‘Does the human skin microbiome adapt to antibiotic exposure?’

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ACRONYMS AND ABBREVIATIONS

CoNS	Coagulase-Negative *Staphylococci*
AdR	Adaptive Resistance
AMR	Antimicrobial Resistance
PGA	Poly-γ-glutamic Acid
PNAG	Poly-N-acetylglucosamine
PIA	Polysaccharide Intercellular Adhesin
PAMPs	Pathogen Associated Molecular Pattern
TLRs	Toll-like Receptors
CFU	Colony-Forming Units
EUCAST	European Committee on Antimicrobial Sensitivity Testing
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
WHO	World Health Organisation

Abstract

Staphylococcus epidermidis is a substantial element of the healthy human skin microflora and can become pathogenic to cause infections, for example, septicaemia and endocarditis. The effects of long-term sub-lethal exposure to antibiotics on the skin microbiota are unknown. How does antibiotic adaptation affect the ability of *S. epidermidis* to interact with host immune systems, inhibit pathogens and keep a healthy microflora? This project aims to characterise five *S. epidermidis* bacteria samples isolated from the skin, before and after exposure to antibiotics thus comparing their antibiotic susceptibility pattern (disc diffusion assay), biofilm formation (crystal violet microtitre plate assay) and microbial virulence (*G. mellonella* infection assay). Serially passaged antibiotic-adapted strains were also assessed for the following characteristics: colony and cell morphology, cross-resistance (to other antibiotics), growth rate and competitive fitness ability. *S. epidermidis* parent strains were firstly susceptible to antibiotics such as erythromycin and doxycycline, and later formed adaptive resistance. Erythromycin adapted strains were found to be resistant to erythromycin and cross- resistant to doxycycline based on both disk diffusion assay and MIC results. Doxycycline adapted strains formed resistance to doxycycline based on both disk diffusion assay and MIC results. Significant differences (p <0.05) were observed in the mean relative biofilm units and *G. mellonella* infection assay survival rates of antibiotic-adapted strains of *S. epidermidis.* Erythromycin adapted strain had significantly enhanced fitness advantage (w value- 1.35, p value- 0.0427) and were significantly virulent (p-value of 0.0065) when compared to the parent strain. Doxycycline adapted strain showed significantly lower fitness advantage (w value- 0.38, p-value 0.0005) yet, they were significantly virulent (p-value of 0.0059) in comparison to the parent strain. These findings highlight the implications of adaptive resistance to antibiotics in the treatment of *S. epidermidis* infections, and therefore necessitate the development of more sophisticated future treatments for bacterial infections. It is important for the private and public healthcare sectors worldwide to invest in the advancement of new anti-infectives to combat the growing antibiotic resistance.

Chapter 1

1.0 Introduction

1.1 Skin microbiome and *Staphylococcus epidermidis*

The human skin microflora has a vital role to play in health and disease. The growing arsenal of experimental methods are rapidly reaching new horizons in the research of human to microbe and microbe to microbe interactions. The skin covers around two square metres of the human body while maintaining the body integrity, it is the biggest organ in regard to surface area. The skin allows the body to interact with its surroundings and provide protection from several physical, mechanical, biological and chemical assaults (Leonel *et al.* 2019). In addition to this, the skin allows the human body to remain "healthy" by inhibiting external pathogenic microbes from entering and preventing the body from losing critical internal fluids. The skin also keeps individuals healthy by transmitting sensations and balancing body temperature (Leonel *et al.* 2019). The skin constantly regenerate cells, hence, it is a self-renewing organ. The skin involves an outer layer, epidermidis, a subjacent connective tissue, dermis, and subcutaneous tissue (Grice & Segre, 2011). Every skin site is composed of several components, including melanocytes, keratinocytes and Langerhans cells in the epidermis, fibroblasts, pericytes, immune cells, nerve endings and endothelial cells in the dermis, and adipocytes in the subcutaneous tissue (Grice & Segre, 2011).

An ecogenomic study discovered that *Staphylococcus* and *Corynebacterium* species are the prevailing microbes colonising moist skin sites, culture data also suggests that these micro-organisms favour high humidity skin sites (Grice & Segre, 2011) (Figure 1). These high humidity areas involve the axillary vault, umbilicus (navel), the gluteal crease (topmost part of the fold between the buttocks), the inguinal crease (side of the groin), the popliteal fossa (behind the knee), the antecubital fossa (inner elbow) and the sole of the foot (Figure 1). *Staphylococci* inhabit aerobic sites on the skin and may utilise the urea present in sweat for nitrogen. Processing of apocrine sweat by *Staphylococci* (and other axillary vault microbes) causes the malodour characteristic connected to humans sweat (Figure 1) (Grice & Segre, 2011). One of the major *Staphylococci* species identified on human skin is *Staphylococcus epidermidis,* commonly inhabiting nares, axillae and the head (Dreno *et al*., 2016). *Staphylococcus epidermidis* is the most dominant Gram-positive, coagulase-negative cocci commensal on the healthy human skin microflora. *S. epidermidis* is responsible for about 60-70% of all coagulase-negative *Staphylococcal* species on the stratum corneum (Dreno *et al.,* 2016).

Non-bacteria microbes have also been identified from the skin (Figure 2A). *Malassezia* species are the most frequently isolated fungal species, found mainly in sebaceous areas. The Demodex mites

including *Demodex folliculorum* and *Demodex brevis* are microscopic arthropods and are a part of the normal skin microflora (Figure 2A) (Grice & Segre, 2011). Sebum provide Domodex mites nourishment, hence, mites are abundantly found after puberty, choosing to colonise sebaceous regions of the face. Demodex mites can similarly feed off epithelial cell lining the pilosebaceous unit and feed on microorganisms that inhabit the same area e.g. *Propionibacterium acnes*. The function of viruses as a skin commensal has not yet been researched, research is restricted by the current microbiological and molecular means to isolate and classify viruses (Figure 2A) (Grice & Segre, 2011).

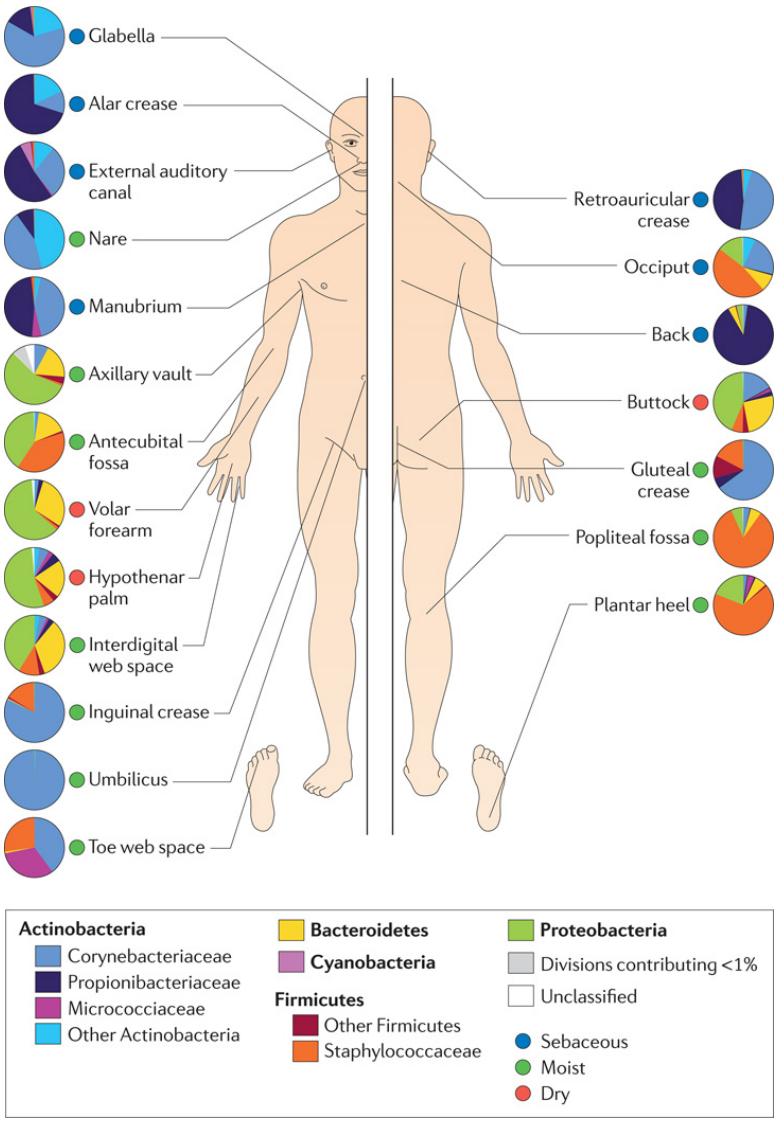

Figure 1. Human skin sites with bacteria Microbes that are considered as skin commensals involve: *Coryneforms* of the phylum *Actinobacteria* (the genera *Corynebacterium, Propionibacterium* and Brevibacterium) and the genus *Micrococcus* (Grice & Segre, 2011). Bacteria family classification is shown alongside the phyla in bold. Different skin sites: sebaceous or oily (blue circle), moist (green circles) and dry or flat skin (red circle). The sebaceous sites are: alar crease (edge of the nostril);

glabella (amongst the eyebrows); auditory canal (within the ear); occiput (behind the scalp); retroauricular crease (behind the ear); manubrium (top chest area); and back. Moist sites are: axillary vault (armpit); nare (within the nostril); antecubital fossa (inner elbow); interdigital web space (area between the middle and ring fingers); inguinal crease (side of the groin); gluteal crease (upper area of the crease between the buttocks); popliteal fossa (back of the knee); plantar heel (underneath the heel of the foot); toe web space; and umbilicus (navel). Dry sites are: hypothenar palm (palm of the hand proximal to the little finger); volar forearm (inner area of the mid-forearm); and buttock (Grice & Segre, 2011).

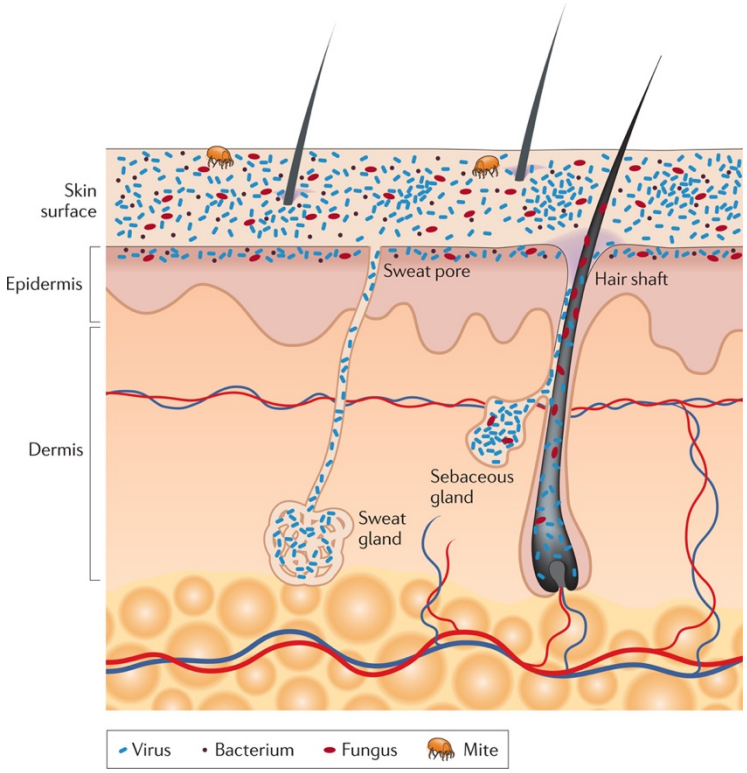

Figure 2A. Skin anatomy illustration with skin commensals and appendages (Grice & Segre, 2011). Commensal *Staphylococcus epidermidis* remain in the skin epidermis where there are no blood vessels. If *S. epidermidis* reach the blood vessels in the dermis, it can spread to other areas of the body and cause disease. Microbes (bacteria, viruses and fungi) and mites coat skin surface area and inhabit deep inside the gland and hair. Rod and round bacteria e.g. *Proteobacteria* and *Staphylococcus* species, respectively. These microbial populations are acutely intertwined between themselves and other microorganisms. Fungi that exist in a state of commensalism on the skin e.g. *Malassezia* species grow both as dividing filamentous hypha and as singular cells. Elements of virus can exist both freely and in bacterial cells. *Demodex folliculorum* and *Demodex brevis* skin mites are one of the tiniest arthropods and live inside or close to hair follicles. Hair follicles, sweat

glands and sebaceous glands are few examples of skin appendages illustrated (Grice & Segre, 2011). The skin is usually cool, acidic and dry, variation of the skin microflora is reliant on the cutaneous site thickness, folds, density of hair follicles and glands. The outermost layer of the epidermis is the stratum corneum, of which 90% epidermis cells are made up of terminally differentiated keratinocytes (corneocytes). Squames (enucleated keratinocytes) comprises of keratin fibrils that are crosslinked. The keratin can retain large volumes of water between the fibers. Cornified envelopes are situated in the lipid bilayers, producing the 'bricks and mortar' of the epidermidis. Squames migrate from the basal layer of the skin to the outer skin surface and shed, this process could take four weeks (Grice & Segre, 2011).

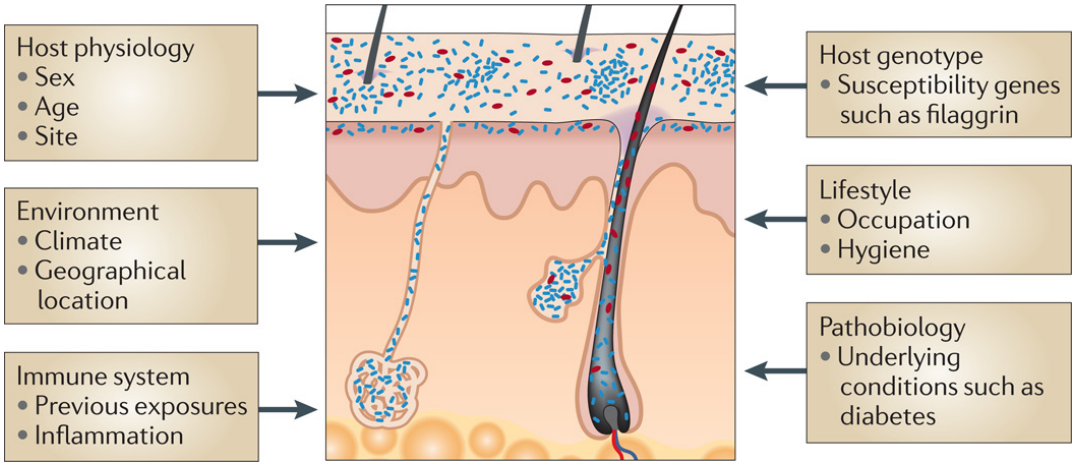

Figure 2B. Factors contributing to variation in the skin microbiome (Grice & Segre, 2011)**.** Factors such as age, anatomical location and gender can influence the variation of microflora in skin. The skin has approximately 12 to 16 layers of corneocytes, and each corneocyte has an average thickness of 1 micrometer, depending on the age, anatomical site, and UV radiation exposure (Grice & Segre, 2011)**.** Male and female skin environment differ physiologically and anatomically, for example, producing different concentrations of sweat, sebum and hormone for different skin sites, this partly accounts for the bacterial variances seen between both sexes (Grice & Segre, 2011). Environment specific to an individual including clothing choice, occupation and drug usage, may regulate skin microflora colonisation. Skin care products, soaps, body/face wash products, moisturisers and cosmetics, are all possible factors that influence the skin microflora variations. These products can modify the skin barrier condition, however, how it may affect the skin microflora is unclear. Measureable skin culture samples highlighted that high-humidity and high-temperature correlates to increased amounts of commensal microbes on the axillary vaults, back, and feet compared to high-temperature and low humidity environment (Grice & Segre, 2011).

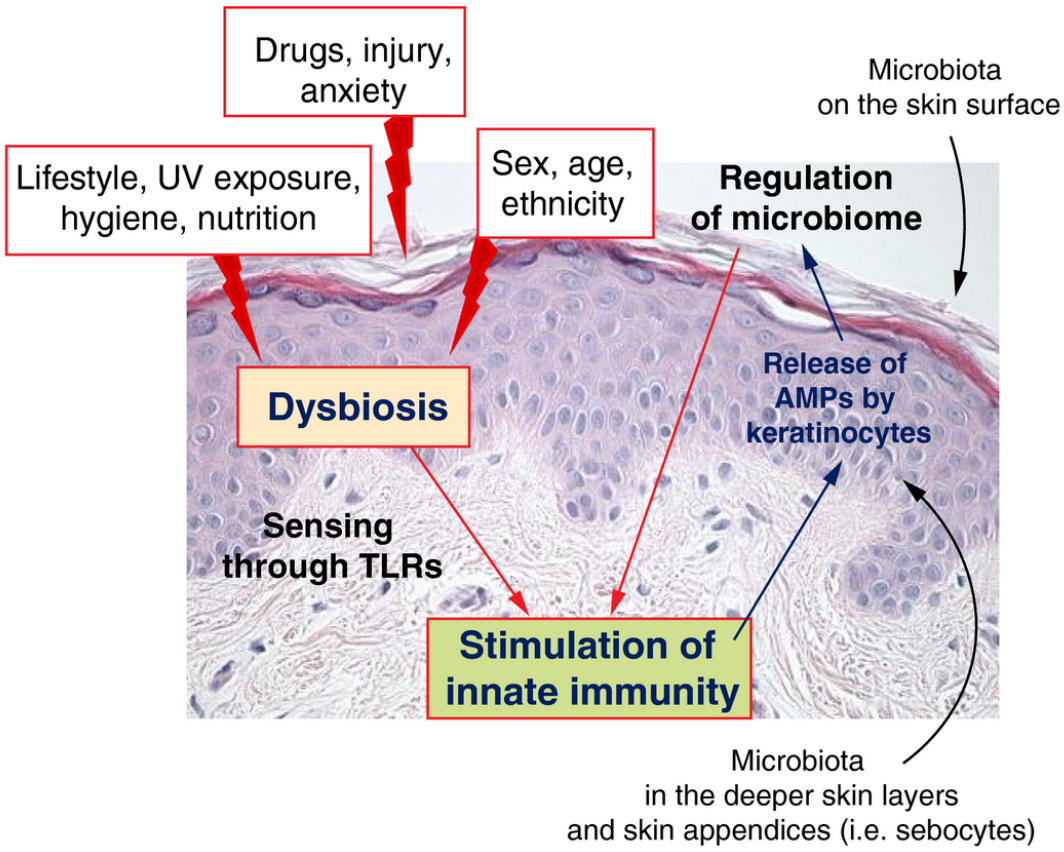

Figure 3. Stimulation of innate immunity. Agents that may possibly cause dysbiosis and the human host innate immune response of the skin (Dreno *et al.,* 2016).

The human skin is an organ which functions as a highly complex barrier while symbiotically interacting with bacterial populations via adaptive and innate immune system signals. Bacteria provide important nutrients, affecting cellular metabolism and strengthen the immune system (Figure 3) (Grice & Segre, 2011; Baldwin *et al.,* 2017). Loss of protective bacteria intensifies inflammatory skin diseases (Williams & Gallo, 2017). The skin is always exposed to countless exogenous and endogenous elements which influence symbiosis, possibly leading to inflamed skin, skin allergies, infections, cutaneous tumour formation or autoimmune illnesses (Bukhari, 2015; Leonel *et al.,* 2019). The gastrointestinal tract and faeces microflora have been researched and documented for several years while the skin or scalp microflora has not been investigated till recently (Bukhari, 2015).

In a recent study, it has been reported that *S. epidermidis* strains obtained from healthy patients' skin could induce a gathering of CD8+ T lymphocytes in the skin via dendritic cells presentation of antigens (Linehan *et al.,* 2018). However, *S. epidermidis* strains that stimulate accumulation of CD8+ T

lymphocytes in the skin were absent from adult humans with skin disease (Linehan *et al.,* 2018). Linehan *et al.* (2018) analysed *S. epidermidis* genome and carried out *in vitro* experiments to find major histocompatibility complex Ib (MHCIb) on dendritic cells, the cells displayed N-formyl methionine peptides, which are secreted by *S. epidermidis*, to CD8+ T lymphocytes. The researchers completed RNA sequencing of CD8+ T lymphocytes which were induced by or linked to *S. epidermidis.* After evaluating the global transcriptome, results revealed that CD8+ T lymphocytes can up-regulate various genes linked with immune regulation and tissue repair (Grice & Segre, 2011; Linehan *et al.,* 2018). In addition to this, Linehan and colleagues also investigated the effects of *S. epidermidis* on skin injury via punch biopsy method. Interestingly, *S. epidermidis* stimulated CD8+ T cells, which facilitated re-epithelisation of the injured skin and promoted wound healing.

