy when resistance cannot occur. Coculturing *S. lugdunensis* 1007 and *S. aureus* USA300 on BHI agar was established with monoculture controls and conditions for experimental evolution against the antimicrobial producer were similar to previous experiments. *S. lugdunensis* 1007 was confirmed to inhibit growth of *S. aureus* USA300 by causing a zone with a diameter of 1± 0.5 mm (Chapter 3).

The growth yields from monoculture or for coculture were calculated using the Miles and Misra technique with species colonies distinguished by morphology and pigmentation. The competition experiment was maintained for fifteen days. Monoculture showed a different dynamic of growth than the coculture. For monoculture *S. aureus* USA300 yields were maintained for fifteen days at ~ 3.5 x 10^9 cfu ml^{-1}, while *S. lugdunensis* 1007 had lower cell yields of ~ 2.6 x 10^9 cfu ml^{-1} (P value < 0.001). For coculture, *S. aureus* USA300 had a greater yield on the first day at ~ 2.5 x 10^9 cfu ml^{-1} compared to *S. lugdunensis* 1007 at ~ 6 x 10^8 cfu ml^{-1} (P value < 0.001). However, at day two and three of coculture, *S. aureus* USA300 had a significantly decreased population by day four at ~ 2.8 x 10^8 cfu ml^{-1} that increased sharply at day five and six to ~ 3.6 x 10^9 cfu ml^{-1} and then was maintained ~ 2-3 x10^9 cfu ml^{-1} up to day 15. On the other hand, coculture of *S. lugdunensis* 1007 showed its greatest population yield

from day one to four with ~ 6 x 10^8 - 1.2 x 10^9 cfu ml^{-1} before decreasing to an experimental minimum yield of ~ 2-4 x 10^8 cfu ml^{-1}, up to day 15 (Fig 4.7).

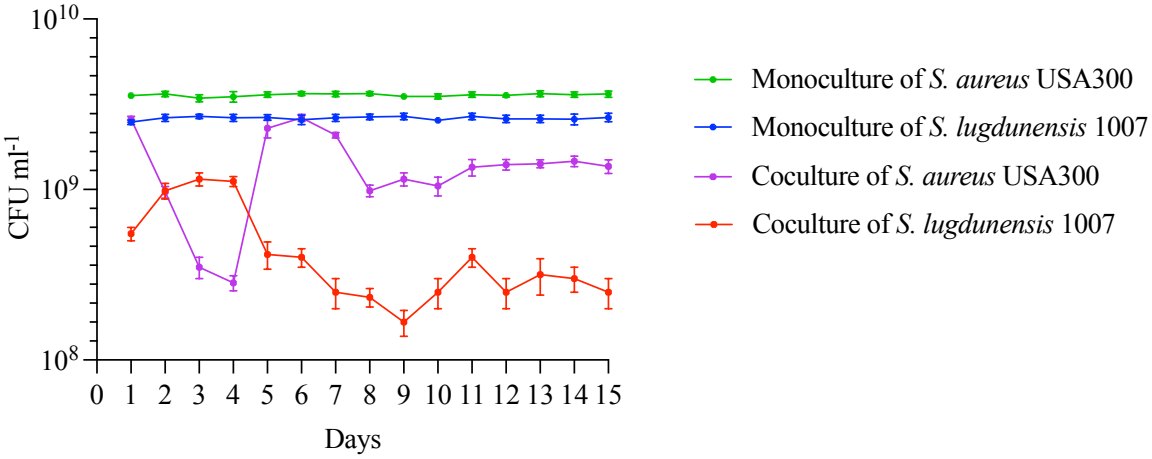

Figure 4. 7. Competition dynamic between antimicrobial producer *S. lugdunensis* 1007 and S. *aureus* USA300. Strains were cultured in monoculture and coculture on BHI agar for 24 h at 37°C and transferred daily onto new agar. Viable counts were used to determine population changes. Monoculture of *S. aureus* USA300/control (green circle). *S. aureus* USA300 when cocultured with *S. lugdunensis* 1007 (purple square). Monoculture of *S. lugdunensis* 1007/control (blue circle). *S. lugdunensis* 1007 when cocultured with *S. aureus* USA300 (red square). Data are presented as the mean +/- STDEV of triplicate of one experiment across the three replicates with t-test analysis for statistical significance effect. Y-axis was presented in log 10 scale.

4.3.10 *S. lugdunensis* 1007 does not select resistance in *S. aureus* USA300

Deferred growth inhibition assays were used to investigate multiple colonies that were taken from daily competition. On days one through fifteen of competition, growth of tested colonies of *S. aureus* USA300 (USA300-D-15) was inhibited by *S. lugdunensis* 1007 and showed no

change in the zone of inhibition (Fig 4.8 B) matching the control/WT, which had a 1 ± 0.5 mm zone (Fig 4.8 A).

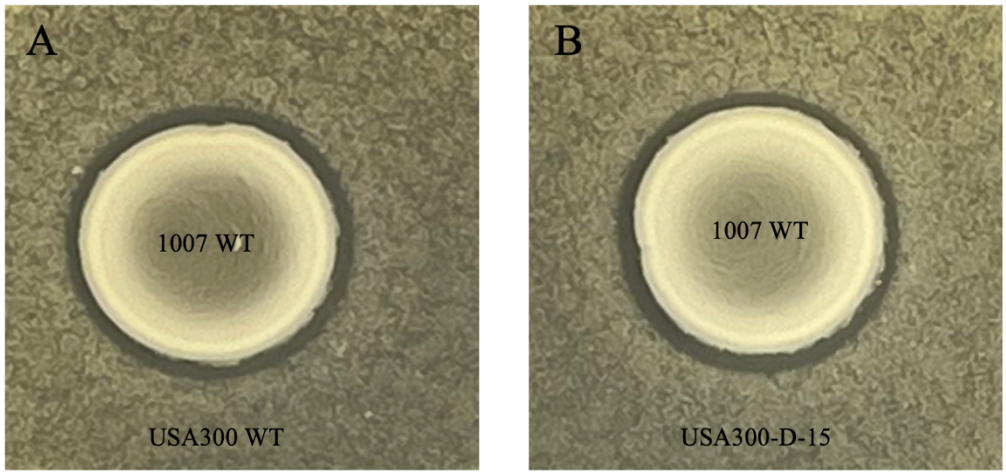

Figure 4. 8. *S. aureus* USA300 displayed no resistance to antimicrobial producer *S. lugdunensis* 1007. Deferred growth inhibition assay was used to determine the antimicrobial activities. For A, B the antimicrobial producer *S. lugdunensis* 1007 WT (inhibitor) was cultured as a spot of O/C on BHI agar plate and after 24 h of incubation at 37ºC, the plate was sprayed with either WT or *S. aureus* USA300-D-15. (A) *S. lugdunensis* 1007 WT showed a zone of inhibition (1± 0.5 mm) against *S. aureus* USA300 WT. (B) *S. aureus* USA300-D-15 that was isolated from day fifteen of competition showed same zone size as *S. aureus*USA300 WT.

4.3.11 Whole Genome Sequencing

In this experiment, *S. aureus* USA300 was unable to develop resistance to antimicrobial producer *S. lugdunensis* 1007. However, to evaluate any genetic changes that could account for the improved growth yields of USA300 during the experiment, three clones selected from three biological triplicates of either WT, non-evolved *S. aureus* USA300 isolated from the competition were DNA sequenced using the Illumina platform methods to identify mutations selected in the experimental evolution. Three clones were sequenced from each selected day (Table 4.6).

Table 4. 6. The chosen clones and days.

Strain /Sample ID	Interference competition between	Sequence number of clones	Day of competition
S. aureus USA300 WT-day-0	None	3	0
S. aureus USA300 WT-day-15	None	3	15
S. aureus USA300-no resistance-day-15	1007 WT and USA300 WT	3	15

4.3.12 Genome sequence changes

Genomic sequence variants were identified using the Snippy tool as described in chapter 2. Reads were aligned to *S. aureus* USA300 WT-day-0. Although *S. aureus* USA300 was unable to develop resistance to antimicrobial producer *S. lugdunensis* 1007, it was decided to sequence three clones of *S. aureus* USA300-non-evolved resistance-day-15 to understand the incremental improvement in the fitness of *S. aureus* USA300 from fourth day of the competition (Fig. 4.7).

Five mutations found as non-synonymous variant in *S. aureus* USA300-non-evolved resistance-day-15 (Table 4.7). Among these three SNPs were identified: a gene encoding a DUF4889 containing protein of unknown function, *ygaZ* gene encoding a membrane protein responsible for regulating the passage of nutrients into the cell (Sueki et al., 2020) and a gene encoding a hypothetical protein. SNPs in the *agrA* and *yezG*_1genes were found in clone 2.

As previously done, three clones of *S. aureus* USA300 WT-day-15 were sequenced as a control to distinguish the mutation from the WT. Five non-synonymous sequence variants were

detected in the controls (Table 4.8). First, a mutation in the gene encoding L-cystine absorption protein (TcyP), which is known to be crucial for *S. aureus* pathogenicity in terms of nutritional sulphur acquisition (Lensmire et al., 2020). Additionally, SNPs of *yhdG_1*, *yezG_1* and a hypothetical protein (ID: 02700) were found in the WT and shared by *S. aureus* USA300-non-evolved resistance-day-15. Therefore, it was suggested that SNPs in genes encoding the three proteins DUF 4889, ygaZ and/or hypothetical protein might be responsible for improving the fitness of *S. aureus* USA300 bacteria during day 4 to day 15. This is based on these SNPs being found in all sequenced clones of *S. aureus* USA300-non-evolved resistance-day-15

Table 4. 7. SNPs and INDELs were found in the *S. aureus* USA300-no resistance-day-15.

SNPs and INDELs were detected in three clones (1,2,3) after fifteen days of interference competition with antimicrobial producer *S. lugdunensis* 1007. NCBI protein BLAST was used to provide mor information on the hypothetical proteins and the number in bracket indicates the percentage of protein identity. Asterisk corresponds with a termination codon. Shading colour indicates shared SNPs that were found in all the clones.