One of the functions of the skin microbiota is to avoid unwanted pathogenic bacteria from colonising the skin, this maintains an environmental equilibrium in each skin niche. The configuration of the skin microflora may largely influence medical progression for treating cancer, as cancer that use procarcinogenic or anticarcinogenic actions mainly rely on a balanced skin microflora (Garrett, 2015; Nakatsuji, *et al*. 2018). Skin microbiota can add to carcinogenesis, by augmenting or reducing a host's risk, this can be categorised into three general groups: (i) modification of the host cell proliferation to death ratio (ii) influencing host immune system function and (iii) manipulation of host metabolism (Garrett, 2015). Thus, it is vital to keep a balanced skin inhabitant populace to also preserve a healthy gut microbiota (Dreno *et al.*, 2016).

For microbes to colonise the human skin they must resist environmental factors (injury, UV exposure, anxiety, and hygiene) which constantly change compared to the protected areas of the body (Dreno *et al.,* 2016) (Figure 1 &3). The skin microenvironment undergoes constant fluctuation in mechanical stress, pH value, salt concentration and osmolarity. The commensal bacteria must adapt to the dry and moist skin conditions. There are only few known bacteria that can withstand high salt concentrations or other extremities on human epithelia. Non-halophilic *Staphylococci* are known to have an extraordinarily high capacity to resist such influences, especially high levels of salt (Espadinha *et al*, 2019). *S. epidermidis* is unaffected by the harsh surroundings that usually occur in its natural habitat due to the genes it may possess. For instance, *S. epidermidis* can survive extreme salt and osmotic pressures via six transport systems for osmoprotectants and eight sodium ion / proton exchangers (Dreno *et al.,* 2016). The ability to form biofilms help the bacteria to survive harsh conditions significantly. Over the years a large amount of osmoprotective elements have been expressed and reported in *S. epidermidis* biofilms (Mirzaei *et al.,* 2017). Also, *S. epidermidis* is anticipated to not only have unharmful interaction with the host but have a probiotic function as well. This may exist to avoid more infectious bacteria colonising the skin such as *Staphylococcus aureus*.

Nevertheless, no distinct evidence has been found to verify that *S. epidermidis* is capable of secreting factors that may influence colonisation of other microbes *in vivo* (Mirzaei *et al*., 2017). Certain variants of *S. epidermidis* yield differing lantibiotics including, Pep5, 15X, epidermin and epilancins K7, these lantibiotics are highly toxic and deadly for several Gram-positive bacteria (Espadinha *et al*, 2019).

Healthy skin microflora may influence the host cells leading to a stronger host defence system against other pathogenic microbes. *S. epidermidis* can induce endogenous antimicrobial peptides (AMPs) e.g. β defensins, hence, reinforcing the host defence system against *S. aureus* (Noreen *et al.,* 2015). *S. epidermidis* is capable of activating mast cell-induced anti-viral host defence mechanism, for it to control inflammatory responses throughout the wound healing process, while encouraging the production of stratum corneum AMPs and inducing cutaneous T-cell development (Noreen *et al.,* 2015). *S. epidermidis* hence work to protect the skin by communicating with the host immune system. Furthermore, the skin microbiota may symbolise as a filter for the environmental agents interacting with or breeching the skin. There is also evidence available on how the biological determined host immune system has a strong hold on the assembly of the microflora (Noreen *et al.,* 2015).

Alternatively, once the existence of the skin microbiome is sensed via Toll like receptors (TLRs), epidermal Langerhans cells can induce naïve T cells to pedestal a Th17 reaction, this allows keratinocytes to control the AMPs emission (Noreen *et al.,* 2015). Consequently, other than human innate immune system and dendritic cells in the epidermis, TLRs also appear to instruct the adaptive immune response, thus encouraging the complicated regulation of the bacteria growing in the skin (Noreen *et al.,* 2015).

1.2 Commensal *S. epidermidis* preventing skin cancer

Nakatsuji *et al*. (2018) have recently identified certain variants of *S. epidermidis* that produce 6-N-hydroxyaminopurine (6-HAP) with the capacity to block DNA polymerase activity. Intravenous inoculation of 6-HAP inhibited melanoma B16F10 progression from occurring in mice and there was no sign of toxicosis. Mice was also colonised with 6-HAP producing *S. epidermidis* strain, resulting in a low prevalence of ultraviolet-induced tumours compared to mice colonised with a non - 6-HAP producing *S. epidermidis* control strain. 6-HAP producing *S. epidermidis* strains were found in several healthy human skin microbiota indicating that the skin microflora of particular individuals may provide protection against skin neoplasia (Nakatsuji *et al*. 2018).

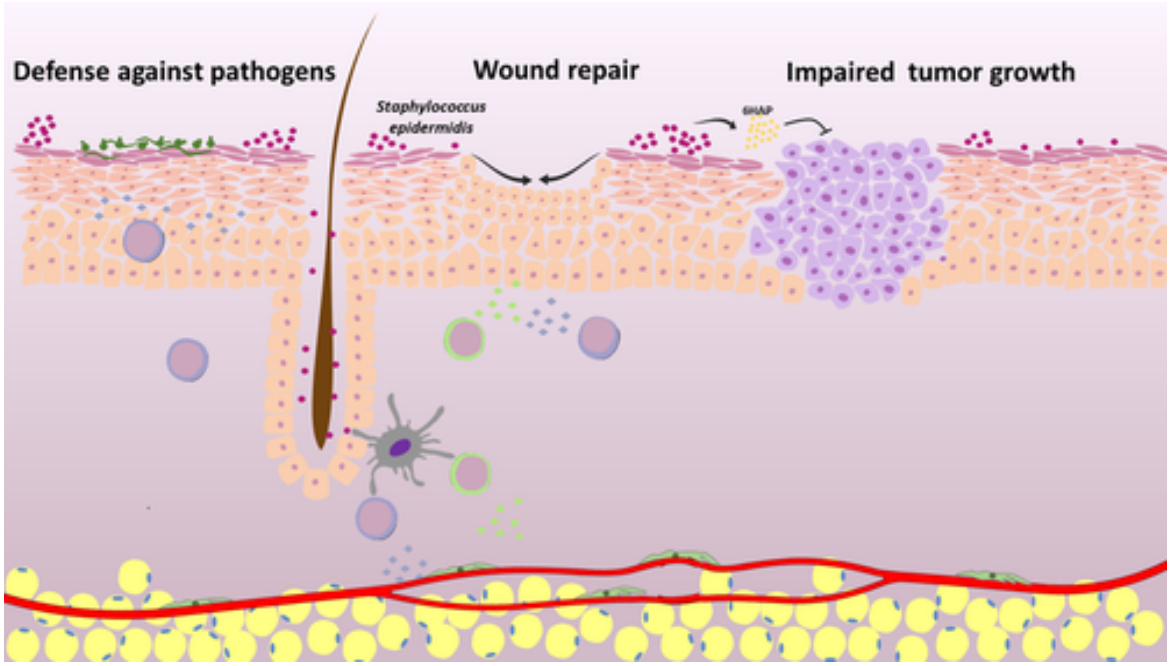

Figure 4. *S. epidermidis* stimulate wound recovery and tumour deterioration in the skin (Leonel *et al.,* 2019). Recent studies suggest novel roles for *S. epidermidis* in the skin microenvironment (Linehan *et al.,* 2018; Nakatsuji *et al.*, 2018). *S. epidermidis* support wound healing through an assemblage of CD8+ T lymphocytes in the skin and inhibit cutaneous tumour development via 6-N-hydroxyaminopurine secretion. Future research will demonstrate detailed cellular and molecular mechanisms entailed in the relations between skin microbiota and numerous factors of the skin microenvironment (Grice & Segre, 2011; Leonel *et al.,* 2019).

1.3 The influence of gut microbiome on skin microbiome

Several lines of evidence have indicated that there is a bidirectional communication between the gut microbiome and the skin microflora as various studies connect healthy gastrointestinal to skin homeostasis and allostasis (Levkovich *et al*., 2013). Gastrointestinal illnesses and differences in the gastrointestinal system usually show signs and symptoms on the skin (O'Neill *et al.,* 2016). The gut microflora commonly indicate change in the pathophysiology of various inflammatory diseases (Shah *et al.*, 2013; Thrash *et al.*, 2013; Gloster *et al.,* 2016).The gut microbiome has a great impact on the gastrointestinal system and can be used for healing purposes by supplementing or prescribing probiotics. Furthermore, the gut microbiota has been observed to have an effect on the skin microbiota (Schwarz *et al.,* 2017).

Moreover, the short chain fatty acids produced by fermentation of fiber in the gastrointestinal tract, acetate, butyrate and propionate are known to have an important function in controlling the

prevalence of specific skin microflora description, which later have an effect on the skin immune defence processes (Shu *et al*., 2013). Propionic acid may display extreme levels of antibacterial activity against the most prevailing community-acquired methicillin-resistant *Staphylococcus aureus* (Samuelson *et al.,* 2015). Certain bacteria strains which are symbiotic skin inhabitants namely *S. epidermidis* and *P. Acnes* are identified as the most tolerant species compared to the other skin microflora microbes. Overall, the study found encouraging evidence to support the theory of an active interactive system between the human gastrointestinal tract and the skin.

The gut to skin co-operation theory formulated by Arck *et al*. (2010) mentioned a possible gut to brain to skin co-operation, hence, the advantages of oral prebiotics and skin probiotics is explored. Additionally, as well as the oral probiotic created for the human skin, a recent group of emollients and moisturisers involving lysates of bacteria e.g. *Vitreoscilla filiformis* or *Lactobacillus* have been created. The current probiotic invention has been constructed to help manage diseases of the human skin for example, acne or atopic dermatitis. It does this by re-establishing the epidermal barrier, the microbiota of the human skin and regulating the activation of innate immune defence system.

1.4 *S. epidermidis* – An opportunistic pathogen

Nosocomial infections are 41% of the time caused by coagulase-negative *Staphylococci*. Little is known about *S. epidermidis* clonality, pathogenic capabilities including its virulence factors and the prevalence of antibiotic resistance genes such as, mecA, IS256, qacA/B, and icaAB (Mirzaei *et al.,* 2017). Moreover, it is an opportunistic pathogen, as it can only become pathogenic by breaching the host innate immune system (Brown *et al.,* 2012; Yen & Papin, 2017). *Staphylococci* are commonly found on human and other mammals' skin and mucous membranes. Although *S. epidermidis* is not likely to cause terminal disease, treatment for *S. epidermidis* infections have a very low successful rate, hence, a heavy burden on the public health system (Duell *et al.,* 2012; Otto, 2012).

S. epidermidis species show a high level of diversity with seventy-four identified sequence types (ST). The clonal complex entails many of the identified isolates, this includes the most commonly found ST2 isolate (Espadinha *et al*, 2019). Every ST2 isolate comprises of IS256 insertion sequences and *ica* genes which aid ST2 to spread successfully and *S. epidermidis* to be invasive (Mirzaei *et al.,* 2017). Many of the ST2 isolates have the *in vitro* ability to allow *S. epidermidis* to form biofilms. There is information available on *S. epidermidis* genome, this includes biofilm-negative ATCC122288 and the biofilm-positive clinical isolate RP62A9 (Grice & Segre, 2011; Espadinha *et al.*, 2019).

There is little evidence available on the lifestyle of the opportunistic pathogen *S. epidermidis*, however, *S. epidermidis* has recently attracted a lot of attention due to its infectious capabilities

(Brown *et al.,* 2012). Coagulase-negative *Staphylococci* (CoNS) group differentiates *Staphylococcus aureus* with other less or non-infective *Staphylococci* species (Zmantar *et al.,* 2017). *S. haemolyticus* and *S. epidermidis* are a large part of the CoNS group amongst other nosocomial pathogens. Nevertheless, previous research on species identification for CoNS infections found that most of them were caused by *S. epidermidis* (Otto, 2012). *S. epidermidis* commonly causes infections through medical devices like central intravenous or peripheral catheters. *S. epidermidis* infections usually transfer from the skin of a patient, individual, practitioner or employee of health institutions through the inserting process of medical devices (Zmantar *et al.,* 2017). High medical device usage directly correlates to the increasing number of *S. epidermidis* infections. Which are now known to report a minimum of 22% of bacteraemia in USA critical care patients, occurring in 4–5/1000 central intravenous catheter insertions (Espadinha *et al*, 2019; Sandoval-Motta & Aldana, 2016).

There is a large amount of *S. epidermidis* on human skin with similar amounts colonising catheter surfaces (Mirzaei *et al*., 2017). Also*, S. epidermidis* are involved in vascular graft, ventriculoperitoneal medical device, surgical site, cardiac implant and prosthetic joint infection (Mansson *et al.,* 2018). Finally, second to *S. aureus*, *S. epidermidis* has an increased percentage of intracardiac abscesses (38%), with 13% of prosthetic valve endocarditis (PVE) infections, and 24% mortality. Yet, PVE and other deadly infections are infrequent amongst *S. epidermidis* infections, overall *S. epidermidis* infections are characterised as mainly chronic and subacute (Mansson *et al.,* 2018).

Fundamental genetic or pathobiological differences in stratum corneum elements result in dysbacteriosis. Overall, affecting the colonising microbial population size and the diversity of species colonies. Imbalanced microbiota upsets the function of the epidermal barrier and intensifies long-term skin illnesses including acne, psoriasis and eczema. For instance, *S. epidermidis* is a species inhabiting the human skin commensal microflora as an opportunistic pathogen within immunocompromised patients (Figure 3) (Brown *et al.,* 2012; Conlan *et al.*, 2012; Dreno *et al*., 2016).

Why is it beneficial for *S. epidermidis* to have a low level of virulence and not cause life- threatening infections compared to the more virulence *Staphylococcus* species, *S. aureus?* It is assumed that *S. epidermidis* is more transmittable than *S. aureus,* based on a mathematical model formed by Massey *et al.* (2006). This model showed that avirulent variants out-competed virulent subtypes of *Staphylococcal* species. *S. aureus* has low asymptomatic transmission opposed to *S. epidermidis* high level of asymptomatic transmission. Massey *et al*. (2006) found that *S. epidermidis* broadly colonises human skin whereas *S. aureus* mostly colonises the nares region. They also found that *S. epidermidis* is a major part of the entire human population skin microflora in contrast to *S. aureus* species colonising few individuals.

Quorum sensing is a regulation of gene expression where the bacterial cell density influences bacterial gene expression (Figure 3 & 5) (Yan & Bassler, 2019). The bacterium must secrete an autoinducer and sense a signal via a regulatory receptor protein to communicate between cells. When there is a high level of microbial population density, autoinducers amass to a vital threshold value and induce the activation of certain genes and operons (Mirzaei *et al*., 2017). Quorum-sensing occurs in most bacteria, *Staphylococci* secrete post-translational modified protein named accessory gene regulator (Agr) (Espadinha *et al*, 2019; Hellmark *et al*., 2013) (Figure 5). There is a possibility of specific genetic influence on bacterial communication and colonisation (Figure 2) including quorum-sensing signals that cross-inhibit other microbes (Figure 2). Even though quorum-sensing system is advantageous to *S. epidermidis* species compared to *S. aureus in vitro*, there is no proof that it plays a part *in vivo* (Espadinha *et al*, 2019; Sandoval-Motta & Aldana, 2016).

1.5 Agr quorum-sensing system

Accessory gene regulator (Agr) and LuxS quorum-sensing systems are important for *Staphylococcus* intercellular interaction, biofilm growth and pheromone autoinducer peptide (AIP) production (Cheung *et al.,* 2014). The Agr system consist of two operons that are from transcribed promoter P2 and P3 to regulate bacterial cell density (Figure 5) (Cheung *et al.,* 2014). AIP production starts in exponential phase of microbial growth (Mazhar & McAuliffe, 2014). The pheromone AIP is released by *Staphylococci* cell density and sensed by the same group of microorganisms, allowing the expression of biofilm formation. Throughout exponential growth phase the P2 and P3 promoter is switched off with low level of both operon transcription and AIP secretion due to promoter leakage (Figure 5A) (Mazhar & McAuliffe, 2014). The promoter P2 can transcribe a range of sensor proteins, including (1) a membrane transport protein (AgrB); (2) a AIP precursor (AgrD) which processes and exports AIP via transmembrane protein AgrB; (3) a membrane-spanning sensor (AgrC) is the cognate receptor of AgrD-induced AIP; and (4) a transcription factor (AgrA) that is possibly stimulated by AgrC (Cheung *et al*., 2014). When cell density growth increases, the bacteria population and extracellular AIP concentration also increases, hence, increasing the chance of AIP binding to cognate AgrC receptor (Figure 5B) (Mazhar & McAuliffe, 2014). Once AIP is bound to AgrC receptor, AgrA response regulator is activated, a process that comprises of dephosphorylation (Figure 5B). Activated AgrA (DNA-binding protein) bind to both promoters P2 and P3 in *agr* operon (*agr*BDCA), RNAII and RNAIII are then transcribed, respectively (Mazhar & McAuliffe, 2014). RNAII *agr*B, D, C, A genes are transcribed to encode AgrB, D, C, A, and RNAIII *hld* gene is transcribed to encode δ-hemolysin (also known as δ-toxin or δ-PSM). δ-Hemolysin is a haemolytic peptide which is a membrane-active protein toxic to eukaryotic cells. RNAIII controls gene expression through various methods, for example, repressing

translation by blocking mRNA from binding to its target site. Agr can also express exoproteins during stationary phase of growth (high cell density) (Cheung *et al.,* 2014).

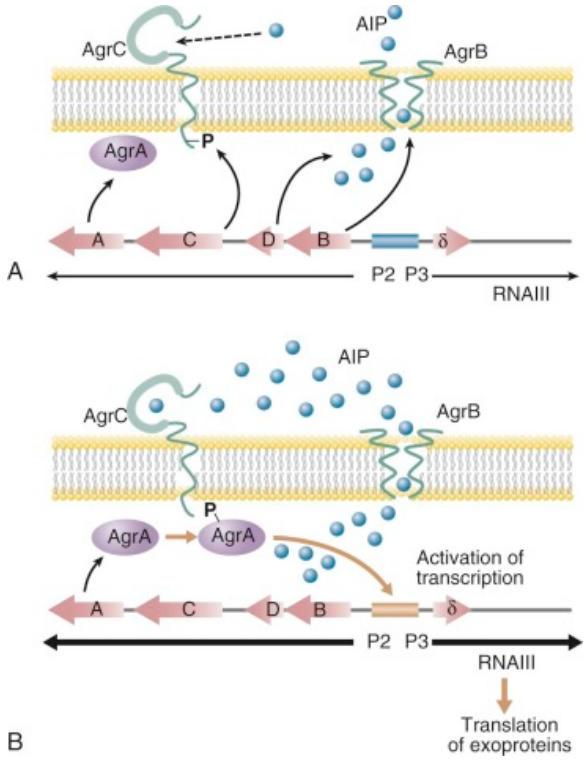

Figure 5. A & B. Schematic diagram of *Staphylococcus* Agr quorum-sensing system (Mazhar & McAuliffe, 2014).

The quorum-sensing system Agr is not very active however the system controls and expresses numerous hostile virulence factors, including the proinflammatory phenol-soluble modulins (PSM), *psm* genes are regulated by direct binding of AgrA to their promoters (Figure 5B) (Cheung *et al.,* 2014). *S. epidermidis* are known to produce six PSMs that are all genome-encoded; PSMα, PSMβ1, PSMβ2, PSMδ, PSMε, and δ-toxin. After δ-toxin, β-PSMs (43–45 amino acid length) are the main types of PSMs found in *S. epidermidis,* they are the key players in biofilm formation (1.4) and detachment (see 1.5) (Cheung *et al.,* 2010; Cheung *et al.,* 2014). Enhanced production of β-type PSMs over α-type PSMs are observed in *S. epidermidis* during biofilm formation (Wang *et al.,* 2011), but how differential production of α- versus β-type PSMs is attained systematically is unknown.

There are physiological changes in biofilms of *S. epidermidis* and within the quorum-sensing *agr* system that protects the bacteria via two mechanisms (Siddhiqui *et al.,* 2018). Firstly, *S. epidermidis* sensitivity level is decreased for destructive substances including cytokines, antimicrobial molecules and antibiotics (Hellmark *et al*., 2013). The second mechanism is to stop the destructive molecule from

being hostile and switch it to a harmless state, this reduces inflammation and stops the host immune system from identifying the pathogen. Hence, the host defence system is completely evaded by *S. epidermidis*, the *S. epidermidis* biofilm appear to be dependent on several physiological alterations. This underlines the significance of *S. epidermidis* biofilm being able to evade host defence system during cutaneous colonisation and biofilm related infections (Siddhiqui *et al.,* 2018).

The acquired immune system comprises of highly specific systemic cells and processes to eliminate *S. epidermidis* colonisation. Nevertheless, there is not much information on how the acquired immune system can achieve this specifically for pathogenic *S. epidermidis* (Espadinha *et al*, 2019; Mirzaei *et al*., 2017). One way how *S. epidermidis* may go unidentified and remain protected from antibodies is by using exopolymers (Cheung *et al.,* 2010). Some studies suggest that the host immune system may have evolved to react in a symbiotic way to colonising microbes (Hooper *et al.,* 2012; Belkaid & Hand, 2014; Le *et al.,* 2018).