Non-synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Detected in clones
NMEDEPAD_00868	Hypothetical protein- Domains of unknown function (DUF) 4889 domain-containing protein (99 %)	947691	Pro68Ser	G to A	1,2,3
NMEDEPAD_01453	Inner membrane protein *ygaZ*	329199	Ala219Ser	C to A	1,2,3
NMEDEPAD_02149	Accessory gene regulator A *agrA*	146749	Arg218*	C to T	2
NMEDEPAD_02274	Putative antitoxin *yezG_1*	144	Arg39Ser	A to C	2
NMEDEPAD_02700	Hypothetical protein (100 %)	124	Ter34Glnext	T to C	1,2,3

Table 4. 8. SNPs and INDELs were found in the *S. aureus* USA300 WT-day-15.

SNPs and INDELs were detected in three clones of *S. aureus* USA300 after fifteen days of passaging on BHI Agar. NCBI protein BLAST was used to provide mor information on the hypothetical proteins and the number in bracket indicates the percentage of protein identity. Shading colour indicates shared SNPs that were found in all the clones.

Non-synonymous					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Detected in clones No
NMEDEPAD_00066	Putative amino acid permease *yhdG_1*	79518	Ser379Gly	A to G	1,2,3
NMEDEPAD_02274	Putative antitoxin *yezG_1*	144	Arg39Ser	T to C	1
NMEDEPAD_02354	L-cystine uptake protein *tcyP*	80456	Gly132Val	C to A	2
NMEDEPAD_02598	Pur operon repressor *purR*	24658	Val229Gly	A to C	1,2,3
NMEDEPAD_02700	Hypothetical protein (100 %)	124	Ter34Glnext	T to C	2,3

4.4 Discussion

In the previous chapter, it was found that *S. aureus* evolved resistance to an undescribed antimicrobial produced by *S. capitis*. The data revealed that *S. aureus* potentially used novel and multiple genetic mechanisms to compete with *S. capitis*. In this chapter, selection for resistance of *S. aureus* to epifadin producer *S. epidermidis* IVK83 (Torres Salazar et al., 2023) was investigated to build on previous studies. Additionally, competition between *S. aureus* USA300 and antimicrobial producer *S. lugdunensis* 1007 was studied.

4.4.1 The effect of epifadin producer S. epidermidis IVK83 on *S. aureus* USA300

Many bacteria, including CoNS, produce antimicrobial peptides to interfere with the growth of other bacteria to promote their own survival within bacterial communities (Paharik *et al*., 2017; Wang, Gu and Breukink, 2020). Given the inhibitory effect of *S. epidermidis* IVK83 upon *S. aureus* USA300 (inhibition zone of 0.5 mm) (Fig 3.2), the effect of *S. epidermidis* IVK83 epifadin producer, and Δ*efiTP* epifadin mutant with disruption of genes responsible for its biosynthesis (Torres Salazar et al., 2023) that produced no growth inhibition (Fig 4.4 B) was examined during competition by coculturing the strains separately with *S. aureus* USA300 on BHI agar. In monocultures, the population sizes of both USA300 and IVK83 were larger than when grown together indicating that these species compete and that there were finite resources in the BHI growth medium being used. The results clearly showed that the inhibitory activity of IVK83 when it was cultured with *S. aureus* contributed to its dominance over *S. aureus* in the day one stages of the competition. However, from the day two, USA300 produced a significantly greater population while IVK83 started to decline significantly until the fourth-day conclusion of competition followed by its absence at day five, (Fig 4.1), suggesting that USA300 evolved resistance after continued exposure to epifadin producer *S. epidermidis* IVK83.

4.4.2 Resistance of *S. aureus* USA300 to epifadin producer *S. epidermidis* IVK83

To colonise a host and survive, *S. aureus* must resist antimicrobials called bacteriocins produced by commensal bacteria (Kawada-Matsuo et al., 2013). Epifadin producer *S. epidermidis* IVK83 and *S. aureus* USA300 were cultured together on BHI agar to select for resistance. WGS was performed to investigate genetic changes in the genomes of clones selected from different days of the competition experiment.

In *S. aureus* USA300, selected clones of day three of the competition associated to resistance to epifadin producer *S. epidermidis* IVK83. SNPs of *apt* and *desK* were identified in the three clones (table 4.2). However, when competition between *S. epidermidis* IVK83 Δ*efiTP* and *S. aureus* USA300 was done, *S. aureus* USA300 selected of day three of the competition had susceptibility to *S. epidermidis* IVK83 Δ*efiTP* (Fig 4.4 C) and none of the above mutations were found (table 4.4). This outcome suggesting that SNPs of *apt* and *desK* are likely to be responsible to resistance to epifadin antimicrobial produced by *S. epidermidis* IVK83. Although several other SNPs were recognised in *S. aureus* USA300 WT selected from day three of the monoculture/control that were associated with the absence of epifadin antimicrobial produced by *S. epidermidis* IVK83 (Table 4.3), the cause of these mutants suggested to be as an adaption of the daily passage on BHI agar or random genetic drift (Kawecki et al., 2012).

It was reported previously that an *apt* mutation was present in a laboratory-derived VISA strain derived from an MRSA strain (Hattangady et al., 2015). Recently, *apt* mutations were found to contribute to reduced vancomycin susceptibility in vancomycin-intermediate *S. aureus* (VISA) strains (Lamichhane-Khadka et al., 2021). In this study *apt* mutation was found to contribute with resistance to epifadin antimicrobial produced *S. epidermidis* IVK83. The gene *apt* encodes the purine that influences bacterial intracellular survival. The purine biosynthesis contributes a significant role to DNA replication and RNA production to promote protein

synthesis (Sivapragasam and Grove 2019). It is possible that *S. aureus* may use *apt* mutation to the resistance of epifadin, however, more studies are needed to clarify this mechanism.

One of the most identified and studied mechanisms of antimicrobial resistance in *S. aureus* are two-component signal transduction systems (TCSs) (Coates-Brown *et al.,* 2018). *S. aureus* uses TCS to adapt to stressful environmental conditions. DesKR or TCS-7 is an uncharacterised TCS, a putative sensor kinase with the histidine kinase. In *B. subtilis*, DesKR was reported to play a significant role in the response to temperature sensing and regulates a lipid desaturase (Fernández et al*.,* 2020; Bleul, Francois and Wolz, 2021). Although there is limited information, DesKR was found in *S. aureus* and associated with adaptation to lower temperature conditions (Kim et al*.,* 2016; Fernández *et al.,* 2020; Bleul, Francois and Wolz, 2021). Ghabban, (2019) working in the Horsburgh research group at University of Liverpool reported *desK* and *desR* mutations in *S. aureus* were associated with antimicrobial resistance to epifadin antimicrobial produced by *S. epidermidis* B155. In this study, from experimental evolution, similarly a mutation in *desK* gene appears to contribute with *S. aureus* resistance to epifadin antimicrobial produced *S. epidermidis* IVK83, supporting the idea that *desK* plays a role in resistance to epifadin antimicrobial. The *desK* mutant (SAOUHSC_01313) (Tyr137Asn) reported by Ghabban, (2019) study was detected in *S. aureus* SH1000 resistant to *S. epidermidis* B155 antimicrobial after twelve days of competition between the two strains. While, in this study a sequence variant in *desK* (NMEDEPAD_00749) (Ala363Val) was detected in *S. aureus* USA300 resistant to *S. epidermidis* IVK83 after three days of competition. Although BHI medium was used in both experimental evolution setups, a different competition method was used to achieve the resistance to the inhibitor strain. In the Ghabban, (2019) study, the competition between the strains used serial transfer into a 250 ml flask containing 50 ml BHI liquid medium. While, in this work the competition was created on 25 ml BHI solid medium. It is unclear why *S. aureus* developed resistance to epifadin faster

than it did in Ghabban work. However, possible explanations are that S. aureus selection on a solid medium occurs more rapidly due to exposure to higher amounts of antimicrobials or epifadine which is hydrophobic. Epifadin is also reported to be highly unstable, and this might account for concentration threshold differences.

Further TCS are known for contributing to antimicrobial resistance in *S. aureus*, with BraRS TCS in *S. aureus*, regulating resistance from nisin A, a lantibiotic produced by *Lactococcus lactis* and nukacin ISK-1, produced by *S. warneri* (Kawada-Matsuo et al*.,* 2013; Arii et al*.,* 2019). Here, DesKR TCS contributes to epifadin survival allowing *S. aureus* USA300 to adapt, as identified by Ghabban, 2019 and in this study that association is extended from strain SH1000 to include strain USA300. Most AMPs act by creating pores in bacterial membranes or disrupting peptidoglycan structure or both. This action leads to an influx of small molecules, potential dissipation of the membranes, and finally stopping cellular biosynthesis (Wang et al*.,* 2020). Therefore, *S. aureus* uses TCS to protect itself from these AMPs. In *S. aureus*, TCS such as SaeRS, VraRS, GraRS and BraSR are classified as intramembrane sensor histidine kinases (Bleul et al., 2021). Here, DesKR TCS is associated with antimicrobial resistance, suggesting a possible precedent for association with regulation of membrane.

A higher exponential growth rate of epifadin resistant *S. aureus* USA300 that is associated with *apt* and *desK* mutations suggests increased strain fitness (Fig 4.5). Therefore, a further competition experiment was prepared to determine the ability of *S. aureus* USA300 epifadin resistance to compete with the ancestral strain *S. epidermidis* IVK83. *S. aureus* USA300 epifadin resistant strain showed increased competitiveness with ancestral *S. epidermidis* IVK83. The ability was determined by an increase of cell number of epifadin resistant *S. aureus* USA300 that outcompeted the ancestral strain over three days (Fig 4.6). This ability could be proposed to result from the *apt* or *desK* mutations possessed by *S. aureus* USA300 epifadin-

resistant and further genetic analysis and strain construction can be performed to unravel the contribution of the *apt* locus.

4.4.3 Interference of antimicrobial producer *S. lugdunensis* 1007 with *S. aureus* USA300

Infections caused by highly antibiotic-resistant bacteria have greatly increased in recent years and represent a major cause of morbidity and mortality worldwide (Haddadin, 2022). Although there is an urgent need for new antibiotics that are effective against resistant bacteria (Bin Hafeez et al., 2021), very few compounds are in development. Lugdunin is an antimicrobial produced by *S. lugdunensis* IVK28 that was proposed to prevent staphylococcal infections (Zipperer et al., 2016). Therefore, it was decided to further investigate this antimicrobial producing species. From 22 *S. lugdunensis* strains screened for their ability to produce antimicrobial activity against different isolation of *S. aureus*, three were found: *S. lugdunensis* 1007, 211421 and 211633 that showed pronounced inhibitory activities against *S. aureus* (Fig 3.2). To know more about the antimicrobial products encoded by the three strains, antiSMASH was performed. The biosynthetic gene clusters for *S. lugdunensis* 1007, 211421 and 211633 showed 93 % similarity to lugdunin (Table 3.1), suggesting that this cluster corresponds to biosynthesis of lugdunin antimicrobial and supported their study. Thus, one of the strains, *S. lugdunensis* 1007 was selected to investigate its antimicrobial effect upon *S. aureus* USA300. To determine whether *S. aureus* USA300 can evolve mutations that increase competitiveness to the antimicrobial producer *S. lugdunensis* 1007, the two species were cultured together on BHI agar. This strategy was successfully applied to evolved resistance of *S. aureus* to other antimicrobials in this study and previous studies in the lab (Ghabban, 2019; Alrajeh, 2021).