1.6 Sensing of antimicrobial peptides (AMPs)

The most important biotic function antimicrobial peptides (AMPs) play *in vivo* is killing pathogens, including Gram-negative and -positive bacteria, viruses and fungi. Gene expression can occur in various host cells such as mucosal epithelial cells and phagocytic cells to encode AMPs (Marquette & Bechinger, 2018). Cytokines and pathogenic microbes induce gene expression to closely regulate host defense response. *S. epidermidis* cause bacterial lipid membrane leak and collaborate with human AMPs production to decrease the number of these microbes. These AMPs are vital cooperating signals between the host defence system and the microbiome. Almost thirty percent of the transcriptome of general epithelial cells use this process to communicate.

There are two main groups that AMPs belong to, cathelicidins and defensins. The AMPs from these groups with an overall positive charge are called cationic antimicrobial peptides (CAMPs). However, an anionic AMP with a negative net charge has been detected from human sweat called dermcidin. (Espadinha *et al.,* 2019). *S. epidermidis,* the Gram-positive bacteria has been found to have an AMP sensor. The recently identified AMP sensor is made up of three crucial factors, ApsS with a sensory function, ApsR the regulator and ApsX the unique final constituent of unidentified purpose. (Vasilchenko *et al.*, 2019)

As AMPs are the main element of the host innate defence system, to protect the bacteria from AMPs, the bacteria can utilise enzymes such as SepA protease, poly-N-acetylglucosamine (PNAG or PIA polysaccharide intercellular adhesin) and poly-γ-glutamic acid (PGA) (See 1.7.3) (Mirzaei *et al.,* 2016; Espadinha *et al*, 2019). The skin has an environment of extensive mechanical stress making it

important for *Staphylococci* to have biofilm-related proteins and PIA (Mirzaei *et al*., 2017). PIA is a glycan of beta-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues of which 15 % are non-N-acetylated. (Spiliopoulou *et al.,* 2012; Hofferek, 2019). These proteins are vital for colonisation and infecting the host as both commensal and pathogenic *S. epidermidis* strains, both type of strains have the same MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (See 1.7.2) (Geoghegan *et al.,* 2010). This indicates that *S. epidermidis* can be viewed as an accidental pathogen and it is clinically important to research adherence and evasion of host immune system as this can be valuable for killing pathogenic bacteria and treating long-term infections (Espadinha *et al.*, 2019).

Just the way human innate immune system recognises *S. epidermidis* pathogen associated molecular pattern (PAMPs), *S. epidermidis* also identifies dangerous foreign molecules produced by the human host (Joo *et al*., 2015). There is an AMP-sensing system within *S. epidermidis* named Aps. Diverse number of AMPs activate Aps system and cause an up-regulation of AMP-defensive systems. AMP-defensive systems may include VraFG proteins, lysylation of phospholipids by the MprF enzyme and D-alanylation of teichoic acids (Joo, & Otto, 2015). This affects the exterior of the bacteria as it diminishes the negatively charged surface and halts the attraction of positively charged AMPs, efficiently exporting AMPs from the cytoplasm membrane (Figure 8) (Lin *et al*., 2018). Hence, Gram-positive bacteria Aps system plays a similar role to Gram-negative AMP sensor named PhoP/PhoQ, however, the bacteria is not genetically related (Lin *et al*., 2018; Joo & Otto, 2015). The bacteria *S. epidermidis* Aps system is activated and limited to cationic AMPs (Lin *et al*., 2018). Aps system is a three-component regulator, it has an important component of unidentified function namely, ApsX (Joo *et al*., 2015). The response regulator protein ApsR (GraR) and histidine kinase ApsS (GraS) make up the other two components of the Aps system (Figure 8) (Joo *et al*., 2015).

1.7 Biofilm

Many microorganism cells produce and emit extracellular substances in the form of a slime layer, thus creating a hydrophobic biofilm. Biofilms are complicated multicellular assemblage of microbes adhered to a surface (Chua *et al*., 2014). This allows the microbe to adhere to different kind of surfaces including metals, plastics and the human body. The gene *icaADBC* is responsible for the coding of PIA and the polysaccharide capsule used in developing biofilm (Qin *et al*, 2016). The *S. epidermidis* biofilm is made up of groups of cells rooted in extracellular slime material which is hundred and sixty micrometres dense with over fifty cells. The *S. epidermidis* skin biofilm perform as a diffusion barrier to host defence mechanisms and antibiotics (Salgueiro *et al*., 2017).

It is important that *ica* operon express polysaccharides to form biofilms (Chua *et al*., 2014; Mirzaei *et al.,* 2017). Some researchers have proposed that polysaccharide adhesin is efficient for adherence and cell to cell communication during the assembly process of biofilm production (Franca *et al.,* 2016). Other researchers have suggested that adhesion is induced by microbial cell surface proteins and polysaccharides can only control the process of accumulating cells (Guo *et al*., 2019).

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1.7.1 Biofilm formation

A biofilm is formed when it adheres to a surface, thereby accumulating cells to form a multi-layered structure (Figure 6) (Hellmark *et al*., 2013). Hence, for biofilm growth it is important to have adhesive agents for the colonisation of surfaces and for the cells to communicate between each other. Biofilm disruption is required to form fluid-filled channels which are important for the delivery of nutrients to all biofilm cells and allows mature biofilm to have the usual 3D shape (Figure 6 & 7) (Franca *et al.,* 2016). Disruption is also required in the detachment of cell aggregations from the biofilm, this restricts the biofilm from expanding and leading to the spread of infection (Figure 6 & 7). Once the biofilm adheres to a surface, it grows through an intercellular aggregation process which is induced by a diverse variety of surface macromolecules (Chua *et al*., 2014). Commonly, exopolysaccharide and few proteins mainly focus on forming the extracellular biofilm matrix. Additionally, extracellular DNA from lysed cells and teichoic acids may have an accessory role in intercellular aggregation and they are mostly reliant on their polyanionic disposition (Chua *et al*., 2014).

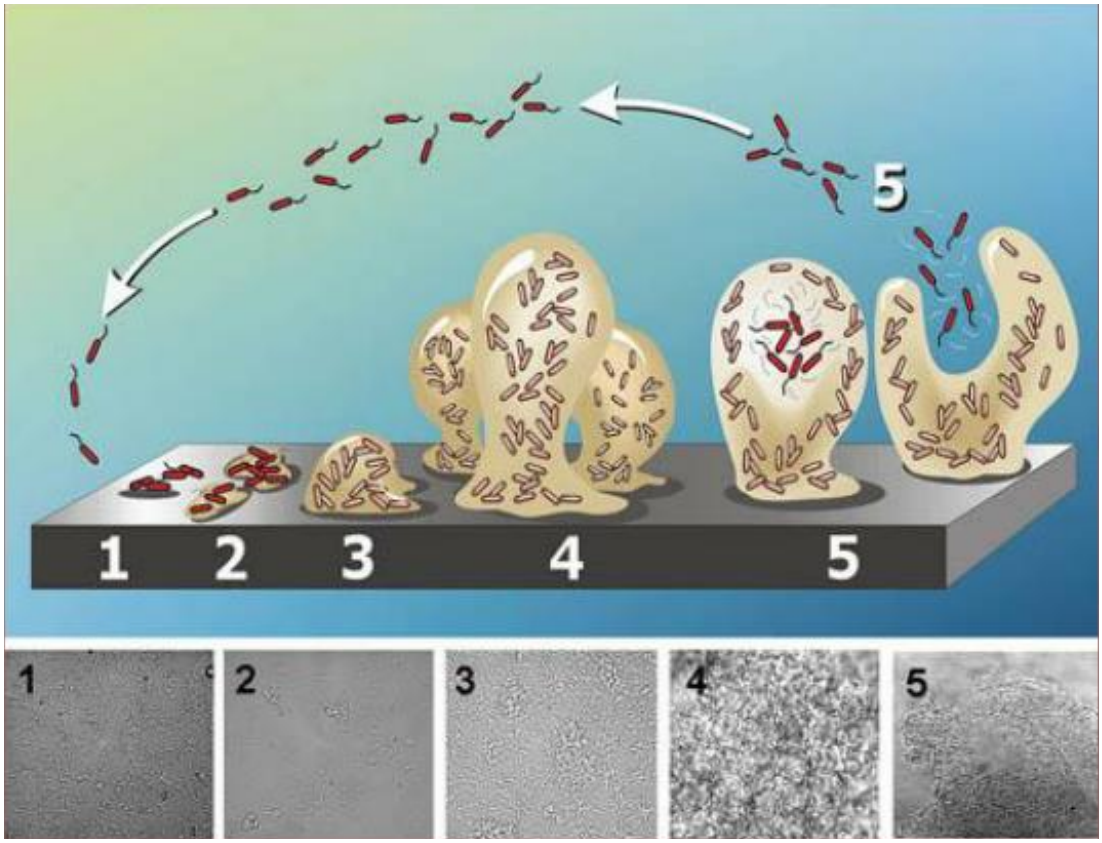

Figure 6. Steps of Biofilm Formation (Monroe, 2007; Aguinaldo, 2015):

1. Bacteria adhere to a surface
2. Bacteria cells replicate and form a single thick polysaccharide matrix layer. Biofilm is not visible at this stage.
3. A 3D biofilm structure is formed by large bacterial colonies secreting exopolysaccharide and quorum-sensing.
4. The exopolysaccharide slime protects the bacteria from harsh environment, antibiotics, toxic chemicals etc. Nutrients are diffused through the matrix. A secondary bacterial species joins the biofilm. The small proximity between both species allows genetic exchange.
5. Depletion of oxygen and nutrients. Due to shear force the planktonic (free-floating) bacteria cells disperse for recolonisation.

Adherence to medical devices including catheters, mostly occurs due to hydrophobic external layer of bacteria (Figure 6 & 7). Certain proteins promote *S. epidermidis* adhering to surfaces, this largely involves surface protein AtlE, which has two functions; to adhere to cells or surfaces and breakdown the cell-wall peptidoglycan of *S. epidermidis* (Chua *et al.*, 2014; Mirzaei *et al.,* 2017). Another protein named Bap/Bhp protein probably promote the hydrophobicity level of the bacteria cell outer layer. *S. epidermidis* mostly cause prosthetic joint infections, when the bacteria accumulate on an implant surface it creates a biofilm and transitions to a stationary phase in bacterial growth due to increased

microbial populace densities (Mansson *et al.,* 2018). During the stationary phase the bacteria grows slowly compared to planktonic bacteria, the bacteria has decreased metabolic activity and, thus, high level of resistance to killing by growth-dependent antibiotics, e.g. cell-wall-active antibiotics (Figure 6 & 7) (Spiliopoulou *et al.,* 2012; Hellmark *et al*., 2013). Additionally, the biofilm decreases the penetrability of antibiotics and host immune components such as complement and antibodies (Figure 6 & 7) (Stipetic *et al.,* 2016).

1.7.2 MSCRAMMS

Matrix proteins within the bacteria rapidly envelope abiotic surfaces such as those present in medical equipment. *S*. *epidermidis* has a large variety of surface proteins that are part of a group named 'microbial surface components recognising adhesive matrix molecules' (MSCRAMMs) and they can potentially communicate with matrix proteins. The MSCRAMMs are likely to have a covalent-bond between them and the microbial exterior via sortase A (Geoghegan *et al.,* 2010). However, MSCRAMMs may be non-covalently bound to the microbial surface polymers e.g. teichoic acids, this concept is yet to be completely understood (Geoghegan *et al.,* 2010). The protein SdrG have been observed to have a covalent bond with fibrinogen and the protein SdrF is covalently bound to collagen (Faustino *et al.,* 2013). Moreover, autolysins Aae and AltE interact non-specifically and have a non-covalent bond with fibrinogen, vitronectin and fibronectin (Salgueiro *et al*., 2017; Becker *et al.,* 2014).

S. epidermidis are able to express three variety of proteins related to the cell surface, namely SdrG, SdrF and SdrH, these involve repeats of serine-aspartate dipeptide (Faustino *et al.,* 2013). The cell surface proteins SdrG and SdrF structure and sequence are almost indistinguishable when compared to the Sdr protein of *S. aureus*, the proteins encompass exceptional 625- and 548-residue A regions at the N termini, succeeded by 110-119-residue B-repeat regions and SD-repeat regions (Faustino *et al.,* 2013). The C termini comprises of hydrophobic amino acid molecules distinctive of cell surface proteins covalently bound to peptidoglycan and LPXTG motifs. However, SdrH comprise small 60-residue A region at its N terminal succeeded by a region with SD-repeats, it has a specific 277-residue C region and a C-terminus hydrophobic fragment. SdrH lack a LPXTG motif (Faustino *et al.,* 2013).

The most commonly researched MSCRAMM of *S. epidermidis* is a protein which binds to fibrinogen namely SdrG (Fbe), it belongs to the aspartate/serine repeat family (Geoghegan *et al.,* 2010; Becker *et al.,* 2014). The three main proteins from the aspartate/serine family exist in several *S. epidermidis* strains including SdrG, SdrF and SdrH. *In vitro*, SdrG is known to be important and enough to encourage *S. epidermidis* to adhere to fibrinogen, also *in vivo* SdrG assists central venous catheter-related infection (Salgueiro *et al*., 2017; Faustino *et al.,* 2013). SdrG can bind to the thrombin cleavage site in the beta chain of fibrinogen via the 'dock, lock and latch' system (Becker *et al.,* 2014; Faustino *et al.,*

2013). This process is meant to cause a highly stable MSCRAMM-substance communication (Geoghegan *et al.,* 2010). This emphasises the significance of SdrG assisting *S. epidermidis* in causing infections, also, there is an increase in the expression of SdrG in an *in vivo* condition with antibodies to SdrG are identified in human blood (Faustino *et al.,* 2013). Not long ago, SdrF has been found promoting infection by encouraging *S. epidermidis* to adhere to ventricular medical devices. Additionally, various other *S. epidermidis* MSCRAMMs are yet to be identified and undergo characterisation to understand the function they play in matrix protein binding process and the pathogenicity of *S. epidermidis* (Geoghegan *et al.,* 2010).

1.7.3 PNAG/PIA

Several strains of *S. epidermidis* create a PNAG homopolymer also known as PIA, which surround and adheres *S. epidermidis* cells within a biofilm (Mirzaei *et al.,* 2016; Spiliopoulou *et al.,* 2012). This protein molecule is different to poly-N-acetylglucosamine polymers observed in flora, for example chitin, this is due to its β 1–6 connection. Recently, PIA/PNAG has also been found in several other microbes such as *Yersinia pestis* and *Escherichia coli*. Biosynthesis of PIA/PNAG is vital for formation of biofilm *in vitro* and it has a substantial influence on *S. epidermidis* infection in animal models (Spiliopoulou *et al.,* 2012). The process of PIA/PNAG production is initiated by a product of a gene namely intercellular adhesion (*ica)* locus (Mirzaei *et al.,* 2016). A chain of reaction from activated N-acetylglucosamine monomers, is formed by IcaD and IcaA where the prolongation relies on the IcaC protein, this is possibly due to the exporting role it is known to play. Once exportation occurs, partial de-acetylation of the N-acetylglucosamine residues is completed by the enzyme IcaB, which is situated on the surface of the cell. Due to de-acetylation the neutral polymer becomes positively charged, this allows PIA/PNAG to bind to surfaces and for it to play a part in numerous biotic interactions, such as *S. epidermidis* evading the host immune defence system (Spiliopoulou *et al.,* 2012). Several universal virulence regulators influence the formation of PIA/PNAG excluding the quorum-sensing regulator *agr* (Figure 5). Although there is limited understanding on what environmental signals regulate the expression of PIA/PNAG, especially *in vivo*, the intricacy of regulation reinforces the significance of PIA/PNAG for *S. epidermidis* physiopathology (Mirzaei *et al.,* 2016).

PIA/PNAG has recently been identified to be insignificant when forming biofilms in all *S. epidermidis* species (Spiliopoulou *et al.,* 2012). This is because *S. epidermidis* strains isolated from biofilm related infections appeared to not have *ica* genes (Mirzaei *et al.,* 2017). Distinct surface proteins involving biofilm-associated protein (Bap/Bhp) and accumulation associated protein (Aap) may induce or predominantly form biofilm in certain strains of *S. epidermidis*. The Aap protein demands zinc ions and proteolytic activation to promote the production of the biofilm. Zinc ions are vital for modular

association of G5 tandem repeats, this underlines that Aap forms fibril-type structures on the microbial outer layer. These domains are seen interacting with N-acetylglucosamine and may possibly bind to PIA/PNAG to form a polysaccharide/protein biofilm system (Mirzaei *et al.,* 2016). A chelating agent was used to prevent the production of biofilm *in vitro*, the result highlighted that strong biofilm forming strains such as *S. epidermidis* RP62A strain are completely reliant on Aap (Otto, 2009). Additionally, monoclonal antibodies produced against Aap stopped *S. epidermidis* RP62A from forming biofilm. Nonetheless, the findings from this report contradicted another report which did not find any protein that induced biofilm formation in the identical strain. Hence, certain proteins may contribute to *S. epidermidis* forming biofilms, however this requires further investigation. Finally, the concept of biofilms being produced only by proteins is not robust as the one on PIA/PNAG that demonstrates both exopolysaccharide and proteins are required to promote *S. epidermidis* biofilm production.

The overall information on molecular processes of *S. epidermidis* regulation and production of biofilm is nearly completely reliant on *in vitro* study (Franca *et al.,* 2016). Animal models have been used to contribute to the pathogenesis of *S. epidermidis* by demonstrating few factors including PIA/PNAG, Fbe, AtlE, SdrF, SdrG and regulators Agr*,* sigB and luxS (Mirzaei *et al.,* 2017; Pereira *et al.,* 2018). Additionally, there is evidence signifying the importance of expressed biofilm elements, *in vivo*. However, there is an imperative requirement for more comprehensive *in vivo* studies to provide complete understanding of *S. epidermidis* biofilm related infections (Franca *et al.,* 2016).

1.7.4 Biofilm in host evasion

Biofilm has a distinctive composition and construction for the development of biofilm resistance to numerous antibiotics and evasion of host defence systems (Franca *et al.,* 2016). *S. epidermidis* show a large variety of genetic variations throughout the biofilm growing process, this includes down-regulation of basic cell mechanisms, for example, nucleic acid, protein biosynthesis and cell wall (Franca *et al.,* 2016). The regulation of gene expression may alter and lead to the inactivity of several antibiotics which target against *S. epidermidis* developing biofilm cells, these involve antibiotics such as, quinolones, aminoglycosides and penicillin (Hellmark *et al*., 2013).

S. epidermidis is unaffected by the harsh surroundings that usually occur in its natural habitat due to the genes the bacteria may possess. For instance, *S. epidermidis* can survive extreme salt and osmotic pressures via six transport systems for osmoprotectants and eight sodium ion / proton exchangers (Dreno *et al.,* 2016). There are only few known bacteria that can withstand high salt concentrations or other extremities on human epithelia, other than *S. epidermidis.* The ability to form biofilms help the bacteria to survive harsh conditions significantly. Over the years a large amount of osmoprotective elements have been expressed and reported in *S. epidermidis* biofilms (Mirzaei *et al*., 2017).

Furthermore, the bacteria must survive host immune defence attacks, especially antibacterial peptides. β-defensin is one of the key antibacterial peptides found mostly at epithelial surfaces. Gram-positive bacteria can act as a chemo-attractant between innate and acquired immune systems (Siddhiqui *et al.,* 2018).

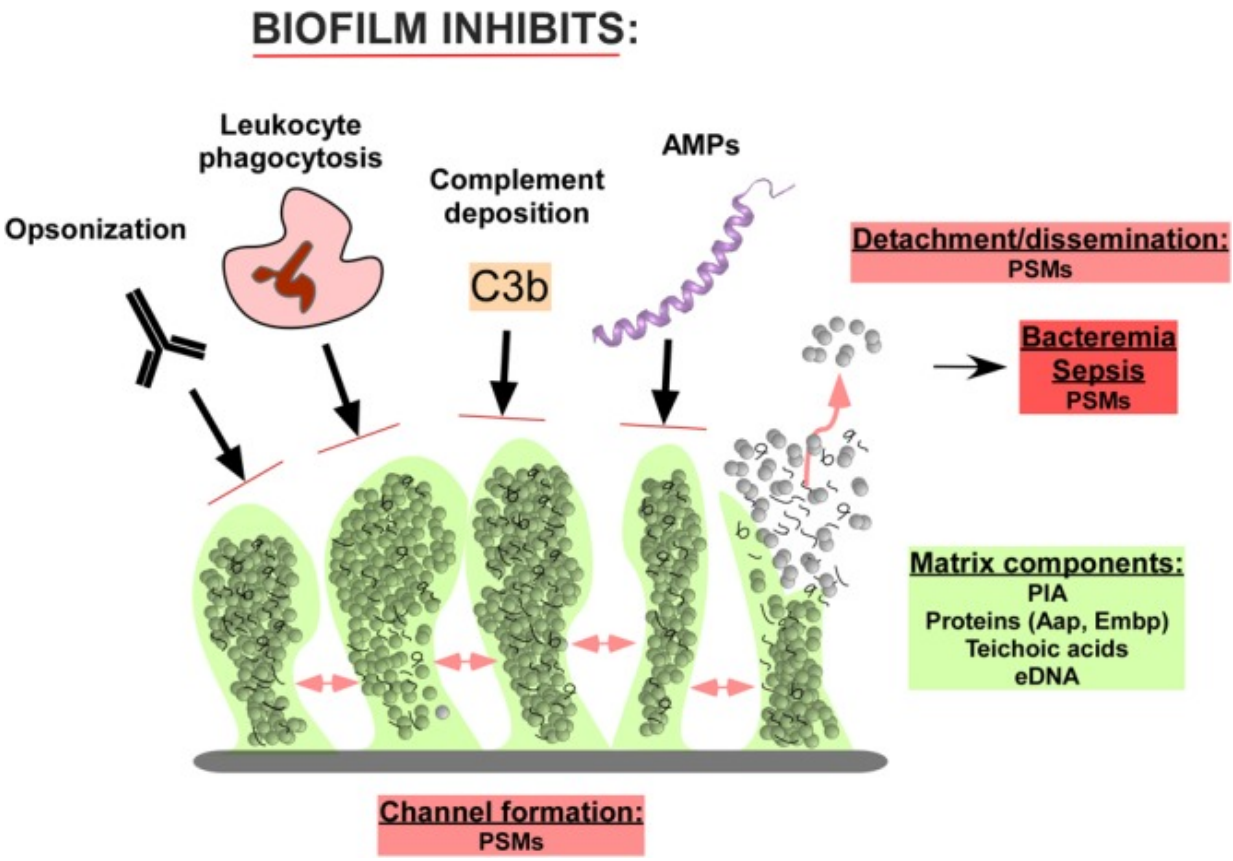

Figure 7. *S. epidermidis* biofilm structure and function in host immune evasion. The biofilm matrix is made up of PIA/PNAG, Aap, Bap/Bhp, extracellular matrix binding protein (Embp), extracellular DNA (eDNA) and teichoic acids. Biofilm channels are produced by PSMs, which eventually cause cell cluster detachment and overall dissemination of the biofilm. PSMs also play a role in bacteremia and sepsis in immunocompromised individuals. Both biofilm structure and matrix provide protection from host defences such as opsonising immunoglobulins binding, complement components and AMPs. Furthermore, there is a drastic reduction in leukocytes attacks (Le *et al.,* 2018).

Brandwein *et al.* (2016) isolated *Staphylococcal* strains from hospital settings and patient body sites, *Staphylococcus aureus* biofilm was found in atopic dermatitis lesions on patient skin sites. Once *S. epidermidis* penetrates through the stratum corneum it must evade the host defence processes by changing its own mechanisms (Brandwein *et al.,* 2016). *S. epidermidis* biofilm adapts to the host immune system and alters its bacterial gene expression involving a decreased metabolism, low

transcription and translation as well as a switch from the aerobic system to fermentation to produce energy (Allen *et al.* 2014). These alterations are possibly due to the reduced level of oxygen within the biofilm and the limited amount of nutrition available. Notably, this causes the biofilm to become dormant and enter the stationary phase of growth, thereby, the antibiotic used against the biofilm becomes ineffective as the biofilm now has low sensitivity for the antibiotic treatment (Brandwein *et al.,* 2016). Likewise, the biofilm is capable of evading the host defence mechanisms as cytokines and antibiotics mainly rely on highly functional cell metabolism and cell-development mechanisms.

1.8 Biofilm detachment

Unlike the intercellular accumulation phase, there is little understanding of *S. epidermidis* biofilm structure and the process of detachment. It is commonly known that *S. epidermidis* biofilm detachment is regulated by the Agr quorum sensing mechanism (Pereira *et al.,* 2018). Biofilms that are not controlled by Agr due to dysfunction are a lot denser and display clear deficiencies during detachment (Boles & Horswill, 2011). A model that had Agr expressed in the outer surface of biofilm was known to encourage cluster of cells detaching from the biofilm surface, hence, regulating biofilm dissemination (Pereira *et al.,* 2018). Similarly, the function of Agr in *S. epidermidis* is restricted to the exposed surfaces of a biofilm, this suggests that both closely related *Staphylococci* species share the same quorum-sensing system to control the detachment of biofilm (Pereira *et al.,* 2018). The mutation of both Agr system or *psm* genes, can support the formation of compact biofilms (Wang *et al*., 2011), however, the biofilm can no longer detach and form biofilm elsewhere (Joo and Otto, 2012).

There are two known processes of biofilm detachment, one of them include the enzymatic breakdown of proteinaceous biofilm elements (exopolymers) and the second process is disrupting the non-covalent interactions by utilising surfactant-like substances. The first process has been observed in *S. aureus,* nevertheless, no evidence has been observed where enzyme is degrading exopolymers in *S. epidermidis* to promote biofilm detachment. Nonetheless, *S. epidermidis* produce lots of exoproteases that have little to none substrate specificity, they may possibly breakdown exopolymers (Boles & Horswill, 2011). On the other hand, *Staphylococcal* strains lack specific enzymes to degrade exopolysaccharide such as PIA/PNAG, whereas, many other microorganisms that produce PIA/PNAG are able of degrading their exopolysaccharide. Furthermore, surfactant-like substances disrupt non-covalent hydrophobic and electrostatic interactions. An example of a non-covalent interaction is the cationic PIA/PNAG interacting with anionic exopolymers or the interaction between the hydrophobic areas of the microbial outer layer. The small amphipathic PSMs including *S. epidermidis* delta-toxin

are suggested to have this detachment mechanism (Boles & Horswill, 2011). *S. epidermidis* exoproteases and PSMs are strongly controlled by Agr, thus, supporting the concept of exoproteases and PSMs having a function in organising the structure of the biofilm (Cheung *et al.,* 2014; Boles & Horswill, 2011).

1.9 Protective exopolymers

S. epidermidis and *Bacillus anthracis* are the only species known to date where PGA plays a role in the spread of disease. Additionally, PGA encourages *S. epidermidis* to grow in high salt concentrations (Chua *et al*., 2014). Numerous halophilic microorganisms PGA have similar function that contribute to osmotolerance and PGA also promote *S. epidermidis* colonisation. Also, *cap* genes are increasingly expressed during biofilm growth. Remarkably, PGA exist in several CoNS, but is missing in *S. aureus* (Cave *et al,* 2019).

Furthermore, exopolysaccharide PIA/PNAG can also protect *S. epidermidis* from human host defence mechanisms such as AMPs, neutrophil phagocytosis, immunoglobulins and complement deposition. Positively charged PIA/PNAG protect *S. epidermidis* from AMPs with either negative or positive charge, this indicates that the activity of PIA/PNAG may not be restricted to electrostatic repulsion of AMPs with identical charge (Yan & Bassler, 2019).

1.10 Pathogen-associated molecular patterns

Pathogen associated molecular pattern (PAMPs) are substances displayed on the bacteria exterior layer, the host innate defence system can identify PAMPs as a foreign molecule via pathogen recognition receptors (PRRs) such as TLRs (Noreen *et al.,* 2015). PAMPs identification leads to the activation of host defence response for example, cytokine is secreted, and phagocytosis occurs. Similar to most Gram-positive bacteria, *S. epidermidis* PAMPs are lipoteichoic acids and lipoproteins. Additionally, *S. epidermidis* have specific unique molecules such as PIA/PNAG which activate the host innate defence mechanism by activating the Toll-like receptor 2 (TLR2) (Noreen *et al.,* 2015). *S. epidermidis* uses PIA/PNAG for host immune evasion by evading PRRs. For instance, it is observed that PIA/PNAG stimulate the Toll-like receptor 2 (TLR2) (Noreen *et al.,* 2015). Nevertheless, this has yet to be confirmed with deletion mutant genomes, also TLR2 stimulators could have been misidentified due to contaminated lipoproteins (Yan & Bassler, 2019). Identification of PIA/PNAG by the host defence mechanism plays a fascinating role of hide-and-seek between the host and the invading bacteria, as *S. epidermidis* uses the molecule for host immune evasion while stimulating the innate immune defence system.

It is imperative to know that lipoproteins were the foundation of the study, which is a strong pro-inflammatory contaminant that may result in incorrect identification of TLR2 stimulants (Noreen *et al.,* 2015). Likewise, pro-inflammatory capabilities of *S. epidermidis* PSMs are yet to be verified using gene deletion mutation or synthetic peptides (Otto, 2014). Nonetheless, the similitude of the confirmed function of *S. epidermidis and S. aureus* PSMs show that the detailed pro-inflammatory influence of *S. epidermidis* PSMs is authentic; however, the activation of TLR2 by PSMs require confirmation (Cheung *et al.,* 2014; Noreen *et al.,* 2015). Lastly, an abnormal short-chain pro-inflammatory lipoteichoic acid has been observed in *S. epidermidis*. On the other hand, the chemical characterisation of the purified element has no indication of teichoic acid-associated polymer, and hence the character of this substance and the reported pro-inflammatory function require confirmation. Thereby, further research is needed for characterisation of *S. epidermidis* substances that stimulate the host immune defence system.

1.11 Evasion of host defences

A pathogen must evade the human body host defence in order to withstand and cause disease. Few host defence systems exist, for example, AMPs on the skin. *S. epidermidis* must survive other host defence responses once it breeches the epithelial barrier. Invading microbes as well as *S. epidermidis* interact with the innate immune system in an unspecified way. The colonising *S. epidermidis* bacteria have numerous mechanisms to avoid being ingested and killed by neutrophils while also evading AMPs.

A pathogen must evade multiple human body natural defences for survival (Otto, 2009). There is limited subset of mechanisms of host defence mechanisms existing on the human skin. *S. epidermidis* must avoid AMPs and survive several other defence mechanisms after penetrating the epithelial barrier (Whitney *et al*., 2010). The innate immune system reacts without being specific to any microbe entering, including *S. epidermidis* (Cheung, *et al.,* 2010). The innate immune system mainly reacts by ingesting microbes using neutrophils, eradicating them with AMPs and reactive oxygen species (Otto, 2009). *S. epidermidis* can avoid being ingested and killed by neutrophils via AMPs resistance mechanism (Figure 6) (Le *et al.,* 2018).

The function of the acquired immune system during *S. epidermidis* infection is not well understood. The human immune system has difficulty killing lifelong *S. epidermidis* infection regardless of the creation of antibodies for *S. epidermidis* exopolymers. This shows that the acquired immune system is ineffective in destroying *S. epidermidis* biofilm. *S. epidermidis* polymers including PIA/PNAG and PGA may cause this, these exopolymers shelter the bacteria from antibody recognition and other important mechanisms of innate host defence. Even with low production of pseudopeptide polymer

PGA, the gene product of *cap* locus is imperative for *S. epidermidis* protection from AMPs and neutrophil phagocytosis. Furthermore, the host defence system may have adapted overtime to not have a strong response against predominant infectious pathogens.

1.11.1 AMP Resistance

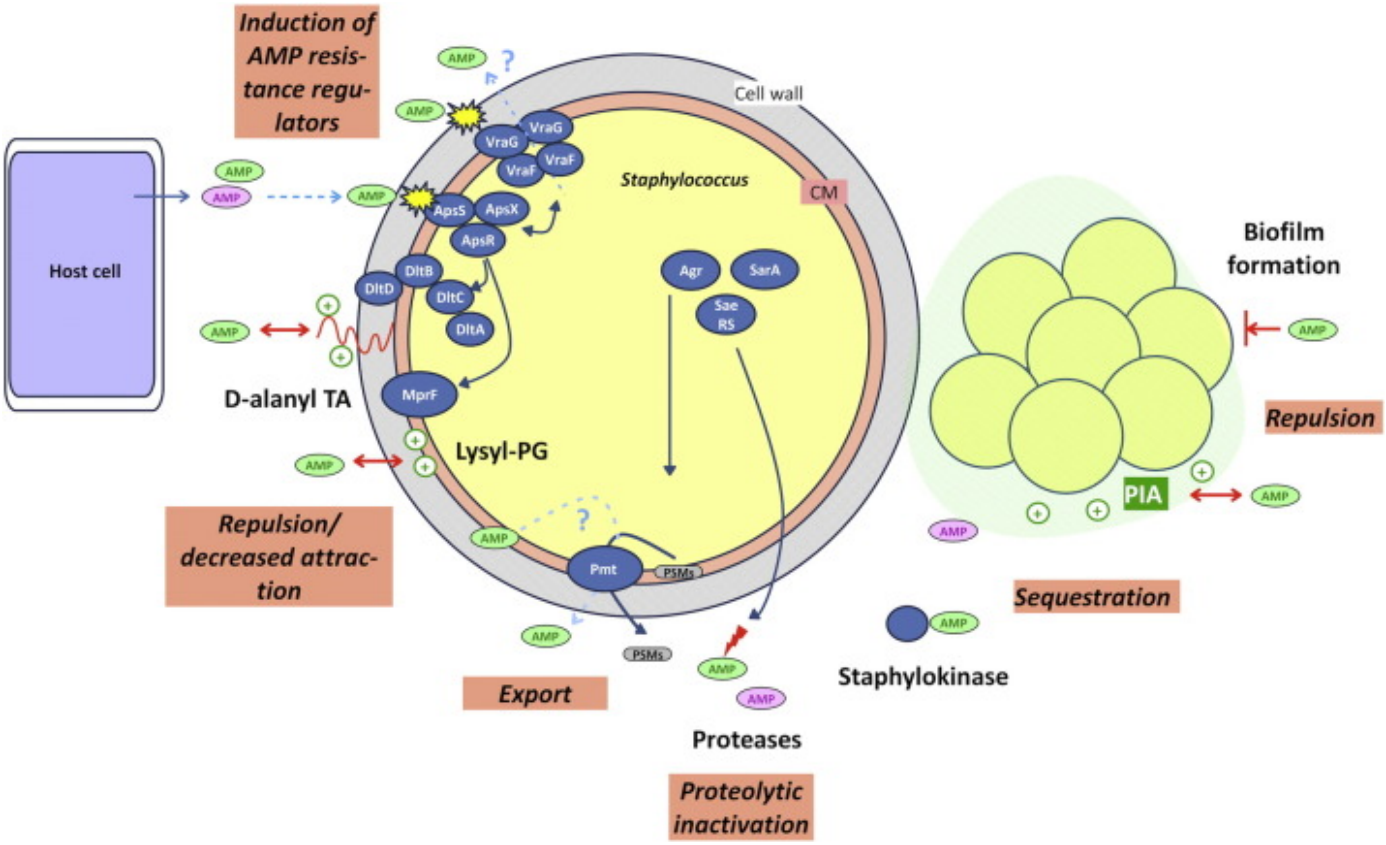

Figure 8. A general demonstration of *Staphylococcus* becoming resistant to AMPs (Joo & Otto, 2015).

AMP hBD3 is an important part of the innate immune system on the epithelial. *S. epidermidis* can develop resistance to AMPs by inducing hBD3 in order to control the three main genes *dltB*, *vraF* and *mprF* (Cheung *et al.,* 2010). Human host cells mainly generate positively charged AMPs (CAMPs, green in diagram) and sometimes negatively charged AMPs (red in diagram), e.g. dermcidin. CAMPs stimulate the Aps/VraFG regulatory system to cause an amplification in Dlt system expression. The *dlt* operon D-alanylates both teichoic acid and MprF, thus, altering lysyl-phosphatidylglycerol. Due to these modifications the cell wall repulses CAMPs or has a low attraction for CAMPs (Figure 8). Aps/VraFG also stimulates VraFG expression, this could possibly involve AMP export. PSM ABC transporter export membrane active AMPs e.g. *Staphylococcal* PSM toxins and maybe AMPs produced

by the host from the cell membrane (CM). The exported proteases that have little substrate specificity tend to destroy both anionic AMPs and CAMPs, regulated by global regulators such as Agr and *Staphylococcal* accessory regulator (SarA) (Figure 8). PSMs and other toxins are degraded by aureolysin, a protease which is also regulated by multiple global regulators, including Agr and SarA. CAMPs are inactivated by staphylokinase, through a sequestration process. This method is observed in surface polymers that have an opposite charge to AMPs e.g. positively charged PIA exopolysaccharide sequestrating negatively charged AMPs. The production of a biofilms adds to AMP resistance through various methods such as reduced penetration. Additionally, the cationic biofilm PIA may repulse CAMPs (Figure 8) (Joo & Otto, 2015).

1.11.2 PSMs

S. epidermidis as well as all the other *Staphylococci* species including the pathogenic *S. aureus* contain genes encoding PSMs and other related peptides (Cheung *et al.,* 2014). These are both hydrophilic and hydrophobic helix shaped small peptides. PSMs are a group of virulence toxins that are soluble in phenols and may contribute to the pathogenicity of *S. epidermidis* (Otto, 2014). Although, PSMs commonly lack bacteriostatic characteristics (Cheung *et al.,* 2014). AMPs as well as PSMs of alpha-type (20–25 amino acids) are cationic, whereas beta-type PSMs (43–45 amino acids) have a net anionic charge (Cheung *et al.,* 2010). The amphipathic α-helix nature of PSMs allow it to have an affinity for lipids, hence, α-type PSMs are more active against eukaryotic cells compared to prokaryotic cells. PSMs with big hydrophobic side chains prevent antibacterial activity by avoiding microbial membrane disruption. Cheung *et al.,* (2014) findings showed that PSMs can have a different effect on prokaryotic cell membrane compared to eukaryotic cells. The α-type PSMs can lyse human cells such as erythrocytes and leukocytes while promote inflammatory responses (Cheung *et al.,* 2014).

1.12 Pathogenicity

It is important for most pathogens to produce toxins to be more virulence such as *S. aureus.* Although, *S. epidermidis* is considered avirulent and a non-enterotoxin producer, Pinheiro, *et al.* (2015) identified enterotoxin genes within *S. epidermidis.* Pinheiro, *et al.* (2015) reported that the most identified common enterotoxin genes were *sea* (53.3%), *seg* (64.5%) and *sei* (67.5%). Also, the *seg* gene in *S. epidermidis* had a positive correlation (p = 0.02) with high toxicity levels. The *agr* group II is possibly linked to *S. epidermidis* producing enterotoxin C, which increases the pathogenicity of *S. epidermidis* variants identified from blood samples, which then leads to severe cases of sepsis (Pereira *et al*., 2018). However, compared to the large production of toxins by *S. aureus, S. epidermidis* produce few toxins,

the major toxin it does produce are namely PSMs. All *S. epidermidis* produce PSMs except *S. epidermidis* strains with natural *agr* dysfunction (Pereira *et al.,* 2018). PSMs are typically α-helical, short and amphipathic (Cheung *et al.,* 2014) and sometimes have cytolytic function but mainly pro-inflammatory function. *S. epidermidis* produce PSMγ/ δ-toxin (a 24-amino acid peptide) and *S. aureus* produces a similar peptide but the positioning of one amino acid varies from the *S. epidermidis* homologue (Cheung *et al.,* 2014). Several reports have found that *S. epidermidis* PSMγ assist in necrotising enterocolitis in new-borns. Few *S. aureus* PSMs have similar function as *S. epidermidis* PSMs of lysing host neutrophils (Cheung *et al.,* 2014). Nevertheless, *S. epidermidis* PSM production mostly constitutes of noncytolytic β-type and mild cytolytic PSMs (Otto, 2014). In conclusion, although *S. epidermidis may* lack highly virulence toxic molecules compared to *S. aureus*, due to the *S. epidermidis* PSM production, *S. epidermidis* has an evolutionary advantage over more virulence *Staphylococci* strains (Otto, 2014). Although *S. epidermidis* toxicity factor has been researched the process of toxicity is not well understood.

Furthermore, other possible virulence substances produced by *S. epidermidis* are now being studied as well as the fibrinogen binding process (Becker *et al.,* 2014). A gene called *fbe* consists of an open reading frame of 3,276 nucleotides which encode a protein named Fbe, which has a molecular weight of ~119 kDa (Salgueiro *et al*., 2017). Biomaterial implants are quickly inhabited by plasma substances and fibrinogen aid bacterial cells adhere to the biomaterial surface. A problem may occur when adhesion occurs on the identical regions of medical implant by pathogenic microorganisms, hence, causing infection (Salgueiro *et al*., 2017; Becker *et al.,* 2014).

1.13 Clinical background

S. epidermidis commonly cause minor skin infections, for example, pimples and boils. These minor infections usually heal without the need for antibiotics. *S. epidermidis* typically infect patients in healthcare settings by adhering to hydrophobic biopolymers of prosthetics, hence, contaminating the equipment used for patient-care and colonising the environmental surroundings (Kleinschmidt *et al.,* 2015). Intravascular implants involving prosthetic heart valves and shunts may carry *S. epidermidis* biofilms and infect patients (Hellmark *et al*., 2013). *S. epidermidis* can infect a large wound and contaminate catheters and prosthetic joints to enter the host bloodstream (Mansson *et al.,* 2018). Pus secretion and high levels of inflammation may occur when a patient uses a contaminated catheter carrying *S. epidermidis*. This makes it tremendously painful for the patient to urinate. *S. epidermidis* is also known to cause illnesses such as septicemia and endocarditis (Onyango *et al*., 2018; Hellmark *et al*., 2013).