During the interference competition experiment, the results showed that the antimicrobial producer *S. lugdunensis* 1007 when cultured with *S. aureus* USA300 was dominant over *S. aureus* USA300 in the first four days of the competition. *S. aureus* USA300 then yielded a

significantly greater population size while *S. lugdunensis* 1007 started to decline significantly from day five of the competition (Fig 4.7). However, during the examination of *S. aureus* USA300 for their resistance using the deferred growth inhibition assay (Libberton et al., 2015) there was no resistance detected as no zone change in comparison to the 1 ± 0.5 mm inhibition zone of the WT (Fig 4.8).

4.4.4 *S. aureus* USA300 was unable to develop resistance to antimicrobial producer *S. lugdunensis* 1007.

S. aureus cannot evolve resistance to lugdunin from *S. lugdunensis*, despite continuous serial passaging in the presence of subinhibitory concentrations of lugdunin over 30 days and on this basis lugdunin was reported to have promise as a potential drug for inhibiting growth of *S. aureus* (Zipperer et al., 2016). In this work, *S. aureus* USA300 showed no selected resistance to the antimicrobial producer *S. lugdunensis* 1007 after fifteen days of competition, supporting the previous study data that *S. aureus* cannot develop resistance to lugdunin or lugdunin-producing commensal bacteria. However, the changes in growth of *S. aureus* USA300 during the experiment and selection of changed yield does allow for further study of competition.

Lugdunin was reported to cause proton leakage in synthetic, protein-free membrane vesicles, suggesting that it does not need to target a proteinaceous molecule to exert its antibacterial activity. However, this atypical mode of action raised the question of whether the bacterial producer strains would also use an unusual mechanism to achieve resistance to lugdunin. *S. lugdunensis* has reported to achieve self-resistance to its product and has potential resistance genes (Schilling et al., 2019). Here, antimicrobial producers of *S. epidermidis,* B14, 273, 039331N1 and *S. capitis* 47, particularly, *S. lugdunensis* 211681 and 211421 showed resistances to antimicrobial producer *S. lugdunensis* 1007 (Fig 3.4), proposing that these antimicrobial producer strains may share a mechanism to protect itself from lugdunin antimicrobial. It was reported that *S. lugdunensis* uses four ABC transporter proteins LugEFGH

for lugdunin secretion and self-resistance. Deletion of the entire gene set (*lugIEFGH*) strongly decreased the MIC to exogenous lugdunin indicating that the genes are involved in producer self-resistance to lugdunin (Krauss et al., 2020). However, it is unclear whether this protection mechanism is associated to the ability to be an antimicrobial producer in other strains. As stated, although there was no resistance linked with *S. aureus* USA300 against *S. lugdunensis* 1007 following experimental evolution, mutations such in the genes *agrA* encoding the quorum sensing TCS sensor kinase and a gene encoding a protein DUF 4889 were detected in *S. aureus* USA300-no resistance-day-15. These mutations are likely explanations for the improved the growth yield of *S. aureus* USA300 from day four to fifteen in the competition. On the other hand, *S. lugdunensis* 1007 had the capacity to compete until the last day, thus sequencing *S. lugdunensis* 1007 may produce further insights of its fitness.

4.5 Conclusion

Additional coculture competition between *S. aureus* USA300 and *S. epidermidis* IVK83 epifadin producer was conducted in this chapter. Antimicrobial epifadin demonstrated experimentally evolved resistance with mutation in the *desK* gene of the two-component system (TCS). Another experimental evolution between *S. lugdunensis* 1007 and *S. aureus* USA300 was performed in a separate coculture study. As a result, USA300 was unable to acquire resistance to *S. lugdunensis*, supporting earlier findings that *S. aureus* was unable to develop resistance to lugdunin while having some mutations that selected improved fitness.

Chapter 5: Biofilm competition between *S. epidermidis* and *S. aureus*

5.1 Introduction

In the last decade, microbial competition has received significant attention and has mostly been studied in *vitro*, either in monoculture or in biofilm. Biofilm formation is a significant virulence factor in *S. aureus* strains contributing to several serious diseases associated with antibiotic resistance (Martínez-García et al*.,* 2019). As an opportunistic pathogen associated with many serious infections*, S. aureus*, a common nasal and skin coloniser, is potentially a much more virulent bacterium than others resident in its niche (Masiuk et al*.,* 2021). Beneficial bacteria colonise the skin, acting as a physical barrier to prevent the invasion of pathogens such as *S. aureus* (Paharik et al*.,* 2017). *S. epidermidis* is the most identified of the coagulase-negative staphylococci (CoNS) and is reported to have antimicrobial activity (Lee et al*.,* 2019). These activities include biofilm-forming abilities to outcompete pathogenic bacteria such as *S. aureus* during nasal colonisation (Liu et al*.,* 2020; Severn and Horswill, 2022). *S. epidermidis* competes with *S. aureus* for stable colonisation of human skin reservoirs by production of antimicrobial factors and perhaps by nutrient limitation. Research aims to understand the relative contributions of host variables, environmental factors, and genetic characteristics of *S. aureus*, as well as competitive interactions with co-colonising bacteria. Although the relationship between *S. aureus* and *S. epidermidis* colonisation has been widely studied, there is still a gap knowledge related to their competitive success (Liu et al*.,* 2020).

5.2 Biofilm formation as a significant feature of staphylococcal infections

Several serious diseases are caused by biofilm-associated *S. aureus* and *S. epidermidis,* such as those associated with medical lines and indwelling devices. *S. aureus* biofilm bacteria are responsible for more than 70% of bone and joint infections (Sultan et al*.,* 2022). These infections are difficult to treat because the thick layer of the biofilm can be highly resistant to antibiotics (Singh et al*.,* 2010; Sultan et al*.,* 2022). The first stage of biofilm formation is attachment can happen on inert or biotic surfaces. This initial attachment is coated in plasma proteins, such as fibronectin, fibrinogen, and vitronectin (Speziale et al., 2014). This initial attachment is typically very weak (Costerton et al*.,* 1999; Davey and O'Toole, 2000). In the second stage (development), attachment activates bacterial surface protein communication, and the cell produces components of an extracellular matrix (EXM), which becomes irreversible. This matrix comprises various molecules, typically polysaccharides, nucleic acids, and proteins (Allison, 2003). The matrix is used to build up and strengthen the bacterial biofilm (Vlastarakos et al*.,* 2007; Mozes and Rouxhet, 2012). Biofilms produced by *S. aureus* and *S. epidermidis* are very similar concerning structure and function. Both *S. aureus* and *S. epidermis* produce a biofilm composed of poly-N-acetyl glucosamine (PNAG) also called PIA (polysaccharide intercellular adhesin). In *vitro*, PNAG/PIA production is important for biofilm formation and is considered a critical factor causing bacterial biofilm infections (Kropec et al*.,* 2005, Otto, 2009). Biofilm production in *S. aureus* is primarily regulated by 12 different genes. These genes include intercellular adhesion (*icaA, B, C* and *D*) genes, fibrinogen-binding proteins (*fib*) gene, clumping factor (*clfA* and *B*), elastin binding protein (*ebps*), laminin binding protein (*eno*), collagen binding protein (*cna*) gene and fibronectin-binding proteins (*fnbA* and *fnbB*) genes (Idrees et al*.,*2021). While *S. epidermidis* biofilm is associated with *fbe, aap, and icaA* genes (Wang et al., 2022).

5.3 Quorum sensing systems and biofilm competition

Quorum sensing (QS) is an essential mechanism contributing to the maturation process. (Singh et al., 2017). In the latter stage (spreading), the EXM is integrated with new layers of cells (Zhang et al., 2009). Bacteria released at this stage from a mature biofilm can grow on new surfaces (Stewart and Franklin, 2008). QS is an important biofilm regulatory mechanism and is intensely studied for control of bacterial pathogenesis (Yarwood et al., 2004; Koch et al., 2014; Le and Otto, 2015). QS has a central role in the regulation of staphylococcal pathogenicity and many studies have sought to control virulence factor expression and biofilm formation in *S. aureus* (Brackman and Coenye, 2015). Cell-cell signalling occurs via secretion of diffusible auto-inducing peptides encoded by the accessory gene regulator (Agr) chromosomal locus responsible for QS (Janzon and Arvidson, 1990; Novick et al., 1993).

The Agr quorum-sensing system is a possible tool of staphylococcal competition. Many studies of competition in staphylococci have focused on Agr QS inhibition via the different structural groups of autoinducing peptide (AIP) present in *S. aureus* and cross-species inhibition with AIP produced by CoNS (Paharik et al., 2017). *S. aureus* can encode one of four allelic Agr gene variants (I-IV) of the Agr locus, which result in the production of AIP signal structures: AIP-I, II, III and IV, while *S. epidermidis* has three Agr variants and AIPs. Within the Agr signalling system, each AIP activates its cognate AgrC receptor to signal cell density. *S. epidermidis* is proposed to compete with *S. aureus* on the skin. This competition is thought to occur because of the ability of *S. epidermidis* to affect AIP, which inhibits *S. aureus* quorum sensing. As a result, more research on AIPs, which are a potential source of competition, is needed to better understand how the skin microbiota interact with one another and the host (Parlet et al., 2019).

5.4 Aims

The aim of this chapter was to develop a model for *S. aureus* biofilm formation that would allow competition effects of *S. epidermidis* in the presence or absence of a second strain of *S. aureus*. The effect of co-resident *S. epidermidis* expressing a specific antimicrobial on the stability of resident *S. aureus* from invading strains of *S. aureus* expressing the same or different Agr group AIPs could then be investigated. This would model the scenario where co-resident staphylococci in the anterior nares encounter an invading *S. aureus* and might enable study of species succession if the resident *S. aureus* is destabilised. The hypothesis was proposed that *S. aureus* utilises a range of specific mechanisms during its interaction with other bacterial species and that the presence of these other species will dictate success during invasion of a niche.