Septicemia or bacteraemia is the poisoning of the blood caused by infectious bacteria (Kleinschmidt *et al.,* 2015). Septicemia is a microbial infection in the bloodstream where the bacteria may enter through the urinary tract, skin or lungs. Septicemia tend to affect the weak immunocompromised neonatal, older individuals and patients that had surgery (Kleinschmidt *et al.,* 2015). Septicemia is a life-threatening disease as the bacteria toxins spread through the bloodstream to the entire body, septicemia must be cured quickly in a healthcare facility, if it is left unchecked septicemia can develop into sepsis (Kleinschmidt *et al.,* 2015). Septicemia symptoms include chills, fever, rapid respiration and fast heart rate. If septicemia is not treated properly or left untouched, the symptoms become more severe. These symptoms may include nausea, red marks on the skin, confusion, shock, lack of concentration and pass low volumes of urine.

Endocarditis is the microbial colonisation of the inside the lining of heart muscles and heart valves (endocardium). Without proper early treatment, endocarditis may impair or destroy the heart valves that may lead to fatal complications. Endocarditis treatment mainly include the usage of antibiotics and sometimes surgery. People with artificial heart valves, damaged heart valves, or other heart defects are at a higher risk of developing endocarditis. Symptoms of endocarditis include, fatigue, aching muscles and joints chills, fever and night sweats, shortness of breath, swelling of feet, legs or abdomen and changed heart murmur (the sound of blood rushing through the heart).

When Sahal & Bilkay (2014) examined blood samples from patients that had cardiovascular surgery as well as wound samples, *S. epidermidis* strains displayed a strong capacity to form biofilms (surgery samples 35% and wound samples 40%), 80% of them were resistant to β-lactam and 100% of them were multi antibiotic resistant (Sahal & Bilkay, 2014). The spread and quick growth of antibiotic resistant *S. epidermidis* microbe in healthcare settings are associated with certain factors for example, increased selective pressure, that occur due to improper and widespread use of antimicrobials mainly in hospitals. Moreover, there are other possible agents that cause dissemination of microbes, which may involve cross transmission amongst patients via inadequate infection and control strategies, bacterial mutation and horizontal resistance genes transfer along with an intricate relationship among nominated antimicrobial substances and microbial resistance.

1.14 Treatment

S. epidermidis infected pus (from a boil or a pimple) is grown and tested against antibiotics in the lab to see which antibiotic kills the bacteria. An actual *Staphylococci* skin infection e.g. a boil may heal after ten to twenty days if it is not treated, but if it is treated with antibiotic then the healing process

speeds up. 80% of catheters infected with *S. epidermidis* are treated with antibiotics including vancomycin without removing the catheter. Two treatments that are frequently favoured in treating other microbes including *S. aureus* is decolonisation and vaccination, however these options are ineffective for *S. epidermidis*. Firstly, there is no vaccine available *for S. epidermidis* infections and lots of studies show that the use of traditional immunisation is problematic for *Staphylococci* infection. Secondly, *S. epidermidis* is a major part of the human microbiota, hence it is difficult for it to be eradicated, if *S. epidermidis* were to be destroyed, quick re-colonisation from other individuals can occur. By excluding *S. epidermidis* from the human microflora it may hypothetically lead to more aggressive pathogens replacing *S. epidermidis*. Therefore, it is generally agreed upon that the most effective way of reducing the number of *S. epidermidis* infections is by preventing the spread of *S. epidermidis* (Szemraj *et al.,* 2019). This consists of medical equipment sterilisation, health care personnel in contact with indwelling medical devices during surgery and patient body parts sterilisation.

It is a challenging burden on the healthcare system to treat infections linked to *S. epidermidis* biofilms. This is due to the bacteria being resistant to antibacterial molecules and the human immune defence mechanism (Hoiby *et al*., 2010). In addition to this, currently the occurrence of virulence microbial strains becoming resistant to several antibiotics is drastically rising. Thus, making it difficult to treat microbial infections which has quickly become the deadliest health threat to all humans (Guridi *et al*., 2015). This difficulty has mostly occurred due to the phenotypic resistance of microorganisms as they act as a 'reservoir' and harbour resistance genes in the bacteria chromosome and/or plasmid DNA.

Lastly, França *et al.* (2016) findings raised significant consternation about the recent approach suggested on clearing *S. epidermidis* biofilm-associated infection. The most commonly proposed treatment for *Staphylococci* biofilm infections is to use enzymes that degrade matrix, for example, dispersin B has the capacity to disperse cells from *Staphylococcal* biofilms attached to a given surface. Nevertheless, the utilisation of enzymes that breakdown biofilm matrix or other molecules eventually cause disassembly of *Staphylococcal* biofilms (Boles & Horswill, 2011), this approach requires careful consideration as cells dispersed from the biofilm may increase inflammation in the host, therefore, increasing the level of disease (Franca *et al*., 2016).

1.15 Prevention

Health care professionals should focus on carrying out preventive measures to reduce the incidence of *S. epidermidis* infections or to completely eliminate the bacteria (Perez & Patel, 2018). These practices involve controlling risk factors, for example reintubation, nasogastric and endotracheal tubes, tracheotomy, poor infection control and hand washing procedures among hospital employees, old antibiotic treatments, contaminated respiratory aids, water or drugs are effective means of preventing *S. epidermidis* infections (Perez & Patel, 2018).

A small synthetic antimicrobial peptide called bactericidal peptide 2 (BP2) is shown to reduce 80% risk of acquiring *S. epidermidis* infection in the hospital via implants in a research carried out by (Perez & Patel, 2018). BP2 has strong bactericidal activity at micromolar concentrations for a broad spectrum of microorganisms, involving antibiotic-resistant bacteria. The staphylocidal activity of BP2 is not affected by physiological salt concentrations and is only slightly affected by the presence of human plasma (Simonetti *et al.,* 2013). BP2 is more active than HNP1-3 and as active as LL-37, a highly potent human antimicrobial peptide. Unlike LL-37 (Mahlapuu *et al.*, 2016) and HNP1-3 (Papot *et al*., 2017), the microbicidal activity of BP2 is not inhibited by physiological salt concentrations, stressing the potential of BP2.

1.16 Antibiotics

An antibiotic is a substance with a low molecular weight (<1000 daltons), for example antimicrobial substances (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019). Initially, microbes generated antibiotics to stop other distinct microorganisms from growing. The word 'antibiotic' has a Greek origin, where 'anti' means 'against' and 'bios' translates to 'life', hence, 'against bacterial life' (Kumbhar & Watve, 2013). The sulfa antibiotic prontosil was the first antibiotic introduced in 1935, it functions as a metabolic precursor to inhibit the enzyme needed for biosynthesis of microbial DNA (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019). Also, all fungi, microfauna and animals produce little peptides that function as antibiotics, namely AMPs. AMPs are the first line of host defence system for inhibiting colonising bacteria. Generally, AMPs enter specific bacteria to disrupt the barrier function. There is an ongoing dispute on the origin and physiological function of antibiotic substances when created naturally by microbes (Kumbhar & Watve, 2013). Some antibiotic substances develop to have a signalling role, such as alternating morphogens for streptomycetes, when the bacteria lack nutrition, the life cycle ends up changing including the function of the antibiotic (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019).

Antibiotics have different molecular ways to eliminate bacteria, for example, inhibiting growth, protein synthesis, nucleic acid synthesis, cell wall synthesis, enzyme activity, specific biochemical processes and altering cell membrane permeability (Kapoor, Saigal & Elongavan, 2017). Some examples are briefly explained in Table 1. Traditionally, antibiotics have been classified as bacteriostats which stop cell growth or bactericides that kill bacteria. Bacteriostats rely mostly on the host immune system to eliminate the bacterial infection. However, bacteriostatic antibiotics can become bactericidal when used at a high concentration, hence, this method of classification is not enough (Kapoor, Saigal & Elongavan, 2017). Another more recent method to classify antibiotics is to observe antibiotic activity, if it is reliant on time, concentration or is it co-dependent (Table 1). Information on how the antibiotic operates is important, however, more data is required on the spectrum of activity (which microbes are susceptible to the antibiotics), level of resistance detected (due to repetitive use) and if the concentrated antibiotic is effective *in vivo* with therapeutic dose rates (Kapoor, Saigal & Elongavan, 2017).

Pharmacokinetics and pharmacodynamics can explain the antibiotic's subsequent effects and association between the dose and body response (Campbell & Cohall, 2017). The pharmacological reaction mainly relies on the antibiotic binding to its target. The antibiotic concentration at the receptor site has a great impact on the effectiveness of the drug (Campbell & Cohall, 2017). The pharmacodynamics of the antibiotic can be influenced by physiological alterations caused by genetic mutations, aging, disease and other antibiotics. A physiological change may transpire due to the capability of disorders to modify receptor binding, alter the amount of binding proteins, or reduce the sensitivity to receptors (Campbell & Cohall, 2017).

Table 1. Antibiotics used in this research

Year of discovery	Year of introduction	Mode of action	Chemical groups	Antibiotics	Site of Action	Bactericidal or Bacteriostatic?	Spectrum of activity
1957	1960	Elimination is reliant on concentration, with a strong post-antibiotic effect.	Nitroimidazole	Metronidazole	Nucleic acid synthesis	Bactericidal	Narrow
1983	1987		Fluoroquinolone	Ciprofloxacin	Nucleic acid synthesis	Bactericidal	Broad
1955	2000	Elimination is reliant on time with little or no post-antibiotic effect.	Penicillin	Amoxicillin,	Cell wall	Bactericidal	Broad
1979	1985			Co-Amoxiclav			
1952	1964		Cephalosporin	Cefalexin	Cell wall	Bactericidal	Broad
1948	1951		Macrolide	Erythromycin	Protein Synthesis	Bacteriostatic	Broad
1956	1972		Diaminopyrimidine	Trimethoprim	Folate synthesis	Bacteriostatic	Broad
1957	1985		Oxazolidinone	Linezolid	Protein synthesis	Both	Narrow
1957	1967	Elimination is reliant on the level of concentration and length of exposure.	Tetracycline	Doxycycline	Protein synthesis	Bacteriostatic	Broad
1980	1990		Ketolide	Clarithromycin,	Protein synthesis	Bacteriostatic	Broad
1980	1981			Azithromycin			

Revised table from Bouchard (2017)

1.17 Antibiotic resistance

At present, there is a universal concern for the era of antibiotics as it is coming to an end (Santiago & Maximino, 2016). It is predicted that medicine in the near future may return to 19th century medicine where one slight injury may cause deadly infection and death (WHO, 2014). For decades antibiotics have been used globally and repeatedly. Since 1935, all infections caused by bacteria have been cured quickly, even if the last generation of bacteria showed resistance to antibiotics (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019). Resistance to antibiotics is inevitable. All animals including humans live with a large variety of bacteria in their gut and skin microflora, most of these microbes namely commensals are highly valuable or completely innocuous (Conlan *et al.*, 2012). Only a small portion of these commensals are capable of becoming harmful based on what niche they live in or what area of the host they occupy (Conlan *et al.*, 2012).

The discovery of antibiotics was and still is a breakthrough in treating bacterial infection, however, due to the overuse of antibiotics there are now antibiotic-resistant strains of pathogenic microbes (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019). This method of treatment lead to non-reversible mutation in new resistant strains of pathogens or commensal bacteria (Conlan *et al.*, 2012; Santiago & Maximino, 2016). This is a global health problem which requires an urgent solution as it may lead to a permanent unbalance skin and gut microflora. This is why there are laws and regulations put in place to limit the use of antibiotics for minor infections (Dreno *et al*., 2016).

The world population of bacteria is approximately 5 x10^{30}, this means every individual on this planet has around 50 tons of bacteria (Namkeleja *et al.,* 2016). Bacteria has the capacity to replicate their DNA and divide rapidly, also, if the bacteria have specific nutrients available to it, it is capable of duplicating every twenty minutes within the gastrointestinal tract (Pfeifer *et al.,* 2019). The DNA transcriptase in a given bacteria make around one mistake in every 10^6 base pairs of replicated DNA (Namkeleja *et al.,* 2016). If 3 to 10 mutations occurred within a bacterial genome for every generation, the bacterial populace of 10^8 in a particular niche would most likely have 300 to 1000 variants. There is a high possibility of one of these mutations to cause resistance to the mode of action of an antibiotic, such as penicillin. When the bacteria are exposed to a strong concentration of antibiotic with sufficient amount to kill the bacteria, the sensitive bacteria will perish. The resistant duplicate bacteria that was once an exceptionally minor mutant in the microbial community, has now access to nutrients and space released by the dying neighbouring bacteria, the resistant duplicate microbe will gradually become widespread in the community. It has been calculated that, when obtained as a group, the sum genes for bacteria found in the soil worldwide go through four separate mutations every 3.4 hours.

Substantial number of mutants are bound to exist in the estimated world population of bacteria (5 x10^{30}) (Namkeleja *et al.,* 2016).

Strong and speedy antibiotic prescriptions work remarkably well when the bacterial population is destroyed completely (Aminov, 2010; Domínguez & Meza-Rodriguez, 2019). However, when this does not occur, the outcome of antibiotic usage becomes disadvantageous. As *S. epidermidis* is a part of the normal microbiota for healthy humans, it has developed resistance to numerous antibiotics that are used frequently, for example, methicillin, novobiocin, clindamycin, and benzyl penicillin (Saffari *et al*., 2016). Consequently, other antibiotics namely vancomycin or rifampin are now used to treat infections.

S. epidermidis contain integrated plasmids that carry genes encoding species-specific LPXTG surface proteins and resistance to cadmium. *S. epidermidis* Genome Island may encode possible virulence factors including numerous phenol-soluble modulins (Xue *et al.,* 2017). *S. epidermidis* contain cap operon (*capABC*) and gamma-glutamyl transpeptidase gene as found on the *B. anthracis* pX02 plasmid, which encodes polyglutamate capsule, a key virulence factor of *Bacillus anthracis* (Xue *et al.,* 2017). Any other phenotypic variances are possibly caused by single nucleotide polymorphisms which can be seen in most cell envelope proteins. Phylogenetic study of *cap* genes in the operon specify that the attainment of this locus may be created by a plasmid-mediated transfer event from the ancestor of *bacilli* to *S. epidermidis*. Nevertheless, several species-specific metabolic functions including polyphosphate synthesis and acetoin dehydrogenase are encoded by complete operons in *S. epidermidis*, this may be due to gene loss by a common ancestor (Xue *et al.,* 2017).

Five key mechanisms have been identified to cause antibiotic resistance, including, the formation of deactivating enzymes, alteration in target sites, initiation of alternative pathway e.g. bypass pathway, the inability to activate antibiotics and antibiotic prohibition from active efflux of target site (MacGowan & Macnaughton, 2017). The main mechanism that cause antibiotic resistance is the chemically modified antibiotic molecule as well as the destroyed antibiotic molecule. Secondly, mutated target sites, altered enzymatic function of the target sites and full substitution or bypass of target sites cause the most antibiotic resistance. Furthermore, majority of antibiotic resistance occur when there is low porosity and deceased amount of efflux pumps. (Munita & Arias, 2016).

The consecutive attainment of resistance in most classes of antimicrobial agents, including tetracycline, macrolides, aminoglycosides, penicillin and chloramphenicol, has made curing and controlling *S. epidermidis* infections increasingly problematic.

During the late 1960s, semisynthetic penicillin and methicillin were extensively used and led to the rise of methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE), which persists today in both community environments and health care settings (Stamatiou *et al.,* 2013). *S. epidermidis* are known to contain specific antibiotic resistant genes, especially for methicillin, which is one of the first antibiotic used to treat *S. epidermidis* infections, approximately 75–90% of *S. epidermidis* isolates found in a hospital setting had methicillin resistant gene, oppose to *S. aureus* isolates with only 40–60% harbouring methicillin resistant gene (Stamatiou *et al.,* 2013). *S. epidermidis* carry mobile genetic elements (MGEs) called *Staphylococcal* cassette chromosome *mec* (SCC*mec*) comprising of *mecA* gene which encode a penicillin-binding protein (PBP) with low methicillin affinity (Rolo *et al*., 2012; Mirzaei *et al.,* 2017). PSM-*mec* (20–25 amino acids), a short α-type PSM is also encoded by SCC*mec* (Cheung *et al.,* 2014). PSM-*mec* has the ability to activate and stimulate neutrophils (Qin *et al.,* 2016) via human formyl peptide receptor 2 (FPR2) interaction, the G protein-coupled receptor is expressed on different types of host immune cells. PSM-*mec* can immediately up-regulate the expression of both CD11b and gp91phox, induce calcium flux, chemotaxis, and IL-8 release in neutrophils (Qin *et al.,* 2016). Not only does *S. epidermidis mecA* gene encode for methicillin resistance but also broad β-lactam resistance (Saffari *et al*., 2016).

In addition to this, ten various SCC*mec* elements have been found in *S. epidermidis.* The most commonly identified SCC*mec* element in *S. epidermidis* is the small SCC*mec* type IV structure which may spread without damaging the fitness of its host bacteria and evade specific antibiotic pressures (Xue *et al.,* 2017). Moreover, *Staphylococcal* variants carry various type of SCC*mec* structures and *S. epidermidis* frequently uptakes and emits *SCCmec* elements.

Furthermore, *S. epidermidis* is resistant to lots of other antibiotics besides methicillin such as erythromycin chloramphenicol, gentamicin, flouroquinolones, rifamycin, sulfonamides, tetracycline and clindamycin (Saffari *et al*., 2016). Also, *S. epidermidis* is less likely to be resistant to tigecycline, streptogramins, and linezolid and is found to be intermediate resistant to vancomycin. The capability of *S. epidermidis* to form biofilm greatly reduces the likelihood of vancomycin and other antibiotics to have an effect (Saffari *et al*., 2016). Bacteria plasmids mostly encode antibiotic resistant genes and are frequently found in methicillin-resistant instead of methicillin-susceptible variants. Endemic nosocomial strains of *S. epidermidis* are usually resistant to both methicillin and other antibiotics (Stamatiou *et al.,* 2013).

The overuse of antibiotic treatment for *S. epidermidis* infection reflect the high-level of antibiotic resistance within *S. epidermidis*. The ubiquity of *S. epidermidis* as a symbiotic microbe on the human skin, makes it an ideal reservoir for antibiotic resistant genes especially the SCC*mec* elements that do

not incur a fitness cost to the host (Xue *et al.,* 2017). Thus, several lines of evidence suggest that *S. epidermidis* cassette chromosome *mec* which encode methicillin resistance are transmitted to *S. aureus* (Gill *et al*., 2005). Also, the uptake of SCC*mec* type IV by community associated methicillin-resistant *S. aureus* (CA-MRSA) has largely influenced public health (Xue *et al.,* 2017). CA- MRSA is a name given for an infected person who has not visited a healthcare facility such as a dialysis centre, nursing home or a hospital. SCC*mec* type IV has allowed the bacteria to remain fit to spread highly virulence toxins while being resistant to methicillin, hence, causing the epidemiology of CA-MRSA strains (De Leo *et al*., 2010). Furthermore, recent evidence suggests that MGEs can be transferred via horizontal gene transfer from *S. epidermidis to* CA-MRSA, which is vital for the spread of disease. Consequently, CA-MRSA infections mainly rely on *S. epidermidis* acting as a reservoir for genetic factors which are then transferred to *S. aureus*, making it important for CA-MRSA to colonise successfully (De Leo *et al.,* 2010).

Although *S. epidermidis* does not carry a large variety of virulence factors, *S. epidermidis* is highly resistant to several antibiotics. In a study carried out by Mendes *et al*. (2012), 50% of *S. epidermidis* strains were found to be resistant to different kinds of antibiotics. Moreover, 70% of *S. epidermidis* isolates from a hospital setting was found to be resistant to Oxacillin (Mendes *et al*., 2012; Flamm *et al.,* 2013). In a study done by Sahal & Bilkay (2014), all *S. epidermidis* strains were sensitive to vancomycin, yet 65% of them exhibited resistance to all β-lactam antibiotics including Penicillin, Oxacillin and Amoxicilin/Clavulonic acid, 60% of all *S. epidermidis* strains were found to be multi antibiotic resistant (Sahal & Bilkay, 2014).

Staphylococcus species have the capacity to adapt quickly and become resistant to one or several other antibiotics. Only 10% of current *Staphylococcal* infections can be treated with penicillin. To cure *Staphylococcal* infections and combat antibiotic resistance, it is important to test for new treatments and interventions. Also, it is vital to research new means for prevention, detection, treatment and to manage *Staphylococcal* infections (Xue *et al.,* 2017). The effectiveness of vancomycin is limited due to the rise of vancomycin-intermediate *S. aureus* and more recently vancomycin-resistant *S. aureus*. The collective occurrences of hypervirulence community-acquired *S. aureus* have become a global concern to the health community and is in a serious need for new methods of control and treatment (Xue *et al.,* 2017).

1.18 The importance of antibiotic research

One possible way to control the rise of microbial resistance is to develop new effective antimicrobial agents instead of unsuccessful drugs (Saxena *et al.,* 2019). Thus, it is important on an international level to research new antibiotic of enhanced activity or unique structure from the limitless source of microbes as resistant bacteria continue to surge. AMR is identified as one of the main threats of the 21st century to global health, food security and development. Antibiotics can be bought over the counter without prescription for human or animal usage, the appearance and widespread of resistance is worsened. Likewise, in countries with no normal treatment guidelines, antibiotics are usually over-prescribed by health employees and over-used by the populace. AMR have repetitively emphasised the vital need for action by national and international organisations (O'Neill, 2014; Davies & Gibbens, 2013).

The second leading cause of global death are infectious diseases, regardless of the advancement made in the identification and development of antibiotics (Silber *et al*., 2016). Infections caused by multi-drug resistant variants show major clinical sign of drug resistance leading to a progressive interest in the research of AMR. Advance screening programs are vital for the selection of various natural and altered chemical compounds to determine new antibiotics active against resistant bacteria (Sanchez *et al*., 2010)**.** Furthermore, there is a growing requirement of antibiotics worldwide. For instance, "marketresearch.com" study highlighted China's need for penicillin which grew exponentially from 1990 to 2000, this trend was predicted to grow and spread over the following years (Dayalan *et al*., 2011).

Several processes of antibiotic resistance commonly develop a fitness cost, which is a significant biological limitation that positively impact progression of resistance. Mutations that confer small amount or no fitness cost are highly likely to survive and grow without antibiotic therapy (Melnyk *et al*., 2015). At the community level, the fitness cost caused by resistance is reversible. However, community-level reversibility rate is slow due to compensatory evolution, co-selection of resistance genes and costless mutations. Furthermore, the larger the fitness cost, the quicker the reversibility (Melnyk *et al*., 2015).

Information about fitness costs and compensatory mutations can be utilised to decrease the possibility of bacteria becoming resistant. This can enable researchers to select antibiotics with resistance mechanisms that have huge fitness costs along with low frequency compensation mutations. Thereby exploiting the in-depth information on physiological aspects of fitness costs, it is possible to select specific novel therapy designs that focus on physiological weak points connected to specific resistance mechanisms (Melnyk *et al*., 2015).

Current research studies emphasise the significance of gathering large amounts of data based on epidemiology of antibiotic resistance (Frieri *et al.*, 2016). This is important as it will help in constructing new control programmes for the evolving universal health crisis, namely, antibiotic resistance. Nosocomial microbial plasmids need to be isolated for molecular characterisation to produce invaluable data to aid in developing novel control strategies for clinical infectious microbes.

1.19 Adaptive resistance (AdR)

Antibiotics provide key defence mechanism against various infectious pathogens that cause disease. Due to this, antibiotics have increasingly been produced and distributed significantly over the last seven decades. This has resulted in the careless overproduction of antibiotics not only for treatment, but largely as food additives for plants, poultry and cattle. The non-selective overuse of antibiotics has led to drastic microbial adaptation, where the microbes have established systems to avoid antibiotic eradicating them.

From the time antibiotics were introduced as antibacterial medium, the starting point of minimum inhibitory concentration (MIC) for several antibiotics has increased dramatically over the years. One example of this 'MIC creep' is erythromycin having a 250-fold MIC upsurge in the last five years. Another example of an antibiotic demonstrating a rise in MIC is ciprofloxacin with a 120-fold MIC increase in nineteen years. However, a combination of events may have caused the mentioned MIC upsurge, nonetheless, the large quantity of construction and distribution of antibiotics has undoubtedly led to the increased MIC.

Microbial communities can become resistant to antibiotics via three known mechanisms; intrinsic, acquired and adaptive resistance (Jose *et al.,* 2016). Intrinsic resistance involves all the innate processes of certain microbes that may inhibit the influence of antibacterial agents, for example, efflux pumps and cell membrane restrain antibiotics from influencing the microbe. Secondly, acquired resistance involves the integration of deoxyribonucelic acid (DNA), possibly through exchange of plasmid genetic material or mutation within the microbe that may develop new capacities to resist strong concentration of antibiotic (Jose *et al.,* 2016). Lastly, adaptive resistance (AdR) requires a universally accepted definition, while Hancock and Férnandez (2012) defined AdR as a brief rise in the capability of a microorganism to resist an antibiotic attack due to gene variations and/or protein expression trigged by the environment. This specific mechanism of antibiotic resistance, oppose to intrinsic or acquired, is reliant on the existence of antibiotics. The AdR requires communication with antibiotics to trigger resistance or fixate the resistant phenotype. However, during certain events AdR may function as a link between the acquired and intrinsic resistance, as heritable mutations or

epigenetic alterations, induced by environmental indicators, may change the expression of innate defence system in turn elevating the microbial resistance to antibiotics (Jose *et al.,* 2016).

The procedure to produce AdR is to firstly expose the microbial community to small doses of antibiotic and then slowly raise the level of concentration. This can be attained by initially exposing the microbial populace to harmless concentrations of antibiotics, thereby activating the innate defence system and preparing the microbial community to survive stronger doses of antibiotics.

One particular feature of AdR is the variation shown in the phenotype of microbes within a populace, this is also identified in closely similar or identical genotypic microbial colonies (Jose *et al.,* 2016). Although the microorganisms share the same genotype, some of them are capable of surviving whereas others die. The isogenic colonies share same ancestors and are exposed to the same surroundings, yet some of the microbes show changes in the phenotype and become resistant. The phenotype alterations can be due to different trends of gene expression. Furthermore, around 20% of microorganisms have the capacity to survive at subinhibitory level of concentration, this indicates that phenotypic variations within a microbial community is not caused by gene mutations. If the phenotypic variations were due to genetic modifications, the expected survival rate would be a lot higher than 20%. The phenotype that displays bacterial resistance has low level of stability and cannot be found in some generations of bacteria when grown without antibiotics. This also indicates that AdR is not due to genetic alterations as that would commonly produce irreversible phenotypes (Jose *et al.,* 2016). Moreover, the resistant phenotype may be due to epigenetic modifications as this is known to be very unstable. The microbial populace displays a gradual increase in resistance and only few of the microbes eventually become resistant, not all of them. This suggests that few microbes must inherit some memory of the process that causes phenotypic resistance.

Although, there has been a lot of progression in the development of antibiotics and in the treatment of bacterial infections, no permanent solution has been found for the complication of bacteria becoming resistant to several antibiotics. Out of all known forms of resistance, AdR is the most problematic as this phenotype is produced by concentration gradients and when in contact with subinhibitory concentration of antibiotics. Both of these mechanisms occur within human and farm animals. Likewise, AdR has been associated with the emergence of various antibiotic resistance, despite there being little understanding of why it occurs and the biological process of AdR (Jose *et al.,* 2016). Some research studies have suggested that epigenetic inheritance, high level of mutations, efflux pumps, gene amplification, structure and phenotypic variations within a population and the production of biofilm may have contributed to the emergence of AdR. Nevertheless, when these notions were researched separately, the researchers failed to stop the quick emergence of AdR or

anticipate the high levels of instability (Jose *et al.,* 2016). Variants of resistant bacteria keep on emerging while the current methods to kill them such as, efflux inhibitors, sequential treatments and combined antibiotics are proving to be highly ineffective (Pizzolato-Cezar *et al.,* 2019).

In a recent study, researchers analysed a collection of global *S. epidermidis* strains (Popescu, 2018). They found that 74% of the isolates from hospital-associated infections belonged to a distinct *S. epidermidis* clonal complex. These isolates were from three separate lineages, most of them were nosocomial with substantially enhanced antibiotic resistance and biofilm formation capacity, thus, signifying (hospital) adaptation resistance. The three *S. epidermidis* lineages share a rpoB mutation which cause resistance to rifampicin (Popescu, 2018). In addition to this, researchers identified a 21.7% increase (2007 - 2013) in teicoplanin-resistant *S. epidermidis* strains in an Australian 800-bed hospital (Popescu, 2018).

Present information on AdR suggest that it is frequently mediated by fitness costs, biological gain-of-function and physiological adaptations. Genetic mutations and phenotypic adaptation can alter bacterial metabolism causing an impaired biological efficiency. To manage adaptive resistant *S. epidermidis* strains, a possible yet an unexplored method can be used to categorise and inhibit metabolic pathways that are important for AdR (Zhong *et al*., 2018). The metabolome commonly refers to the whole set of low-molecular weight molecules (metabolites). Metabolomic analysis is a measurement of metabolites to evaluate the metabolomic processes that are required for the maintenance of growth and normal function of a cell in a specific physiological or developmental stage (Xu *et al.,* 2018). Metabolic profiling is a vital aspect as well as proteomics, transcriptomics and genetics data, to allow a comprehensive representation of the interactions between proteins, transcripts, metabolites and genes in microbes (Xu *et al.,* 2018). Therefore, MS^E was used in this research study to observe and analyse any differences in metabolome before and after antibiotic adaptation.

MS^E is a technique used by Water informatics to acquire accurate data with the strength of mass spectrometry and UltraPerformance LC (Waters, 2011). MS^E only requires one experiment for exact-mass precursor, fragment ion data, sample identification and quantification. Mass spectrometry technique has high sensitivity with an improved in-spectrum dynamic range to detect components, irrespective of the concentration, accurate mass and isotopic pattern is digitally recorded (Waters, 2011). UltraPerformance LC has the capacity to demonstrate advance levels of chromatographic separation and rapidly generate constricted chromatographic peaks. MS^E is easy to set up, does not need a lot of parameters to be adjusted and little method alteration for application with no previous research on the sample components (Waters, 2011).

Microbial fitness and virulence are affected by AdR and genetic systems e.g. genetic co-selection and compensatory mutations (Beceiro *et al*., 2013). Some resistance mutations may experience no fitness costs and persist in the populace without the pressure of antibiotic selection. On the other hand, costs of resistance can be balanced out through second-site mutations which re-establish microbial fitness with no antibiotic exposure (Beceiro *et al*., 2013). The relationship between virulence and resistance reflects the Darwinian model, where specific advantageous traits are selected at some point in time and become permanent. A positive correlation between resistance and virulence can infer a speedy selection of advantageous traits (Beceiro *et al*., 2013). A negative correlation between resistance and virulence (high resistance and low virulence) causes a longer selection period for the specific virulent factors to become permanent in the populace (Melnyk *et al*., 2012). In contrast to this, there can be an increase in virulence with a decrease in resistance. This is where compensatory mutations may occur to produce a positive correlation between resistance and virulence, hence, allowing the bacteria to have a permanent selective advantage. Research on bacterial virulence require an appropriate animal model, for example, insects, as they have a similar mammalian innate immune system (Melnyk *et al*., 2012). Hence, *G. mellonella* virulence assay was used in this study to observe differences between pathogenicity in parent strains and antibiotic adapted strains.

A wax worm that is also known as a caterpillar larva belong to the *G. mellonella* species, from the Order named Lepidoptera and the Pyralidae Family (Tsai *et al.,* 2016). The average length of wax worms is 2-2.5 cm and are cream in colour (Figure 3A). The insect *G. mellonella* and mammals share similar functioning phagasomes, for example, sugranulocytes and plasmatocytes (Tsai *et al.,* 2016). Additionally, *G. mellonella* wax worms produce neutrophils which express distinct proteins that are almost identical to human calreticulins, these proteins usually play a role in non-self-recognition for defence responses induced by cells. *G. mellonella* can be used as an effective virulence model to study pathogenic microbes. *G. mellonella* is the most suitable model to use when researching bacterial infections opposed to other model hosts as researchers do not require ethical authorisation, and the wax worm model is economical due to low-cost while easily sustained with a decreased life span (Melnyk *et al*., 2012). Furthermore, when *G. mellonella* wax worms are differentiated against their invertebrate equivalents such as *Drosophila melanogaster* and *Caenorhabditis elegans*, they are able to survive 37 °C temperature (ideal temperature for most microorganisms to grow), allowing *G. mellonella* to be the most optimum virulence model for bacterial infection research (Tsai *et al.,* 2016).

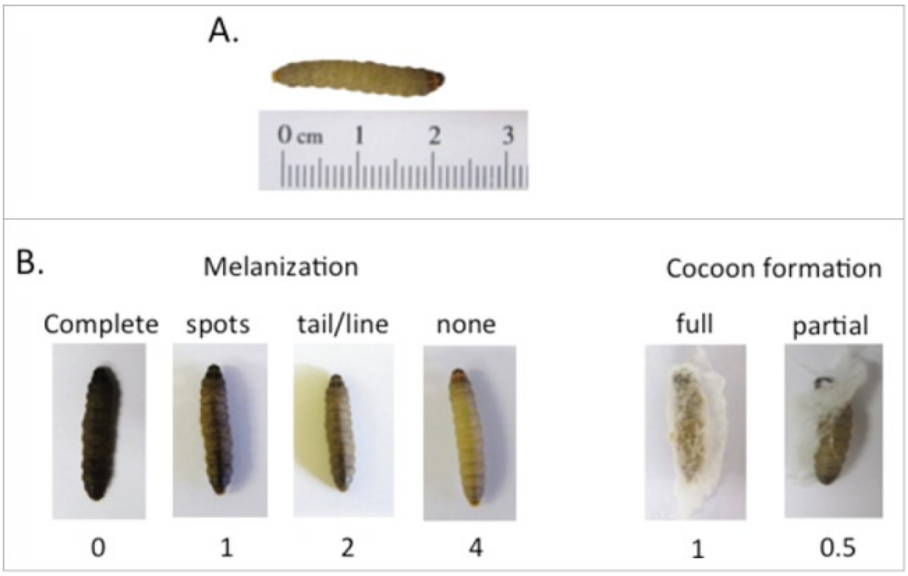

Figure 9. Photographic images of *G. mellonella.* **(A)**. Image of a healthy *G. mellonella* larvae. **(B)**. Images of an infected larvae at different stages of disease. Melanisation, which involves the production and deposition of melanin to encapsulate microbial pathogens at the inoculation spot accompanied by haemolymph coagulation with characteristic black spots on the larvae. Complete melanisation associated with death of the larvae thereafter. In addition, a reduction in cocoon formation is an indicative marker for disease in *G. mellonella* larvae. The numbers represent the assigned health index scores based on the *G. mellonella* Health Index Scoring System (Tsai *et al.,* 2016).

1.20 Unidirectional horizontal gene transfer

S. epidermidis frequently transfer mobile genetic elements to *S. aureus,* excluding toxin genes, still it may be possible for *S. epidermidis* to uptake toxin genes from *S. aureus* through a similar process (Common *et al.,* 2019). Recent studies of clustered regularly interspaced short palindromic repeat sequences (CRISPR) demonstrate short repeats that may stop *S. epidermidis* from acquiring conjugative plasmids and phage from other bacteria (Szemraj *et al.,* 2019). This may explain why the transmission of mobile genetic elements between *S. epidermidis* and *S. aureus* is unidirectional (Águila-Arcos *et al.,* 2017). Clustered regularly interspaced short palindromic repeat sequences are not found in *S. aureus* genomes but have been detected in *S. epidermidis* strains. The role of *S. epidermidis* CRISPR sequence requires a better understanding and further research on how it prevents the uptake of mobile genetic elements (Common *et al.,* 2019). This mechanism may signify why *S. epidermidis* lack a large variety of toxins and are avirulent (Águila-Arcos *et al.,* 2017).

A bacterium can obtain a resistant gene via horizontal gene transfer. Plasmid containing *tra* genes are transferred to other bacteria through a complicated process of conjugation, this is known to be the main cause of new resistant pathogens (Schiwon *et al.,* 2013; Águila-Arcos *et al.,* 2017). Bacteria can directly transfer genetic material to other cells in a biofilm as the biofilm supplies a perfect place to switch genes originated from several microbes. Nonetheless, microbial conjugation may encourage biofilm production as microbial cells need to be present close to each other in order to exchange resistance genes (Donne *et al.,* 2015). The relationship that bacterial conjugation and biofilm share raises the possibility of biofilm infections to disseminate virulence agents.

Aim of the study

The main aim of this study was to characterise bacteria isolated from human skin before and after exposure to antibiotics in terms of their antibiotic susceptibility, biofilm formation, and virulence. To find and emphasise the important role of *S. epidermidis* in human disease by functioning as a reservoir for virulence genes and the possible pathogenic role it may play to cause disease. This will further provide insights in understanding the manner *S. epidermidis* -associated bacteria can adapt, as well as the likely consequences for diagnosis and treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection of parent strains

Five isolates of *Staphylococcus epidermidis* (SE1, SE2, SE3, SE4 and SE5) were obtained from human skin and sub-cultured onto Müller-Hinton Broth (MHB, Oxoid Limited, UK) and Müller-Hinton Agar (MHA, ProLabo, India), and incubated at 37 °C for 24 h (EUCAST, 2019).

2.2 Antibiotic susceptibility testing (disc diffusion assay)

The susceptibility of both parent and antibiotic-adapted strains were determined before and after adaptation to antibiotics according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) guidelines using disc diffusion method. Suspensions of the organisms were prepared in Phosphate Buffer Saline (PBS), compared to a 0.5 McFarland turbidity standard, and spread on MHA. Thereafter, commercially obtained antibiotic discs (Oxoid, United Kingdom) were applied and incubated at 35±1 °C for 24 h according to EUCAST (2019) guidelines. Antibiotics used include: doxycycline (30 µg)**,** ciprofloxacin (5 µg), erythromycin (15 µg), cefalexin (30 µg)**,** amoxicillin (25 µg), trimethoprim (5 µg), clarithromycin (15 µg), linezolid (10 µg), metronidazole (5 µg), azithromycin (15 µg) and co-amoxiclav (30 µg). The entire procedure from preparation of suspension containing the inoculum, the application of the antibiotic discs, and the incubation of plates were all completed within 15 minutes' interval adhering to the 15-15-15-minute rule according to EUCAST (2019). This was followed by the assessment of bacterial growth against antibiotics (antibiogram pattern) by measuring the zones of inhibition and documenting the results using EUCAST Clinical Breakpoint Tables (EUCAST, 2019).

2.3 Selection of antibiotic-adapted isolates

Weekly serial passage exposure experiments were set up using antibiotics that were effective against parent strains through aseptic selection of growth closest to the terminal point of the zones of inhibition, and used to inoculate a subsequent plate, antibiotic was added and incubated for 48 h at 37 °C (a single passage). After each passage, a separate streak was made to ensure culture purity. This

procedure was repeated for 9 passages and zones of inhibition were measured at every passage. Thereafter, frozen stock of the parent strains (designated P0) and antibiotic-adapted strains (P9) were stored at -80 ˚C for further analysis.

2.4 Determination of cross-resistance

After 9 passages, the antibiotic-adapted strains of *S. epidermidis* were spread on MHA and antibiotic discs applied and incubated at 37 ˚C. Individual zones of inhibition were measured and compared with the 9th passaged antibiotic-adapted strains to access cross resistance (EUCAST, 2019).

2.5 Determination of biofilm formation

Biofilm formation was analysed using the crystal violet biofilm assay (O'Toole, 2011). Overnight suspensions of susceptible parent strains and antibiotic-adapted strains of *S. epidermidis* were diluted 1 in 100 in MHB, and 100 µl aliquots of each were transferred to a 96-well plate (4-6 replicate wells per treatment) alongside negative controls (MHB). Plates were incubated aerobically at 37 ˚C for 24 h enclosed within a humidified vessel to reduce evaporation. Optical density (OD) of planktonic bacteria was measured and contents discarded, followed by addition of 125 µl of 1% (wt/vol) crystal violet stain to corresponding wells and incubated at room temperature for 10 minutes. This was removed, and the wells were washed thrice with distilled water and the plates were left to air dry. Thereafter, biofilm-attached crystal violet was extracted with 250 µl ethanol following incubation at room temperature for 10 min, and the absorbance was read using a microplate reader (FLUOstar Omega, BMG LABTECH) at 600 nm. The results obtained were expressed as the mean of biofilm-attached crystal violet absorbance comparative to the absorbance rates subtracted from the negative controls. Relative biofilm-forming units were calculated by dividing the absorbance of biofilm-attached crystal violet by the equivalent planktonic OD. The entire assays were performed in triplicate.

2.6 Determination of relative fitness

Overnight cultures of *S. epidermidis* parent strain (P0) or antibiotic-adapted strain (P9) were diluted 1:10 with the OD of 0.1 at 600 nm. Sterile TSB with 1% glucose (10 ml) was inoculated in triplicate with P0 or P9, alone or in combination. Flasks were incubated at 37 °C shaking at 100 rpm for 24 h. At 24 h, dilutions from each flask were plated onto MHA in triplicate and incubated at 37 °C for 18 h.

Bacterial viable counts were determined after 18 h of incubation, and relative fitness was assessed for bacteria grown independently and in combination, using the equation W = In (RF/RI) / In (SF/SI), where the W refers to relative fitness, RI and SI refer to the numbers of P9 and P0 cells at the start point, respectively, and RF and SF refer to the numbers of P9 and P0 cells at the endpoint (Latimer *et al.,* 2012).