5.5 Results

5.5.1 Agr Typing

The Agr group type (I, II, III, and IV) of *S. aureus* strains was determined, whereby antagonistic activity may play a role in the bacterial competition between CoNs and *S. aureus* in an experimental model. Genomic DNA was isolated from the *S. aureus* strains that was amplified with the Agr group primers (Table 2.3) to reveal that the strains to be included in the experiments belonged to the most common Agr groups I and III (Table 5.1). Twelve strains were tested for their Agr type, but the Agr type II, and IV combination were not achieved. *S. aureus* SH1000, 137 and 184 were nasal isolates (Table 2.1).

Table 5. 1. Agr types of tested *S. aureus* strains

S. aureus strain/number	Agr type
SH1000	I
137	III
184	I

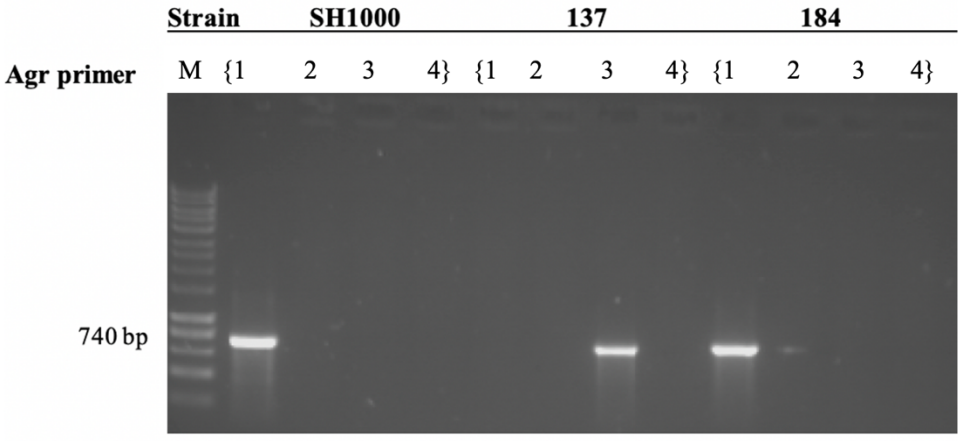

Figure 5. 1. PCR typing of Agr group. Agarose gel electrophoresis (1%) of the PCR products for four different *S. aureus* species amplified with Agr type primers. Well (M) is a molecular size marker (1 kb DNA ladder). In each strain of *S. aureus* SH1000 137 and 184, wells (1) Agr type I, (2) Agr type II, (3) Agr type III and (4) show Agr type IV.

5.5.2 Biofilm formation in different growth media

Growth media with varying glucose and NaCl: LB; LB-GN; BHI; and BHI-GN, were used for the culture of *S. epidermidis* B155, 14 and *S. aureus* SH1000, *S. aureus* 137, and *S. aureus* 184. This investigation was done to select the optimum medium to grow bacterial biofilm in transwell plate. *S. epidermidis* B155, 14 and *S. aureus* SH1000, 137, and 184 showed a high ability for biofilm formation in BHI-GN and was chosen as the optimum medium to grow bacterial biofilm (Fig 5.2).

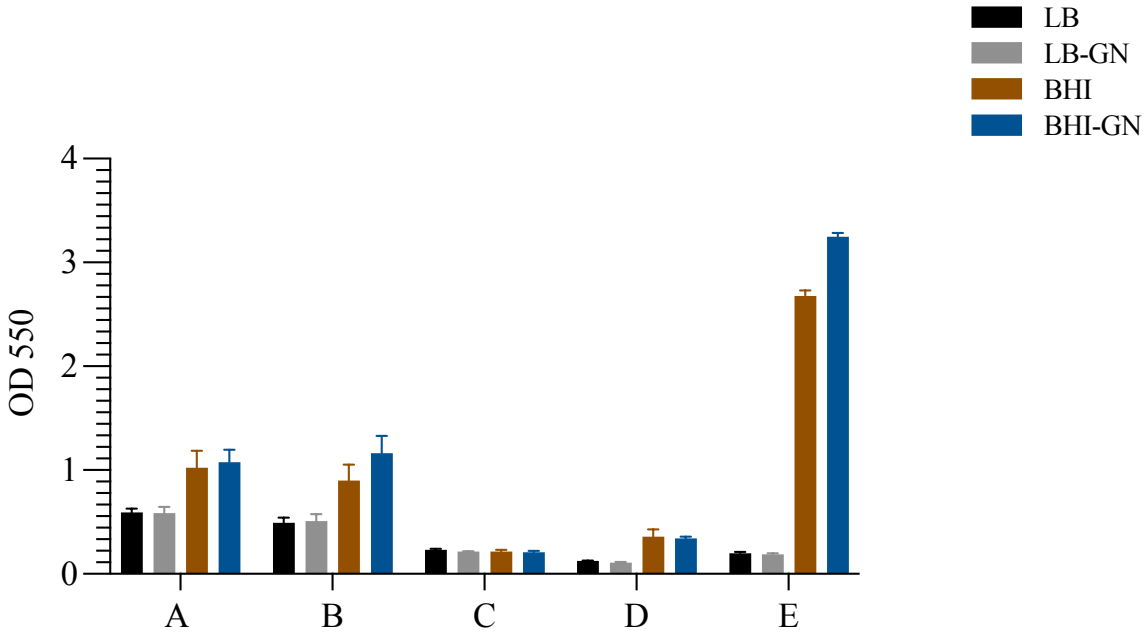

Figure 5. 2. Biofilm formation ability in different growth media. Biofilm formation yield in four growth media for *S. epidermidis* B155 (A). *S. epidermidis* B14 (B). *S. aureus* SH1000 (C) *S. aureus* 137 (D) and *S. aureus* 184 (E). Bars show biofilm formation in LB medium (black), LB-GN (grey), BHI (brown) and BHI-GN (blue). Strains were cultured for 24 h in 1 ml of growth media and yield was measured after washing using crystal violet (CV) assay. Each bar is the mean of three repetitions of culture (mean +/- STDEV).

5.5.3 Transwell Biofilm Competition Assay

To investigate interference of *S. aureus* biofilm formation by *S. epidermidis* in the presence or absence of a second *S. aureus* strain during competition, *S. aureus* was cultured alone in the bottom well of a transwell plate and in the upper well there was either competing *S. epidermidis* alone or with a second *S. aureus* strain .The selected *S. epidermidis* chosen were able to express secreted antimicrobials with activity against *S. aureus* in the plate assays shown below (Fig **5.3).**

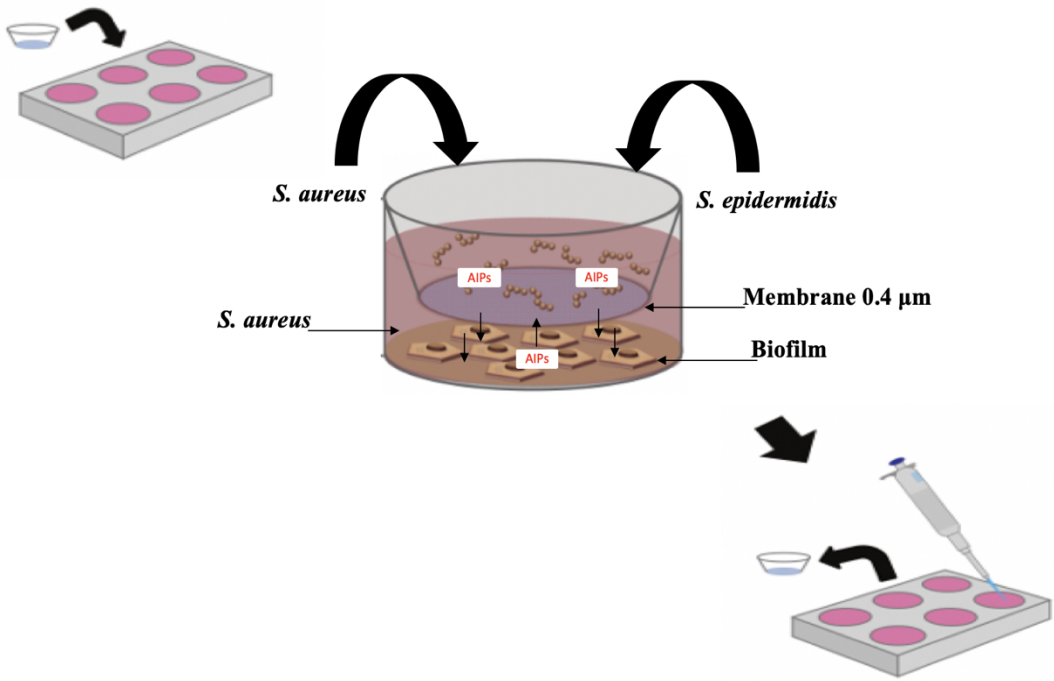

Figure 5. 3. Diagram of a transwell plate. A membrane with 0.4 μm pores separates the upper and lower chambers and bacteria are added to these chambers to investigate interference interactions caused by factors that can include AMPs and AIPs that affect biofilm formation of the strain cultured in the bottom well of the plate. Figure modified from (Flaherty and Lee, 2016).

5.5.4 Competition during biofilm formation

S. epidermidis B155 was chosen as an inhibitor producer to determine its effect on *S. aureus* SH1000 biofilm formation using a transwell biofilm competition assay. *S. epidermidis* B155 expresses epifadin antimicrobial (Ghabban, 2019). Growth of *S. aureus* SH1000 in the lower well was quantified by measuring biofilm after staining or viable counting following culture with either *S. epidermidis* 155 alone or in combination with *S. aureus* 137 in the upper well (Fig 5.4 and 5.5). By comparing with two different controls (absence of *S. epidermidis* or substituting with *S. aureus* in the upper well), the assay showed there was a significant reduction of *S. aureus* SH1000 biofilm in the lower well (P value < 0.05) due to the inhibitory effect of *S. epidermidis* B155 measured by both crystal violet staining and viable counts of disrupted biofilm. In contrast, having both *S. epidermidis* B155 plus *S. aureus* 137 in the top well showed that the biofilm inhibition upon *S. aureus* SH1000 by *S. epidermidis* B155 was negated by the presence of *S. aureus* 137 (Fig 5.4). Two volumes of culture (100 μl and 150 μl with its controls) were used to maintain equivalence between the competition between the two and three strains (table 2.4 in section 2).