2.7 Waxworm infection assay

Microbial virulence of susceptible parent strains and antibiotic-adapted strains (2 per organism) were determined using the *Galleria mellonella* (waxworm) infection assay (Brignoli *et al*., 2019; Latimer *et al.,* 2012; Tsai *et al.,* 2016). Final larval-stages of *G. mellonella* (Livefoods Direct, UK) were kept in the dark at 4 °C for < 7 days, and 18 were randomly allocated to each respective category and incubated at 37 °C for 30 min. Thereafter, overnight suspensions of susceptible parent strains and selected antibiotic-adapted strains were washed twice in PBS and diluted suitably in PBS to attain an OD of 0.1 (5×10^5 - 8×10^5 CFU/ml) at 600 nm. 5 µl of each suspension was inoculated into the hemocele of each larva via the eight left pro-leg with aid of a Hamilton syringe (2.5×10^3 - 4×10^3 CFU per individual). Larvae were incubated in plastic petri dishes at 37 °C, and the number of surviving larvae was documented day-to-day (lifeless larvae were insensitive to touch and appeared dark in colour). A non-treated category and PBS inoculated category were used as negative controls. The experiments were discontinued once the death of a minimum of 2 individuals in a control category was observed. The experiments were performed once for both parent strains and antibiotic-adapted strains.

2.8 Metabolite extraction for MS^E

The process of metabolite extraction from suspended microbial cells involve quenching (by addition of organic solvent followed by extraction of internal metabolites into the extraction solution. The subinhibitory concentration (colonies from the inner circumference of zone of inhibition) as well as zero-inhibitor concentration (colonies from the side of the agar plate) of samples SE1, SE2, SE3 adapted to Amoxicillin, Doxycycline Hydrochloride and Ciprofloxacin were taken using a sterile loop and inoculated into 10 mL of 60% methanol (-48 °C) (in 5, 10 and 20 mL). The samples were centrifuged at 4300 g for 10 min at -8 °C to pellet cellular mass. The cell media was removed and re-centrifuged at 4300 g for 10 min at -8 °C, the residual quenching media was removed again. Extraction can be performed immediately or stored at -80 °C. The TissueLyser adapter set (2 x 24) was stored at -80 °C for 2 h prior to starting disruption and homogenisation, the Eppendorf tubes containing the samples

were pre-cooled on dry ice. Each sample was transferred to 2 mL micrcentrifuge tubes containing 25-50 mg (pretreated nitric acid- washed) glass beads (150-600 µm mean diameter). The TissueLyser was operated for 5 min at 30 Hz. The duration of disruption and homogenisation was extended until no debris was visible. The biomass pellets were suspended in 1 mL of 80% methanol (-48 °C). The dissolved pellets were transferred to chilled 2 mL Eppendorf tubes, flash-frozen in liquid N_2 for 1 min then allowed to melt on wet ice. Upon thawing, the tubes were vortexed for 30 s. The samples were flash-frozen and vortexed two more times. The samples were centrifuged at -9 °C, 14,500 g for 5 min, supernatant was then transferred into fresh 2 mL Eppendorfs. 500 µL of extraction solvent was added to the biomass pellets, to be suspended in 1 mL of 80% methanol (-48 °C). The dissolved pellets were transferred to chilled 2 mL Eppendorf tubes to be flash-frozen in liquid N_2 for 1 min, melted on wet ice and vortexed for 30 s three times. The extracted solution was added to the same final 2 mL Eppendorf. 2 µL of each sample was trasferred into one Eppendorf tube to produce a QC sample. The Eppendorf tubes containing the extracts were left to dry out in the Eppendorf Vacufuge. Solid pellets were immediately stored at -80 °C. The Eppendorf tubes containing samples metabolite extracts were transported on dry ice and securely packaged to Waters for mass spectrometry metabolome analysis (Goodacre *et al*., 2005).

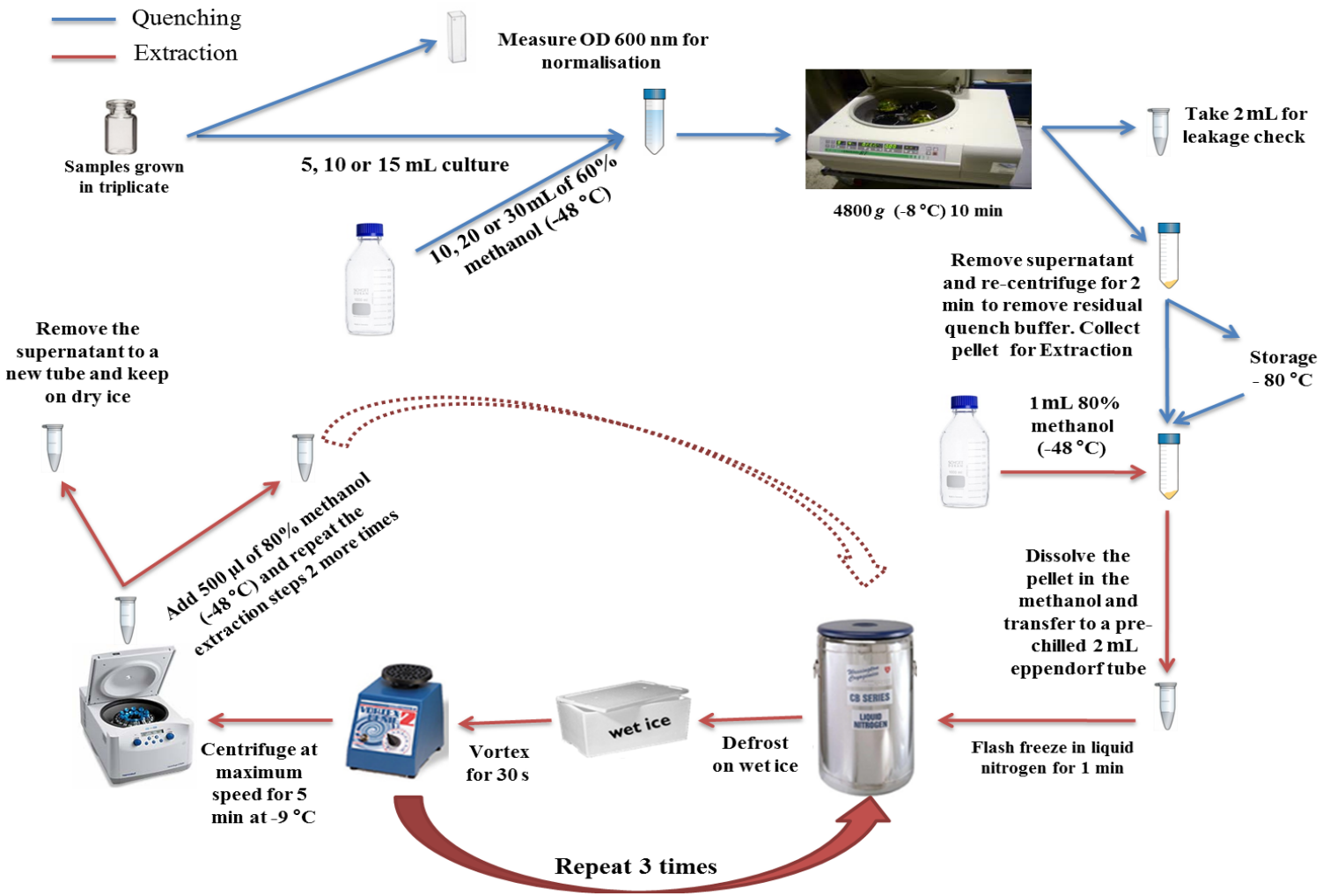

Figure 10. Extraction method. The initial quenching process in blue and the extraction process in red, including the optical density (OD) determination via UV to normalise biomass differences between experimental samples (Goodacre *et al.*, 2005).

2.9 Statistical analysis

Data obtained were analysed using Minitab 17 software and Microsoft Excel 2017. Graphical representations showing antibiotic susceptibility patterns, biofilm formation was plotted using Microsoft Excel 2017, while survival data were plotted using the Kaplan–Meier method (Prism v 7.03). Results were statistically presented as mean ± standard deviation (SD). Differences of $p < 0.05$ were considered statistically significant via One tailed T-test method.

Chapter 3

3.0 Results

3.1 Antibiotic susceptibility pattern

The antibiotic susceptibility patterns of parent strains used in this study was interpreted using EUCAST Breakpoint Table. As indicated by their zone diameters, parent strains of *S. epidermidis* was sensitive to the following antibiotics: doxycycline, ciprofloxacin, erythromycin, cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav (Table 2-6). Furthermore, it is observed that the 9th passage (P9), antibiotic-adapted strains of *S. epidermidis* remained sensitive to cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav, however, it was resistant to doxycycline, ciprofloxacin and erythromycin (Table 2, 3 and 4).

Table 2. Antibiotic susceptibility pattern of Doxycycline adapted strain with zone diameters (sample: SE1-SE5)

SE1		**Zone diameter (mm)**			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Doxycycline - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	15		4	
Ciprofloxacin	5	27		13	
Erythromycin	15	22		0	
Cefalexin	30	35		33	
Amoxicillin	25	31		29	
Trimethoprim	5	25		27	
Clarithromycin	15	34		40	
Linezolid	10	36		33	
Metronidazole	5	0		0	
Azithromycin	15	24		21	
Co-amoxiclav	30	35		39	

SE2		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Doxycycline - adapted strain	
		S	R	S	R
Doxycycline	30	18		8	
Ciprofloxacin	5	26		12	
Erythromycin	15	24		0	
Cefalexin	30	32		30	
Amoxicillin	25	30		27	
Trimethoprim	5	28		25	
Clarithromycin	15	32		25	
Linezolid	10	35		30	
Metronidazole	5		0		0
Azithromycin	15	20		22	
Co-amoxiclav	30	34		36	

SE3		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Doxycycline - adapted strain	
		S	R	S	R
Doxycycline	30	19		10	
Ciprofloxacin	5	23		15	
Erythromycin	15	25		0	
Cefalexin	30	34		30	
Amoxicillin	25	32		29	
Trimethoprim	5	24		22	
Clarithromycin	15	30		27	
Linezolid	10	40		38	
Metronidazole	5		0		0
Azithromycin	15	24		21	
Co-amoxiclav	30	37		39	

SE4		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Doxycycline - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	17			8
Ciprofloxacin	5	26			13
Erythromycin	15	20			0
Cefalexin	30	33		25	
Amoxicillin	25	30		26	
Trimethoprim	5	25		28	
Clarithromycin	15	36		31	
Linezolid	10	38		40	
Metronidazole	5		0		0
Azithromycin	15	18		20	
Co-amoxiclav	30	38		40	

SE5		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Doxycycline - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	14			10
Ciprofloxacin	5	22			12
Erythromycin	15	25			0
Cefalexin	30	36		34	
Amoxicillin	25	31		28	
Trimethoprim	5	26		22	
Clarithromycin	15	31		28	
Linezolid	10	38		35	
Metronidazole	5		0		0
Azithromycin	15	21		25	
Co-amoxiclav	30	38		40	

Doxycycline-adapted strain was observed to have cross- resistance to ciprofloxacin, erythromycin and doxycycline in all five isolates (SE1-SE5) while remaining susceptible to cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav. Doxycycline-adapted strains were resistant to metronidazole before and after being adapted to doxycycline (Table 2).

Table 3. Antibiotic susceptibility pattern of *S. epidermidis* Parent strain and Ciprofloxacin adapted strain (sample: SE1-SE5)

SE1		**Zone diameter (mm)**			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Ciprofloxacin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	15		5	
Ciprofloxacin	5	20		9	
Erythromycin	15	26		10	
Cefalexin	30	37		31	
Amoxicillin	25	26		28	
Trimethoprim	5	22		26	
Clarithromycin	15	33		38	
Linezolid	10	34		31	
Metronidazole	5		0		0
Azithromycin	15	20		19	
Co-amoxiclav	30	32		36	

SE2		**Zone diameter (mm)**			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Ciprofloxacin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	18		7	
Ciprofloxacin	5	26		11	
Erythromycin	15	24		13	
Cefalexin	30	34		29	
Amoxicillin	25	31		26	
Trimethoprim	5	33		30	
Clarithromycin	15	31		29	
Linezolid	10	37		28	
Metronidazole	5		0		0
Azithromycin	15	17		21	
Co-amoxiclav	30	32		34	

SE3		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Ciprofloxacin - adapted strain	
		S	R	S	R
Doxycycline	30	22		13	
Ciprofloxacin	5	27		15	
Erythromycin	15	28		14	
Cefalexin	30	32		28	
Amoxicillin	25	29		30	
Trimethoprim	5	22		20	
Clarithromycin	15	27		25	
Linezolid	10	39		36	
Metronidazole	5	0		0	
Azithromycin	15	22		19	
Co-amoxiclav	30	38		35	

SE4		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Ciprofloxacin - adapted strain	
		S	R	S	R
Doxycycline	30	17		9	
Ciprofloxacin	5	26		12	
Erythromycin	15	20		10	
Cefalexin	30	33		31	
Amoxicillin	25	29		30	
Trimethoprim	5	25		27	
Clarithromycin	15	35		32	
Linezolid	10	39		36	
Metronidazole	5	0		0	
Azithromycin	15	19		20	
Co-amoxiclav	30	38		39	

SE5	Zone diameter (mm)				
Antibiotic	Disc content (µg)	Parent strain		Ciprofloxacin - adapted strain	
		S	R	S	R
Doxycycline	30	16		9	
Ciprofloxacin	5	23		10	
Erythromycin	15	22		11	
Cefalexin	30	34		32	
Amoxicillin	25	32		26	
Trimethoprim	5	28		25	
Clarithromycin	15	32		29	
Linezolid	10	36		32	
Metronidazole	5		0		0
Azithromycin	15	23		24	
Co-amoxiclav	30	39		42	

Ciprofloxacin- adapted strain was observed to have cross- resistance to doxycycline, erythromycin and ciprofloxacin in all five isolates (SE1-SE5) while remaining susceptible to cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav. Ciprofloxacin- adapted strains were resistant to metronidazole before and after being adapted to ciprofloxacin (Table 3).

Table 4. Antibiotic susceptibility pattern of *S. epidermidis* Parent strain and Erythromycin adapted strain (sample: SE1-SE5)

SE1	Zone diameter (mm)				
Antibiotic	**Disc content (µg)**	**Parent strain**		**Erythromycin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	15		5	
Ciprofloxacin	5	27		10	
Erythromycin	15	22		0	
Cefalexin	30	32		26	
Amoxicillin	25	34		32	
Trimethoprim	5	25		27	
Clarithromycin	15	34		40	
Linezolid	10	36		33	
Metronidazole	5		0		0
Azithromycin	15	24		21	
Co-amoxiclav	30	35		39	

SE2		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Erythromycin - adapted strain	
		S	R	S	R
Doxycycline	30	14		6	
Ciprofloxacin	5	28		11	
Erythromycin	15	25		0	
Cefalexin	30	35		29	
Amoxicillin	25	31		29	
Trimethoprim	5	25		22	
Clarithromycin	15	32		29	
Linezolid	10	34		33	
Metronidazole	5		0		0
Azithromycin	15	29		26	
Co-amoxiclav	30	37		36	

SE3		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Erythromycin - adapted strain	
		S	R	S	R
Doxycycline	30	29		12	
Ciprofloxacin	5	25		14	
Erythromycin	15	27		0	
Cefalexin	30	31		39	
Amoxicillin	25	33		27	
Trimethoprim	5	22		21	
Clarithromycin	15	29		24	
Linezolid	10	37		39	
Metronidazole	5		0		0
Azithromycin	15	22		19	
Co-amoxiclav	30	36		38	

SE4		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Erythromycin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	18		8	
Ciprofloxacin	5	26		13	
Erythromycin	15	20		0	
Cefalexin	30	33		31	
Amoxicillin	25	32		34	
Trimethoprim	5	29		30	
Clarithromycin	15	34		30	
Linezolid	10	35		36	
Metronidazole	5		0		0
Azithromycin	15	21		22	
Co-amoxiclav	30	39		37	

SE5		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Erythromycin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	19		5	
Ciprofloxacin	5	21		12	
Erythromycin	15	28		0	
Cefalexin	30	35		32	
Amoxicillin	25	28		26	
Trimethoprim	5	25		20	
Clarithromycin	15	33		27	
Linezolid	10	34		31	
Metronidazole	5		0		0
Azithromycin	15	19		22	
Co-amoxiclav	30	37		39	

Erythromycin - adapted strain was observed to be resistant to erythromycin and have cross- resistance to doxycycline and ciprofloxacin in all five isolates (SE1-SE5) while remaining susceptible to cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav. Erythromycin - adapted strains were resistant to metronidazole before and after being adapted to erythromycin (Table 4).

Table 5. Antibiotic susceptibility pattern of *S. epidermidis* Parent strain and Cefalexin adapted strain (sample: SE1-SE5)

SE1		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Cefalexin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	13		9	
Ciprofloxacin	5	25		21	
Erythromycin	15	23		20	
Cefalexin	30	34		30	
Amoxicillin	25	30		32	
Trimethoprim	5	27		29	
Clarithromycin	15	36		39	
Linezolid	10	35		30	
Metronidazole	5		0		0
Azithromycin	15	22		21	
Co-amoxiclav	30	38		40	

SE2		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Cefalexin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	15		10	
Ciprofloxacin	5	23		19	
Erythromycin	15	24		20	
Cefalexin	30	29		32	
Amoxicillin	25	33		25	
Trimethoprim	5	28		24	
Clarithromycin	15	30		27	
Linezolid	10	33		32	
Metronidazole	5		0		0
Azithromycin	15	19		21	
Co-amoxiclav	30	35		37	

SE3		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Cefalexin - adapted strain	
		S	R	S	R
Doxycycline	30	21		24	
Ciprofloxacin	5	24		20	
Erythromycin	15	27		26	
Cefalexin	30	33		32	
Amoxicillin	25	32		29	
Trimethoprim	5	26		24	
Clarithromycin	15	31		26	
Linezolid	10	37		39	
Metronidazole	5		0		0
Azithromycin	15	23		22	
Co-amoxiclav	30	37		40	

SE4		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Cefalexin - adapted strain	
		S	R	S	R
Doxycycline	30	19		22	
Ciprofloxacin	5	23		24	
Erythromycin	15	19		25	
Cefalexin	30	31		28	
Amoxicillin	25	29		25	
Trimethoprim	5	27		30	
Clarithromycin	15	37		34	
Linezolid	10	39		37	
Metronidazole	5		0		0
Azithromycin	15	22		28	
Co-amoxiclav	30	35		39	

SE5	Zone diameter (mm)				
Antibiotic	Disc content (µg)	Parent strain		Cefalexin - adapted strain	
		S	R	S	R
Doxycycline	30	17		21	
Ciprofloxacin	5	24		26	
Erythromycin	15	26		28	
Cefalexin	30	37		36	
Amoxicillin	25	29		27	
Trimethoprim	5	29		25	
Clarithromycin	15	34		32	
Linezolid	10	39		37	
Metronidazole	5		0		0
Azithromycin	15	19		22	
Co-amoxiclav	30	36		38	

Cefalexin - adapted strains had no cross- resistance in any of the five isolates (SE1-SE5). The adapted strain remained susceptible to doxycycline, ciprofloxacin, erythromycin, cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav. Cefalexin - adapted strains were resistant to metronidazole before and after being adapted to cefalexin (Table 5).

Table 6**.** Antibiotic susceptibility pattern of *S. epidermidis* Parent strain and Amoxicillin adapted strain (sample: SE1-SE5)

SE1	Zone diameter (mm)				
Antibiotic	**Disc content (µg)**	**Parent strain**		**Amoxicillin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	25		28	
Ciprofloxacin	5	30		29	
Erythromycin	15	33		27	
Cefalexin	30	34		32	
Amoxicillin	25	30		28	
Trimethoprim	5	24		26	
Clarithromycin	15	34		39	
Linezolid	10	36		32	
Metronidazole	5		0		0
Azithromycin	15	24		21	
Co-amoxiclav	30	39		36	

SE2		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Amoxicillin - adapted strain	
		S	R	S	R
Doxycycline	30	25		22	
Ciprofloxacin	5	30		24	
Erythromycin	15	26		27	
Cefalexin	30	33		32	
Amoxicillin	25	30		26	
Trimethoprim	5	28		21	
Clarithromycin	15	33		25	
Linezolid	10	36		32	
Metronidazole	5		0		0
Azithromycin	15	22		24	
Co-amoxiclav	30	38		35	

SE3		Zone diameter (mm)			
Antibiotic	Disc content (µg)	Parent strain		Amoxicillin - adapted strain	
		S	R	S	R
Doxycycline	30	27		33	
Ciprofloxacin	5	30		21	
Erythromycin	15	31		29	
Cefalexin	30	31		28	
Amoxicillin	25	33		27	
Trimethoprim	5	22		18	
Clarithromycin	15	28		26	
Linezolid	10	38		40	
Metronidazole	5		0		0
Azithromycin	15	21		19	
Co-amoxiclav	30	34		37	

SE4		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Amoxicillin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	28		27	
Ciprofloxacin	5	24		22	
Erythromycin	15	23		21	
Cefalexin	30	31		29	
Amoxicillin	25	28		27	
Trimethoprim	5	23		22	
Clarithromycin	15	34		31	
Linezolid	10	36		34	
Metronidazole	5		0		0
Azithromycin	15	16		21	
Co-amoxiclav	30	36		39	

SE5		Zone diameter (mm)			
Antibiotic	**Disc content (µg)**	**Parent strain**		**Amoxicillin - adapted strain**	
		S	**R**	**S**	**R**
Doxycycline	30	26		24	
Ciprofloxacin	5	28		26	
Erythromycin	15	29		27	
Cefalexin	30	34		36	
Amoxicillin	25	36		34	
Trimethoprim	5	29		27	
Clarithromycin	15	33		30	
Linezolid	10	37		35	
Metronidazole	5		0		0
Azithromycin	15	19		21	
Co-amoxiclav	30	40		37	

Amoxicillin - adapted strains had no cross- resistance in any of the five isolates (SE1-SE5). The adapted strain remained susceptible to doxycycline, ciprofloxacin, erythromycin, cefalexin, amoxicillin, trimethoprim, clarithromycin, linezolid, azithromycin and co-amoxiclav. Amoxicillin - adapted strains were resistant to metronidazole before and after being adapted to Amoxicillin (Table 6).