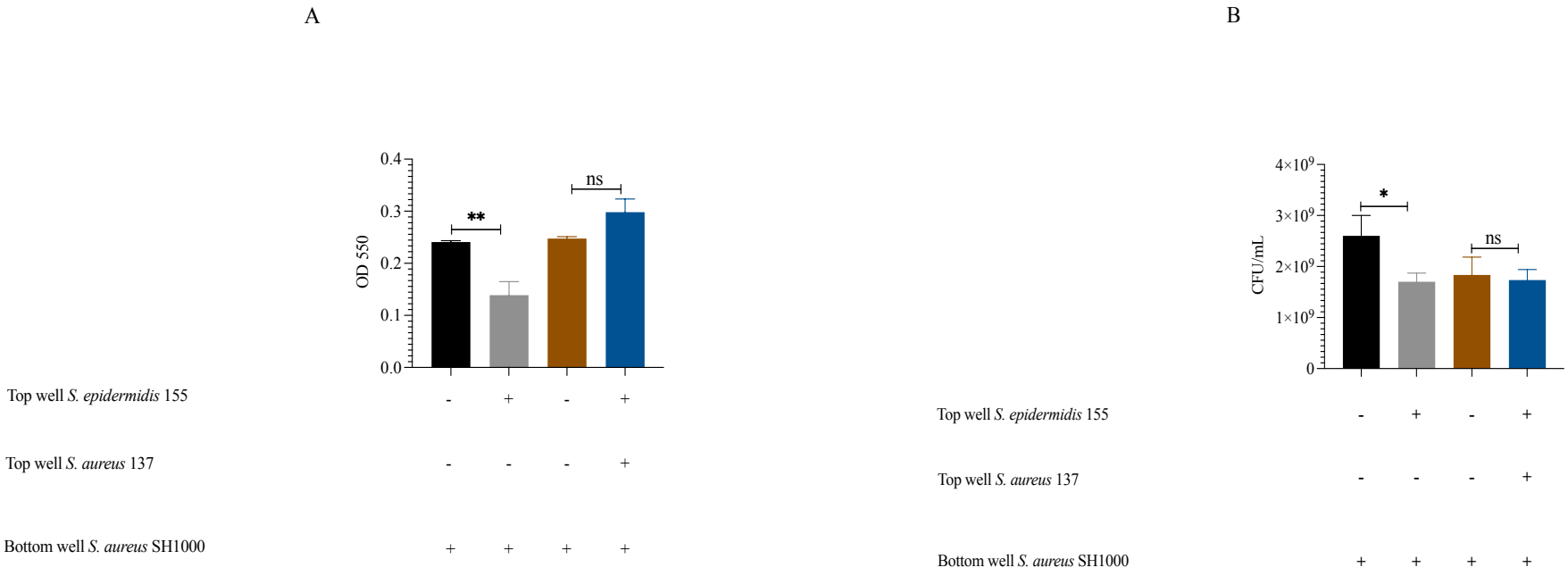

Figure 5. 4. *S. aureus* SH1000 biofilm formation and viable counts from transwell competition with *S. epidermidis* B155 epifadin producer in the absence and presence of *S. aureus* 137. Biofilm was quantified by crystal violet (A) and viable counting of disrupted biofilm (B). *S. aureus* SH1000 alone controls were either 50 µl (black bar) or 150 µl (brown bar) volumes to maintain equivalence. *S. aureus* SH1000 was cultured in the bottom well of a transwell plate with *S. epidermidis* B155 on the top (grey bars in A, B). *S. aureus* SH1000 after competition with both *S. epidermidis* B155 plus *S. aureus* 137 (blue bars in A, B). Strains were cultured in BHI-GN for 24 h. Data are presented as mean +/- STDEV across the three replicates with t-test analysis for statistical significance effect.

S. aureus SH1000 and *S. aureus* 137 were switched between top and bottom wells in a subsequent experiment to examine whether the biofilm inhibitory effect of *S. epidermidis* B155 expressing epifadin antimicrobial was observed on *S. aureus* 137. *S. aureus* SH1000 was included as the second *S. aureus* strain by adding to the top well to see if a similar negation of *S. epidermidis* inhibition was observed. Using crystal violet staining (Fig 5.5 A), an inhibitory effect of *S. epidermidis* B155 was revealed by a significant decrease of *S. aureus* 137 biofilm compared with its control (P value < 0.05). In contrast, viable counting of disrupted biofilm (Fig 5.5 B) showed there were no significant effects of *S. epidermidis* on biofilm *S. aureus* 137 cell numbers. This could indicate strain-specific effects such as the relative growth yields of *S. epidermidis* in the two experiments.

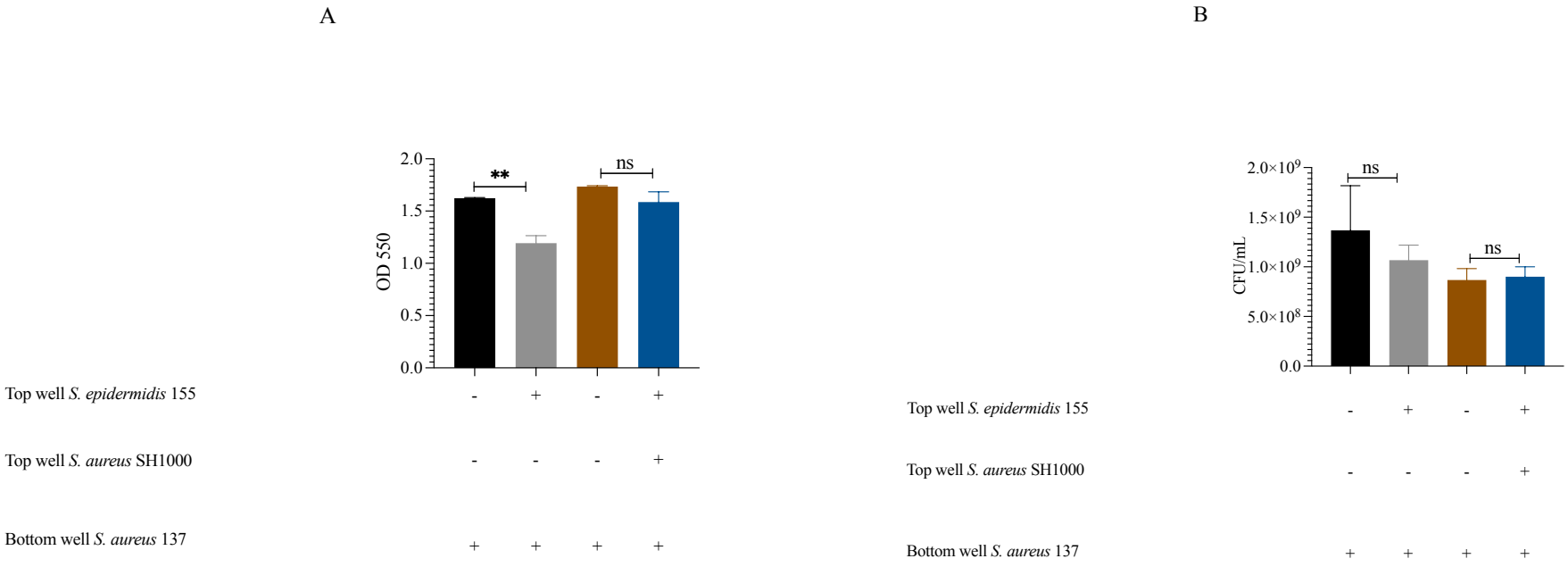

Figure 5. 5. *S. aureus* 137 biofilm formation and viable counts from transwell competition with *S. epidermidis* B155 epifadin producer in the absence and presence of *S. aureus* SH1000. Biofilm was quantified by crystal violet (A) and viable counting of disrupted biofilm (B). *S. aureus* 137 alone controls were either 50 µl (black bars) and 150 µl (brown bars) in A, B, volumes to maintain equivalence. *S. aureus* 137 was cultured in the bottom well of a transwell plate with *S. epidermidis* B155 on the top (grey bars in A, B). *S. aureus* 137 after competition with both *S. epidermidis* B155 plus *S. aureus* SH1000 (blue bars in A, B). Strains were cultured in BHI-GN for 24 h. Data are presented as a triple mean +/- STDEV across the three replicates with t-test analysis for statistical significance effect.

5.5.6 Biofilm competition with an alternative *S. epidermidis* inhibitor producer

To extend the inhibition effects of *S. epidermidis* epifadin producer B155 on biofilm formation of strains *S. aureus* SH1000 and *S. aureus* 137, *S. epidermidis* B14 was substituted for *S. epidermidis* B155. *S. epidermidis* B14 expresses an unknown antimicrobial (Alrajeh, 2021). According to the antiSMASH, *S. epidermidis* B14 had a 37 % similarity to Epidermin in region one and an 81 % similarity to Gallidermin in region six. *S. aureus* SH1000 was substituted by *S. aureus* 184. The inhibition effect of *S. epidermidis* B14 on *S. aureus* 184 biofilm formation was quantified by crystal violet staining and viable counting using the competition transwell biofilm assay (Fig 5.6 and Fig 5.7). From crystal violet staining, compared with the controls, *S. epidermidis* B14 caused a significant biofilm reduction of *S. aureus* 184 biofilm (P value < 0.05) (Fig 5.6 A) and this inhibition was supported by reduced viable counts of disrupted biofilm (P value < 0.05) (Fig 5.5 B).

Adding *S. aureus* 137 to the top well with *S. epidermidis* B14 identified there was significant biofilm inhibitory influence upon *S. aureus* 184 by *S. epidermidis* B14 based on crystal violet staining (P value 0.05) (Fig 5.6 A) of the biofilm but was not supported by viable counts (Fig 5.6 B) of disrupted biofilm.

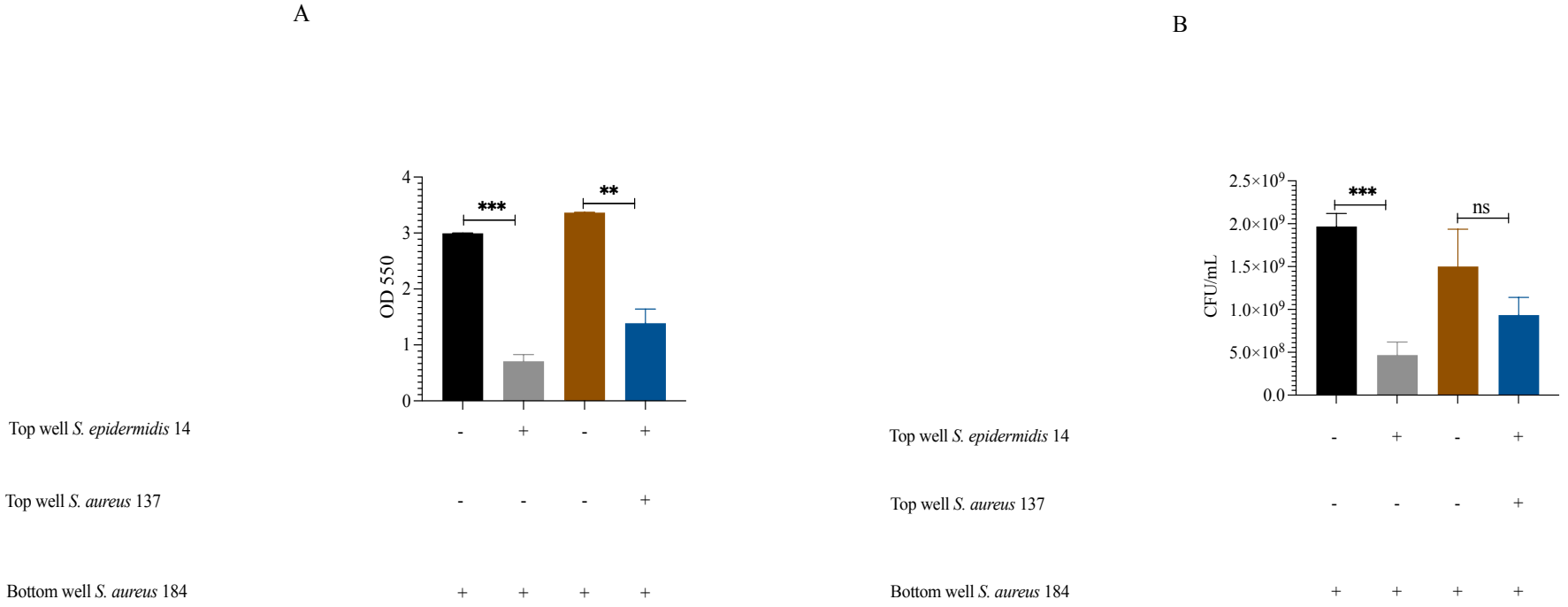

Figure 5. 6. *S. aureus* 184 biofilm formation and viable counts from transwell competition with *S. epidermidis* B14 in the absence and presence of *S. aureus* 137. Biofilm was quantified by crystal violet (A) and viable counting of disrupted biofilm (B). *S. aureus* 184 alone controls were either 50 µl (black bars) and 150 µl (brown bars) in A, B, volumes to maintain equivalence. *S. aureus* 184 was cultured in the bottom well of a transwell plate with *S. epidermidis* B14 on the top (grey bars in A, B). *S. aureus* 184 after competition with both *S. epidermidis* B14 plus *S. aureus* 137 (blue bars in A, B). Strains were cultured in BHI-GN for 24 h. Data are presented as a triple mean +/- STDEV across the three replicates with t-test analysis for statistical significance effect.

Similar to the experiment described above with *S. epidermidis* B155, *S. aureus* 184 and *S. aureus* 137 were switched between top and bottom wells in a subsequent experiment to examine whether the biofilm inhibitory effect of *S. epidermidis* 14 expressing antimicrobial was also observed on *S. aureus* 137. In contrast with the earlier experiment, by comparing with the controls, there was no significant effect of *S. epidermidis* 14 on *S. aureus* 137 biofilm formation and no significant effect on biofilm in the bottom well when *S. aureus* 184 was added to the top well with *S. epidermidis* 14 (Fig 5.7).

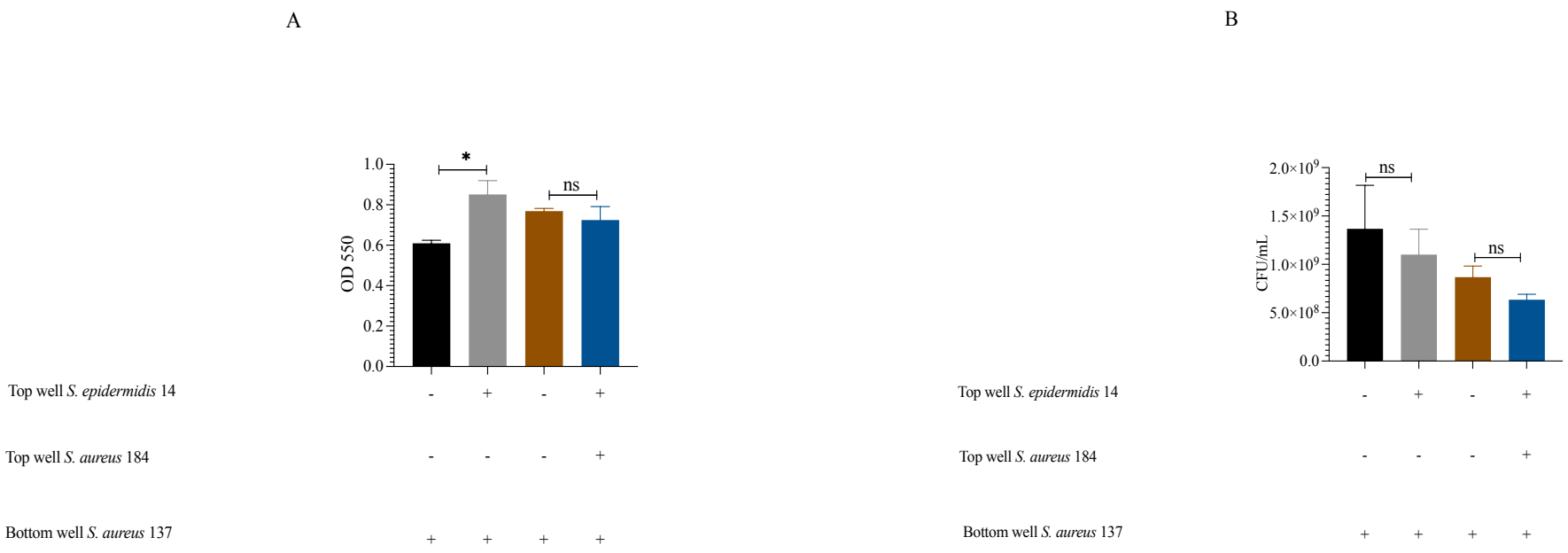

Figure 5. 7. *S. aureus* 137 biofilm formation and viable counts from transwell competition with *S. epidermidis* B14 in the absence and presence of *S. aureus* 184. Biofilm was quantified by crystal violet (A) and viable counting of disrupted biofilm (B). *S. aureus* 137 alone controls were either 50 µl (black bars) and 150 µl (brown bars) in A, B, volumes to maintain equivalence. *S. aureus* 137 was cultured in the bottom well of a transwell plate with *S. epidermidis* B14 on the top (grey bars in A, B). *S. aureus* 137 after competition with both *S. epidermidis* B14 plus *S. aureus* 184 (blue bars in A, B). Strains were cultured in BHI-GN for 24 h. Data are presented as a triple mean +/- STDEV across the three replicates with t-test analysis for statistical significance effect.

5.6 Discussion

The study has determined several *S. epidermidis* isolates act as competitors to inhibit *S. aureus* biofilm formation. The isolates were used to examine the effects of antimicrobials secreted by the competitor species on biofilms and found the effects from both the antimicrobial production on the target species were strain specific and required particular combinations to identify the biofilm inhibition successfully. The hypothesis was that *S. epidermidis* co-resident with *S. aureus* (mimicking the anterior nares as a niche) would be influenced by the presence of a second resident *S. aureus* strain. The experiments were designed to investigate the interference effect of resident *S. epidermidis* expressing specific antimicrobials with activity against *S. aureus* biofilm formation while considering a second *S. aureus* expressing the same or different *agr* group AIPs. BHI supplemented with 1% glucose and 4% NaCl was found to be optimal for the cocultures and was reported as suitable for staphylococcal biofilm studies previously (Fitzpatrick et al., 2006). This growth medium was used to determine the strains best suited to the experimental setup with respect to biofilm formation and study of its interference by *S. epidermidis* (Fig 5.2).

5.6.1 Transwell biofilm assay with bacterial competition

Transwell assay systems have been used in several reported studies, for example to examine the effects of a haemolytic toxin on host membrane integrity, cellular viability, and cellular signalling responses (Flaherty and Lee, 2016). In this research, the advantage of this technique is the potential of examining the effect of secreted factors during competition including antimicrobials and AIPs together with respect to their effects upon another strain's biofilm formation. The 0.4 μm porous membrane of the transwell connects the upper and lower chambers but allows only nutrients and secreted factors to exchange between the two chambers

(Fig 5.3), while preventing the passage of bacteria (Flaherty and Lee, 2016). Bases on the biofilm formation, the assay was shown to be effective with respect to competition and there were clear responses of the biofilm forming species in the lower chamber to the species cultured in the upper well. Although the contributions of the third strain competition was unclear, the transwell system could be used to simplify the model and test the effects of secreted antimicrobials on biofilm formation.

5.6.2 Competition effects on biofilm formation in *vitro*

S. epidermidis is capable of inhibiting *S. aureus* and one of the well-characterised mechanisms is to act via Agr autoinducing competition to affect nasal or skin colonisation (Paharik et al., 2017; Brown and Horswill, 2019; Glatthardt et al., 2020). Therefore, to characterise the potential for its role in the competition model used here, the Agr type of *S. aureus* was determined and with further work its potential contribution to growth inhibition alongside antimicrobials producing *S. epidermidis* can be examined. *S. epidermidis* B155 was included as a producer of epifadin (Ghabban, 2019). *S. epidermidis* B14 was a pronounced inhibitor of *S. aureus* 184 therefore was included in the experiments and expresses unknown producer (Alrajeh, 2021). Although knowing the Agr type of all the combinations tested can be important to understanding the possible interactions between all the Agr groups, the Agr types of *S. epidermidis* 155 and 14 have not been identified in this work. Moreover, not all group type Agr was obtained for *S. aureus* strains (Table 5.1). Therefore, only combinations of *S. epidermidis* with *S. aureus* Agr I and III were tested and overall, the number of strains tested was limited but with a determination to identify if these experiments could provide insights. This incomplete identification of many variables in the experiments made the model complex.

In the transwell competition, *S. epidermidis* B155 was active at significantly inhibiting biofilm growth of *S. aureus* SH1000 (Agr I) or *S. aureus* 137 (Agr III) in separate experiments (Fig 5.4). To test the effect of an imposed second strain of *S. aureus* in the top transwell reservoir that was cultured together with the *S. epidermidis* B155, it was found that *S. aureus* 137 prevented inhibition of *S. aureus* SH1000 biofilm growth (Fig 5.4). To investigate this phenomenon further, the *S. aureus* strains were switched around and *S. aureus* SH1000 was added with the *S. epidermidis* B155 strain to reveal that inhibition of *S. aureus* 137 biofilm was also similarly prevented (Fig 5.5). The outcome of this pair of experiments was the suggestion that either *S. epidermidis* growth was severely impaired by the added strain to limit inhibitor production or there was some antagonism that prevented biofilm inhibition, which could be AIP-mediated between the AIP of *S. epidermidis* and *S. aureus* but the basis for this was not resolved.

To probe this phenomenon more broadly, a further strain *S. epidermidis* B14 that expresses unknown antimicrobial was used for competition and was shown to have a significant inhibitory effect on *S. aureus* 184 biofilm formation. When *S. aureus* 137 was added to the top well for culture with *S. epidermidis* B14, it was evident that there remained a significant inhibition of *S. aureus* 184 biofilm growth by staining but not viable counting (Fig 5.6). The strain locations were switched in the transwell and *S. epidermidis* 14 was tested alone or with the *S. aureus* 184 strain against 137 *S. aureus*. In this case, *S. epidermidis* B14 had no quantifiable inhibition effects (Fig 5.7). Some research has demonstrated that all *agr* system of *S. aureus* can be inhibited by *S. epidermidis* AIP I (Glatthardt et al., 2020). Therefore, further experiments are needed to investigate the AIP types of *S. epidermidis* B155 and 14.

From the studies that were performed it can be determined that there are inhibitory effects of *S. epidermidis* antimicrobials on *S. aureus* biofilm formation. The inhibition effects are, however, strain-specific and the influence of a second *S. aureus* as a competing strain with *S.*

epidermidis did not provide consistent phenomena to pursue the line of investigation to answer the experimental hypothesis.

5.7 Conclusion

The effect of co-resident *S. epidermidis* epifadin producer 155 and unidentified antimicrobial producer 14 *S. epidermidis* on the stability of resident *S. aureus* SH1000 137 and 184 expressing the same or different Agr group AIPs was studied using a transwell biofilm competition assay. As a result, the model could be used to test the effects of secreted antimicrobials on biofilm formation. Overall, in competition between two strains, *S. epidermidis* epifadin producer 155 and *S. epidermidis* 14 have significant effects on biofilm formation of *S. aureus*. However, the effect and role of the Agr groups were unclear when three strains competed and when the strains switched between the upper and bottom wells. It is recommended that testing more Agr group AIPs could provide a better understanding of the function of Agr groups in biofilm competition.

Chapter 6: General discussion and future research

6.1 Main discoveries and suggested further work

Bacterial infections are becoming increasingly difficult to treat due to widespread antibiotic resistance among pathogens, especially *S. aureus* (Foster, 2017). Several studies have provided evidence that skin microorganisms can be a potential source of novel compounds and it is a promising area for discovery of new antibiotics that may help treat drug-resistant infections (Herman and Herman, 2018; Wang et al., 2020). Therefore, the aim of this study was to increase our knowledge how *S. aureus* competes with antimicrobial producing commensal staphylococci.

Nasal carriage has long been understood to be an important risk factor of *S. aureus* infections (Sakr et al., 2018). Skin and soft tissue infections (SSTIs) are most frequently caused by *S. aureus* in the US and the UK (Larsen et al., 2008; Hindy et al., 2021; Juwita et al., 2022). *S. aureus* is notorious for emerging antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) has become one of the most prevalent antibiotic resistant pathogens worldwide. Vancomycin is one of the few antibiotics that is still mostly successfully used to treat MRSA. The difficulty of dwindling options has increased, nevertheless, with the emergence of vancomycin-resistant *S. aureus* (VRSA) (Watkins et al., 2019). According to the Centers for Disease Control and Prevention (CDC), "the human race is now in the post-antibiotic era". While, the World Health Organization (WHO), declared that the crisis of antibiotic resistance is becoming worse (Ovchinnikov et al., 2020). Consequently, finding novel antimicrobials with the ability to outcompete pathogens in the skin environment became necessary and our need to understand acquired and innate resistance is growing.

It is well known that commensal staphylococci secrete a variety of antimicrobials to compete with one another and prevent the growth of other bacteria. These antimicrobials peptides, which are also called bacteriocins produced by coagulase-negative staphylococcal (CoNS)

often inhibit the growth of MRSA isolates (Mahlapuu et al., 2016; Zheng et al., 2017). This initial focus of this study was to examine the antimicrobial activity of CoNS isolates that targeted *S. aureus*. In **Chapter three**, the results showed that strains *S. capitis* 47, and *S. lugdunensis* 211681, 211482 and 1007 were inhibitory towards all *S. aureus* tested, including strains SH1000, B032, 137, 184, 34 and USA300. This supported previous studies that reported that CoNS are known to produce a wide range of antimicrobials to interfere with each other and impede growth of other bacteria such as *S. aureus* (Horswill, 2019). Moreover, there were clear phenotypic differences, showing that not all the tested CoNS strains were capable of inhibiting *S. aureus*. This may be caused by the lack of genes for inhibitor biosynthesis or by a non-specific inhibitor that may target other bacteria. More experiments are needed to test whether these inhibitors produced by the CoNS tested can target other species, for instance *S. epidermidis* which also are becoming more antibiotic-resistant and can lead to disease and death (Adam et al., 2022).

In recent studies, our group showed that *Staphylococcus* species can evolve during their interaction with respect to antimicrobial producers (Libberton et al., 2015; Ghabban, 2019; Alrajeh, 2021). Therefore, and for further research, it was predicted that *S. aureus* would develop resistance to the antimicrobial producers under pressure of competition between the species. Knowing more about these antimicrobials, especially their resistance, can be achieved by considering the resistance mutations (Belhout et al., 2022). The main objectives of this research were to determine if *S. aureus* species can evolve while interacting with an inhibitor producer and to understand the genetic bases of competition and resistance to AMPs. For this aim, the unknown antimicrobial producer *S. capitis* 47 was cocultured with *S. aureus* SH1000 and 184 strains separately on BHI agar plates to identify the possible species interactions and resistance. Similarly, competition was established between the epifadin antimicrobial producer *S. epidermidis* IVK83 (Torres Salazar et al., 2023) and *S. aureus* USA300 to study

the selection of resistance to epifadin antimicrobial. On healthy skin, CoNS have been reported to interact and compete with *S. aureus* using AMPs for the purpose of colonisation and survival. However, this healthy balance can be quickly affected by the expansion of the opportunistic pathogen *S. aureus*, which is the leading cause of skin infections (Parlet et al., 2019). In this study, the dynamic competition clearly supports the reported idea that skin microorganisms, particularly CoNS species, compete with pathogenic strains such as *S. aureus* for human skin (Parlet et al., 2019).

The study here found that *S. aureus* strains SH1000, 184 and USA300 evolved resistance against unknown antimicrobial producer *S. capitis* 47 and epifadin antimicrobial producer *S. epidermidis* IVK83. This confirms the hypothesis that *S. aureus* within communities can survive the antimicrobials produced by commensal bacteria (Kawada-Matsuo et al*.,* 2013), at least with certain antimicrobials.

This limitation is highlighted where *S. aureus* USA300 did not evolve resistance to antimicrobial producer *S. lungdunensis* 1007 even after fifteen days of competition. This result supports the reported studies that *S. aureus* cannot develop resistance to certain antimicrobials produced by CoNS species, and in particular lugdunin or lugdunin-producing commensal bacteria (Zipperer et al., 2016). From these studies, lugdunin offers encouraging evidence to screen the human microbiome for new antibiotics to treat *S. aureus* infections. More studies are needed to discover these potential antimicrobial leads.

It was reported that *S. aureus* isolates become resistant to antimicrobials of various classes at different rates and require differing fundamental resistance mechanisms as well (Watkins et al., 2019). There are two ways that bacteria develop antibiotic resistance: horizontal gene transfer and spontaneous mutation (Baym et al., 2016). It is well known that staphylococcal resistance to penicillin is mediated by *blaZ* gene that encodes β-lactamase. Moreover, *mecA* gene is responsible for methicillin resistance and the *vanA* gene is needed for

vancomycin resistance. Acquisition of these resistance genes are the result of their successful horizontal transfer (HGT) that enables the interchange of bacterial genetic material, particularly genes for antibiotic resistance (Watkins et al., 2019). According to recent research, staphylococci isolated from the human nasal microbiome usually include biosynthetic gene clusters that code for secondary metabolites such bacteriocins (unpublished data).

The study of bacteriocins as antimicrobials, such as nisin, galidermin, epifadin, and lugdunin to help treat drug-resistant infections is already underway (Zipperer et al., 2016; Herman and Herman, 2018; Wang et al., 2020; Torres Salazar et al., 2023). The failure to overcome the antibiotic resistance over the past years has been partially due to the understanding that target-based strategies do not consider the essential bacterial resistance mechanisms such as biofilms, multidrug efflux pumps that contribute to bacterial resistance in *vivo* (Lowy et al., 2003). Therefore, this study was performed to help understand the mechanisms of resistance to these antimicrobials.

Spontaneous mutations can modify the antibiotic target, its expression level, or the regulation of resistance genes, such as those encoding efflux pumps (Baym et al., 2016). In **chapter three**, this study reported single-nucleotide mutations related to an efflux pump of *S. aureus* SH1000 and 184 regulated by the *cro/cI* gene after experimental evolution with an unknown antimicrobial produced by *S. capitis.* Development of resistance can be accrued in single cells by mutations that alter drug binding sites or by increasing expression of endogenous efflux pumps (Ylmaz and Aslantaş, 2017). Efflux pumps are typical physiological features of bacteria and are expressed in antimicrobial sensitive strains (Lekshmi et al., 2018). Drug resistance can result from the activation and expression of genes that code for an efflux system, which dramatically increases the ability to remove antibiotics from the cell (Zarate et al., 2019). To date, there are five main classes of efflux pumps known in *S. aureus* according to their structural protein families. Under these families are more than 15 efflux pumps described so

far. The main pumps included are: first the ATP-binding cassette (ABC) family that includes MsrA, AbcA, Sav1866, VgaA and VgaB; second, the major facilitator superfamily (MFS) such as NorA, NorB, NorC, MdeA, SdrM, and LmrS and QacA/B; third, the multidrug and toxic compound extrusion (MATE) protein family, e.g. MepA; fourth, the small multidrug resistance (SMR) family including SepA, QacG, QacH, QacJ and QacC (also known as QacD, Smr, or Ebr); last, the resistance-nodulation division (RND) family, such as FarE (Foster, 2016; Jang, 2016; Dashtbani-Roozbehani & Brown, 2021). While some of these efflux pumps, such as MsrA, VgaA/B, QacA/B, QacG, QacH, QacJ and QacD, are encoded on plasmids. others are encoded on the bacterial chromosome, e.g., AbcA, Sav1866, NorA, NorB, NorC, MdeA, MepA, SdrM, LmrS, SepA, and FarE (Hassanzadeh et al., 2017; Dashtbani-Roozbehani & Brown, 2021).

In the **fourth chapter**, a mutation in the TCS *desK* gene was selected during competition of *S. aureus* USA300 with the antimicrobial epifadin producer *S. epidermidis* IVK83. The finding supports the association between DesKR TCS and epifadin discovered by Ghabban (2019). Several two-component signal transduction systems (TCS) also control antimicrobial resistance in *S. aureus* (Coates-Brown et al., 2018) in addition to those TCS involved in processes like cell proliferation and pathogenicity (Utsumi and Igarashi, 2012). Mutations in TCS genes helped to identify several *S. aureus* resistance pathways such as GraRS and BraRS (Bleul, Francois and Wolz, 2021). Prior to the study of Ghabban, (2019) DesKR TCS has not previously been described as an antimicrobial resistance mechanism in staphylococci. Thus, confirmation that the mutation in the *desK* gene of *S. aureus* USA300, contributes to epifadin survival has increased our understanding of further resistance mechanisms that *S. aureus* can use. Altogether, the mutations of *desK, Cro/CI* and *pbuG* genes add to our understanding of *S. aureus* resistance mechanism that are important for providing survival from species in the skin microbiome.

Several promising models for finding new drug targets already exist. One strategy is to combine high-throughput screening, chemical modification, and efficacy testing in animals with genetic data for potential therapeutic targets. Crystal structure analysis of drug targets, which allows the study of specific interactions of a given drug with its protein target, can be used to study and modify these antimicrobials to make them more effective. One example of crystal structure of drug targets is modifications of B-lactams that bind the active site of PBP2a (Lowy, 2003). The use of purine nucleosides such as xanthosine and guanosine to reduce cdi-AMP, important for B-lactam resistance can improve the effect of oxacillin is another example of maintaining the efficacy of existing antimicrobials (Nolan et al., 2022).

In addition to acquiring antimicrobial resistance genes, *S. aureus'* ability to produce biofilms makes infections increasingly difficult to treat (Kamaruzzaman et al., 2018). Increased biofilm formation in some *S. aureus* strains has been linked with inactivation of accessory gene regulator (Agr) (Suligoy et al., 2019). However, multiple investigations, have revealed that the commensal *S. epidermidis* secretes molecules that may be able to block *agr* as a novel treatment for *S. aureus* infections. (Tan et al., 2018). *S. aureus* can encode one of four variants of the Agr locus, which result in the production of autoinducing peptide (AIP) signal structures: AIP-I, II, III and IV (Parlet et al., 2019). Most S. *aureus* clinical isolates, including all four *agr* types, have shown a significant reduction in biofilm formation under effect of molecules produced by *S. epidermidis* (Glatthardt et al., 2020). Research has demonstrated that all *agr* system of *S. aureus* can only be inhibited by *S. epidermidis* AIP I but not IV *agr* types (Otto et al., 2001; Williams et al., 2019). In **chapter five**, the effect of *S. epidermidis* B155 epifadin producer on the stability of resident *S. aureus* from invasive strains of *S. aureus* expressing the same or different group Agr AIPs was investigated as model scenario of *S. aureus* that interact with other bacteria in the anterior nares. The strain-specific aspect and the influence of a second *S. aureus* as a competing strain with *S. epidermidis* was not studied. The results showed that

epifadin producer *S. epidermidis* B155 and *S. epidermidis* 14 (expressing an unknown antimicrobial (Alrajeh, 2021)) had a significant inhibition effect on biofilm formation of *S. aureus* that have different type groups of AIP- I III.

To conclude, *S. aureus* mutations were identified in the Cro/CI repressor and *pbuG* genes and in the two-component system DesKR genes indicating that this bacterium has additional mechanisms that contribute to interspecific competition. The main objective of this study and following studies should be to look for further microbiome antimicrobials with unique targets and new mechanisms of action due to the rise in skin diseases and the resistance of skin pathogens such MRSA to new antibiotics. More research will help to understand the composition and dynamics of microbial communities in human skin, which can be a rich source of antimicrobials.

6.2 Limitations of this study

This research advances knowledge of microbial relationships between staphylococci that colonise human skin. The competition between two bacteria in *vitro* can give a close idea of interactions on human skin in *vivo*. However, this competition, especially the evolution of *S. aureus* under effect of other staphylococci can be more complex in *vivo* due to the other factors such as other strains, environmental conditions. Although these few experiments may not explain the whole situation, studying the antimicrobial resistance mutations will always be useful to understand the bacteria resistance mechanisms. Secondly, in this study *S. aureus* SH1000, 148 and USA300 were used as model of skin isolates. It may be necessary to consider additional strains and more relevant environmental setups. Finally, the coronavirus (COVID-19) pandemic has affected this study throughout 2020/2021. Due to the lockdown, there was less time to do desired experiments. Although there was a 6-month extension from the university, unfortunately there was no personal funding from the sponsor to cover these 6 months.

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Appendix

Appendix table A 1. Synonymous variant identified in *S. aureus* SH1000 resistance of D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig, and D-8-W-N-Pig. SNPs and INDELs were detected in three clones (1,2,3) after either two or eight days of interference competition with antimicrobial producer *S. capitis* 47. NCBI BLAST was used to provide mor information on the hypothetical proteins and the number in bracket indicates percentage sequence identity.

Synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Day of change/ detected in clones No
JEJILOIK _01489	Betaine aldehyde dehydrogenase *gbsA*	8262	Ile167Ie	T to A	D-2-G/1,2,3
JEJILOIK _00332	Type VII secretion system protein *essG_1*	266	Asn78Asn	T to C	D-2-G-Pig/1,2,3 D-8-G-Pig/1,2,3
JEJILOIK_01934	hypothetical protein- transposase (100 %)	26021	Phe31Phe	G to A	D-8-L-G-Pig/1,2,3
JEJILOIK_00490	Serine-aspartate repeat-containing protein C *sdrC*	53496	Asp843Asp	G to A	D-8-W-N-Pig/1

Appendix table A 2. Synonymous variant identified in passaged *S. aureus* SH1000 of D-8-L-G-Pig, and D-8-W-N-Pig.

SNPs and INDELs were detected in three clones (1,2,3) after eight days of passaging on BHI Agar. NCBI BLAST was used to provide more information on the hypothetical proteins and the number in brackets indicates percentage identity.

Synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Day of change/ detected in clones No
JEJILOIK_00332	Type VII secretion system protein *essG_1*	311	Tyr93Tyr	T to C	WT-D-8-L-G-Pig/1,2,3 WT-D-8-W-N-Pig/1,2
JEJILOIK_00755	Purine nucleoside phosphorylase *deoD1*	34931	Gly23Gly	G to A	WT-D-8-W-N-Pig/1,2,3
JEJILOIK_01093	hypothetical protein- heavy metal-associated domain-containing protein (69 %)	41732	Ser55Ser	T to C	WT-D-8-W-N-Pig/1,2,3
JEJILOIK_01489	Betaine aldehyde dehydrogenase *gbsA*	8262	Ile167Ile	T to A	WT-D-8-L-G-Pig/2,3 WT-D-8-W-N-Pig/2,3
JEJILOIK_01934	hypothetical protein- transposase (100 %)	26021	Phe31Phe	G to A	WT-D-8-L-G-Pig/1,2 WT-D-8-W-N-Pig/1,3

Appendix table A 3. Synonymous variant identified in *S. aureus* 184 resistance of D-2-G-Pig, D-8-G-Pig, D-8-L-G-Pig, and D-8-W-N-Pig. SNPs and INDELs were detected in three clones (1,2,3) after either two or eight days of interference competition with antimicrobial producer S. capitis 47. NCBI BLAST was used to provide mor information on the hypothetical proteins and the number in bracket indicates percentage sequence identity.

Synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Day of change/ detected in clones No
PILBBNHO_01439	Hypothetical protein-transposase (100 %)	144932	Phe31Phe	G to A	D-2-G-Pig/1 D-8-G-Pig/3 D-8-L-G-Pig/1 D-8-W-N-Pig/1,2,3
PILBBNHO_00086	5-oxoprolinase subunit C *pxpC_1*	84754	Val112Val	G to T	D-8-G-Pig/1,2,3 D-2-G-Pig/3
PILBBNHO_01823	Hypothetical protein-LPXTG-anchored repetitive surface protein *sasC*	78988	Asn446Asn	G to A	D-8-W-N-Pig/2,3
PILBBNHO_02329	Nisin biosynthesis protein *nisB*	12025	Ala32Ala	C to T	D-8-L-G-Pig/1,2,3
PILBBNHO_01398	Nitrite reductase [NAD(P)H] *nasD*	100716	Arg61His	C to A	D-8-W-N-Pig/2

Appendix table A 4. Synonymous variant identified in passaged *S. aureus* 184 of D-8-L-G-Pig, and D-8-W-N-Pig. SNPs and INDELs were detected in three clones (1,2,3) after eight days of passaging on BHI Agar. NCBI BLAST was used to provide more information on the hypothetical proteins and the number in brackets indicates percentage identity.

Synonymous variant					
Gene ID (Prokka)	Gene product description	Genome position	Codon change	Base change	Day of change/ detected in clones No
PILBBNHO_01439	Hypothetical protein- transposase (100 %)	144932	Phe31Phe	G to A	WT D-8-L-G-Pig/3 WT D-8-W-N-Pig/1