3.2 MIC

Table 7. Difference in MIC between Parent and P9 strains (SE1 –SE5)

Sample SE1 vs Antibiotic:	Parent strain MIC mg/mL	Passage '9' strain MIC mg/mL	Resistant Y/N
Amoxicillin	4	2	
Doxycycline	16	64	
Ciprofloxacin	2	8	
Erythromycin	2	≥128	Y
Cefalexin	2	4	
Sample SE2 vs Antibiotic:	Parent strain MIC mg/mL	Passage '9' strain MIC mg/mL	Resistant Y/N
Amoxicillin	4	16	
Doxycycline	16	64	
Ciprofloxacin	2	8	
Erythromycin	2	≥128	Y
Cefalexin	2	8	
Sample SE3 vs Antibiotic:	Parent strain MIC mg/mL	Passage '9' strain MIC mg/mL	Resistant Y/N
Amoxicillin	4	8	
Doxycycline	16	64	
Ciprofloxacin	2	8	
Erythromycin	2	≥128	Y
Cefalexin	2	4	
Sample SE4 vs Antibiotic:	Parent strain MIC mg/mL	Passage '9' strain MIC mg/mL	Resistant Y/N
Amoxicillin	4	2	
Doxycycline	16	64	
Ciprofloxacin	2	8	
Erythromycin	2	≥128	Y
Cefalexin	2	4	
Sample SE5 vs Antibiotic:	Parent strain MIC mg/mL	Passage '9' strain MIC mg/mL	Resistant Y/N
Amoxicillin	4	8	
Doxycycline	4	16	
Ciprofloxacin	2	8	
Erythromycin	2	≥128	Y
Cefalaxin	2	4	

3.3 Cross resistance

Table 8. Cross resistance based on MIC between P9 strains (SE1 –SE5)

Cross resistant MIC tests. Sample (passage 9) vs antibiotic	Ciprofloxacin MIC mg/mL	Doxycycline MIC mg/mL	Erythromycin MIC mg/mL	Cefalexin MIC mg/mL
SE1 amoxi	8	32	8	8
SE2 amoxi	8	8	8	8
SE3 amoxi	8	8	8	8
SE4 amoxi	8	8	8	8
SE5 amoxi	8	8	8	8
Cross resistant MIC tests. Sample (passage 9) vs antibiotic	Amoxicillin MIC mg/mL	Doxycycline MIC mg/mL	Erythromycin MIC mg/mL	Cefalexin MIC mg/mL
SE1 cipro	8	8	8	8
SE2 cipro	8	8	8	8
SE3 cipro	8	8	8	8
SE4 cipro	8	8	8	8
SE5 cipro	8	8	8	64
Cross resistant MIC tests. Sample (passage 9) vs antibiotic	Ciprofloxacin MIC mg/mL	Amoxicillin MIC mg/mL	Erythromycin MIC mg/mL	Cefalexin MIC mg/mL
SE1 doxy	32	64	32	32
SE2 doxy	32	32	64	32
SE3 doxy	32	32	32	32
SE4 doxy	32	32	32	32
SE5 doxy	32	32	32	32
Cross resistant MIC tests. Sample (passage 9) vs antibiotic	Ciprofloxacin MIC mg/mL	Doxycycline MIC mg/mL	Amoxicillin MIC mg/mL	Cefalexin MIC mg/mL
SE1 eryth	2	4	4	2
SE2 eryth	2	16	8	32
SE3 eryth	2	4	4	32
SE4 eryth	2	16	16	2
SE5 eryth	2	16	4	2
Cross resistant MIC tests. Sample (passage 9) vs antibiotic	Ciprofloxacin MIC mg/mL	Doxycycline MIC mg/mL	Erythromycin MIC mg/mL	Amoxicillin MIC mg/mL
SE1 cefal	2	2	2	2
SE2 cefal	2	2	2	2
SE3 cefal	2	2	≥128	2
SE4 cefal	2	2	≥128	2
SE5 cefal	2	2	≥128	2

SE_{ERYTH} strains from all five samples exhibited a large increase in MIC sensitivity to erythromycin by a 64-fold increase. High amount of cross-resistance (4- to 64-fold) was observed in SE_{ERYTH} MICs from SE2 and SE3 samples when exposed to cefalexin. Also, SE4 and SE5 SE_{ERYTH} MICs revealed high level of cross-resistance (8- to 64-fold) when exposed to doxycycline. SE_{DOX} MICs from SE1-SE5 showed an average amount of increase in resistance to doxycycline by 2- to 8-fold. In addition to this, SE_{DOX} MICs displayed slight cross-resistance to erythromycin, ciprofloxacin, amoxicillin and cefalexin with MICs of 32 mg/mL. However, SE_{DOX} MIC from sample SE1 revealed high cross-resistance (2-to 64- fold) when exposed to amoxicillin and SE2 SE_{DOX} MIC also showed high level (2-to 64- fold) of cross-resistance when exposed to erythromycin. Moreover, SE1-SE5 MICs of SE_{CIPRO} displayed a moderate 4-fold increase in resistance to ciprofloxacin. SE_{CIPRO} strain from sample SE5 showed high level (2-to 64- fold) of cross-resistance when exposed to cefalexin. SE_{CEFAL} from samples SE3, SE4 and SE5 revealed high cross-resistance (64- fold) when exposed to erythromycin. SE_{AMOXI} strain from sample SE1 presented high cross-resistance to doxycycline MIC by 4- to 64-fold.

All antibiotic-adapted strains of *S. epidermidis* exhibited cross-resistance: 8 to doxycycline (DOX), 5 to ciprofloxacin (CIPRO), 10 to erythromycin (ERYTH), 3 to cefalexin (CEFAL), and 1 to amoxicillin (AMOXI) (Table 9). In sample SE1, SE_{DOX} formed cross resistance to amoxicillin and the strain SE_{AMOXI} formed cross resistance to doxycycline. In sample SE2, SE_{DOX} formed cross resistance to erythromycin, SE_{ERYTH} formed cross resistance to cefalexin and SE_{CEFAL} formed cross resistance to erythromycin. In sample SE3, SE_{ERYTH} formed cross resistance to cefalexin and SE_{CEFAL} formed cross resistance to erythromycin. In sample SE4, SE_{ERYTH} formed cross resistance to doxycycline and SE_{CEFAL} formed cross resistance to erythromycin. In sample SE5, SE_{CIPRO} formed cross resistance to cefalexin, SE_{ERYTH} formed cross resistance to doxycycline and SE_{CEFAL} formed cross resistance to erythromycin (Table 9).

Table 9. Cross resistance of antibiotic-adapted strains to antibiotics based on MIC

SE1	**DOX**	**CIPRO**	**ERYTH**	**CEFAL**	**AMOXI**
SE	S	S	S	S	S
SE_{DOX}	R	S	S	S	R
SE_{CIPRO}	S	R	S	S	S
SE_{ERYTH}	S	S	R	S	S
SE_{CEFAL}	S	S	S	S	S
SE_{AMOXI}	R	S	S	S	S
SE2	**DOX**	**CIPRO**	**ERYTH**	**CEFAL**	**AMOXI**
SE	S	S	S	S	S
SE_{DOX}	R	S	R	S	S
SE_{CIPRO}	S	R	S	S	S
SE_{ERYTH}	S	S	R	R	S
SE_{CEFAL}	S	S	R	S	S
SE_{AMOXI}	S	S	S	S	S
SE3	**DOX**	**CIPRO**	**ERYTH**	**CEFAL**	**AMOXI**
SE	S	S	S	S	S
SE_{DOX}	R	S	S	S	S
SE_{CIPRO}	S	R	S	S	S
SE_{ERYTH}	S	S	R	R	S
SE_{CEFAL}	S	S	R	S	S
SE_{AMOXI}	S	S	S	S	S
SE4	**DOX**	**CIPRO**	**ERYTH**	**CEFAL**	**AMOXI**
SE	S	S	S	S	S
SE_{DOX}	R	S	S	S	S
SE_{CIPRO}	S	R	S	S	S
SE_{ERYTH}	R	S	R	S	S
SE_{CEFAL}	S	S	R	S	S
SE_{AMOXI}	S	S	S	S	S
SE5	**DOX**	**CIPRO**	**ERYTH**	**CEFAL**	**AMOXI**
SE	S	S	S	S	S
SE_{DOX}	R	S	S	S	S
SE_{CIPRO}	S	R	S	R	S
SE_{ERYTH}	R	S	R	S	S
SE_{CEFAL}	S	S	R	S	S
SE_{AMOXI}	S	S	S	S	S

S-sensitive R-resistant

3.4 Biofilms

The relative biofilm-forming unit was calculated, showing a significant difference (p <0.05) between the biofilm of SE1 parent strain and antibiotic-adapted strains of S. epidermidis (SEERYTH and SEDOX) (Figure 11. A, B, & C). In sample SE1, there was a decrease in biofilm formation by the following antibiotic-adapted strains (at 24 h SEERYTH = 12.5%; at 48 h SEERYTH = 16%, SEDOX = 35.6%); (at 72 h SEERYTH = 8.4%, SEDOX = 20.6%) compared to the parent strain. On the other hand, sample SE1 displayed an increase in the amount of biofilm formed by the subsequent adapted strains (at 24 h SEDOX =142.7%; at 48 h SECIPRO =11.7%) when compared to the parent strain. From 24 h to 72 h the parent strain continues to grow in large amount overtime and significantly more than SEERYTH biofilm. However, SEERYTH does not have the same progressive trend as the parent strain, there was a decrease in biofilm formation from 24 h to 48 h and an increase in biofilm formation during 72 h growth period (Figure 11. A, B, & C). The 48 h parent strain biofilm was significantly thicker than 48 h SECIPRO biofilm. SEDOX biofilm was significantly denser than the parent S. epidermidis and SEERYTH biofilms when grown for 24 h (Figure 5. A). However, during 48 h and 72 h growth periods, SEDOX biofilms grew significantly the least compared to the parent S. epidermidis and SEERYTH biofilms (Figure 11. B & C).

Sample SE2 antibiotic-adapted strains formed significantly less biofilm (at 48 h SECIPRO = 42.4%; at 72 h SECIPRO = 7%) in comparison to the parent strain. Also, substantial biofilm was significantly formed by the following SE2 adapted strains (at 24 h SECIPRO = 17.6%, SEDOX = 27%; at 48 h SEERYTH = 101.5%, SEDOX = 45%; at 72 h SEERYTH = 64.3%, SEDOX = 188.5%) when compared to the parent strains. 24 h parent strain grew significantly less than SECIPRO and SEDOX. 48 h parent strain grew significantly less than SEERYTH and SEDOX yet grew significantly more than SECIPRO. 72 h parent strain grew significantly more than SECIPRO, but less than SEERYTH and SEDOX biofilms. SEERYTH biofilm grew significantly more than the parent strain, SECIPRO and SEDOX biofilms when grown for 48 h. However, SEERYTH biofilm grew significantly more than SECIPRO biofilm and significantly less than SEDOX biofilm during 72 h growth period. 24 h SECIPRO biofilm grew significantly more than the parent strain yet less than SEDOX biofilm. During 48 h and 72h growth periods, SECIPRO biofilm grew significantly the least compared to parent strain, SEERYTH and SEDOX biofilms. During 24 h and 72 h growth periods, SEDOX biofilm was observed to grow significantly the most compared to parent strain and SEERYTH biofilms. However, during 48 h SEDOX biofilm grew significantly less than SEERYTH, yet significantly more than the parent strain and SECIPRO biofilms.

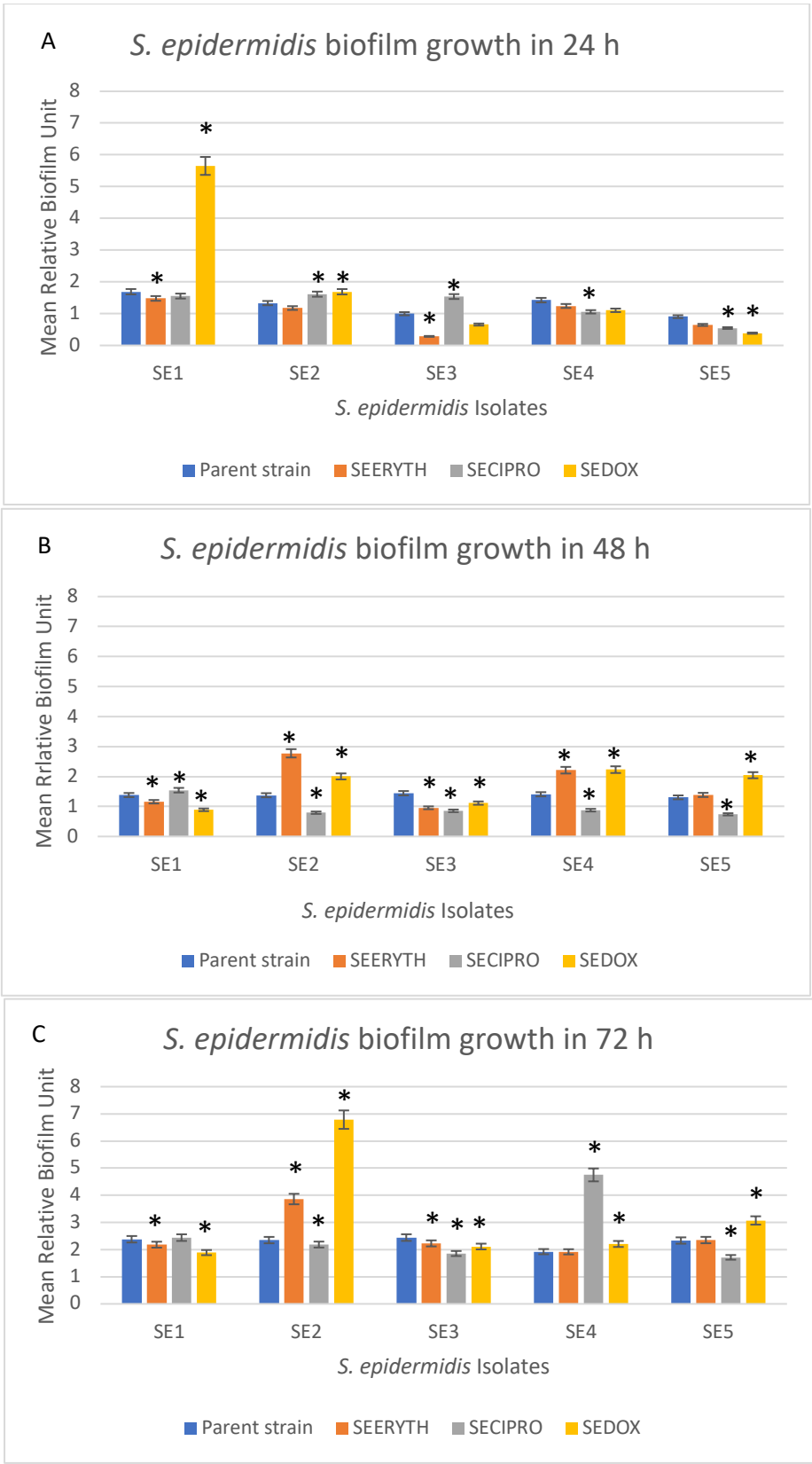

Figure 11. Mean relative biofilm units of five *S. epidermidis* samples (SE1 - SE5) with error bars. Significant differences (p <0.05) are shown between the mean relative biofilm forming capacity of the various antibiotic-adapted strains and parent strains over time.

Less biofilm was significantly formed by the sample SE3 adapted strains (at 24 h SE_{ERYTH} = 71.6%; at 48 h SE_{ERYTH} = 33.7%, SE_{CIPRO} = 40.7%, SE_{DOX} = 23%; at 72 h SE_{ERYTH} = 9%, SE_{CIPRO} = 23.9%, SE_{DOX} = 13.5%) in comparison to the parent strain. In contrast, more biofilm was significantly formed by the resulting SE3 adapted strain (at 24 h SE_{CIPRO} = 54%) opposed to the parent strain. From 24 h to 72 h the parent strain was significantly larger than SE_{ERYTH} biofilm as it continues to grow in larger amounts. However, SE_{ERYTH} biofilm significantly increased in density from 24 h to 72 h. During 24 h, SE_{CIPRO} biofilm significantly grew the most compared to the parent strain and SE_{ERYTH} biofilms. However, from 48 h to 72 h, SE_{CIPRO} significantly grew the least compared to parent strain, SE_{ERYTH} and SE_{DOX} biofilms. SE_{DOX} biofilm displayed a significant increase in growth from 48 h to 72 h, it grew significantly more than SE_{CIPRO} and SE_{ERYTH}.

In sample SE4, biofilm formation was significantly produced far less by the subsequent adapted strains (at 24 h SE_{CIPRO} = 25.7%; at 48 h SE_{CIPRO} = 37.7%) compared to the parent strain. SE4 sample also showed a significant increase in biofilm formation by the following adapted strains (at 48 h SE_{ERYTH} = 57%, SE_{DOX} = 58.4%; at 72 h SE_{DOX} = 14.7%, SE_{CIPRO} = 147%) in comparison to the parent strain. From 24 h to 48 h the parent strain biofilm was observed to grow increasingly and significantly more than SE_{CIPRO}. 48 h SE_{ERYTH} biofilm significantly grew more than parent strain and $SE_{CIPRO.}$ SE_{CIPRO} significantly decreased in biofilm growth from 24 h to 48 h and then increased when grown for 72 h. SE_{CIPRO} biofilm was observed to grow significantly the most during 72 h whereas it hardly grew during 48 h. SE_{DOX} biofilm density remained the same during 48 h to 72 h yet significantly more than the parent strain during both 48 h to 72 h growth periods.

Sample SE5 had significantly smaller amount of biofilm produced by the subsequent adapted strains (at 24 h SE_{CIPRO} = 39.6%, SE_{DOX} = 57.4%; at 48 h SE_{CIPRO} = 43.3; at 72 h SE_{CIPRO} = 26.6%) when compared to the parent strain. Nevertheless, substantial biofilm was significantly formed by the following adapted strains (at 48 h SE_{DOX} = 56.6%; at 72 h SE_{DOX} = 31.7%) in comparison to the parent strain. From 24 h to 72 h the parent strain significantly grew more than SE_{CIPRO}. From 48 h to 72 h SE_{CIPRO} biofilm grew significantly less than SE_{DOX} biofilm but grew significantly more than 24 h SE_{DOX} biofilm. SE_{DOX} biofilm significantly increased in density from 48 h to 72 h.

3.5 Relative fitness: P0 vs P9 strains

The overall productivity (CFU per milliliter) of P9 after 24 h of growth was significantly lower than that of P0. This deficit in growth was substantially more pronounced when the strains were grown in competition (Figure. 12). The relative Darwinian fitness (W) levels of P0 and P9 were compared when grown separately and when in competition with each other (Figure. 12). A relative fitness of 1 implies no fitness effect between strains, a value of below 1 indicates impaired fitness, and a value above 1 specifies enhanced fitness.

The relative fitness (W) of SE_{ERYTH} P9 to P0 during individual growth was 1.02, compared to 1.35 during competition. Thus, in a non-competitive environment SE_{ERYTH} P9 grew 1.8% slower than P0, whereas when in a competitive environment, SE_{ERYTH} P9 grew 22.9% slower than P0. P0 was observed to be significantly fitter than SE_{ERYTH} P9 when grown together.

The relative fitness (W) of SE_{DOX} P9 to P0 during individual growth was 1.26, compared to 0.38 during competition. Thus, in a non-competitive environment SE_{DOX} P9 grew 28.2% slower than P0, whereas when in a competitive environment, SE_{DOX} P9 grew 58.6% slower than P0. P0 was significantly fitter than SE_{DOX} P9 when grown separately and together.

The relative fitness (W) of SE_{CIPRO} P9 to P0 during individual growth was 1.4, compared to 0.45 during competition. Thus, in a non-competitive environment SE_{CIPRO} P9 grew 49.4% slower than P0, whereas when in a competitive environment, SE_{CIPRO} P9 grew 55.9% slower than P0. P0 was significantly fitter than SE_{CIPRO} P9 when grown separately.

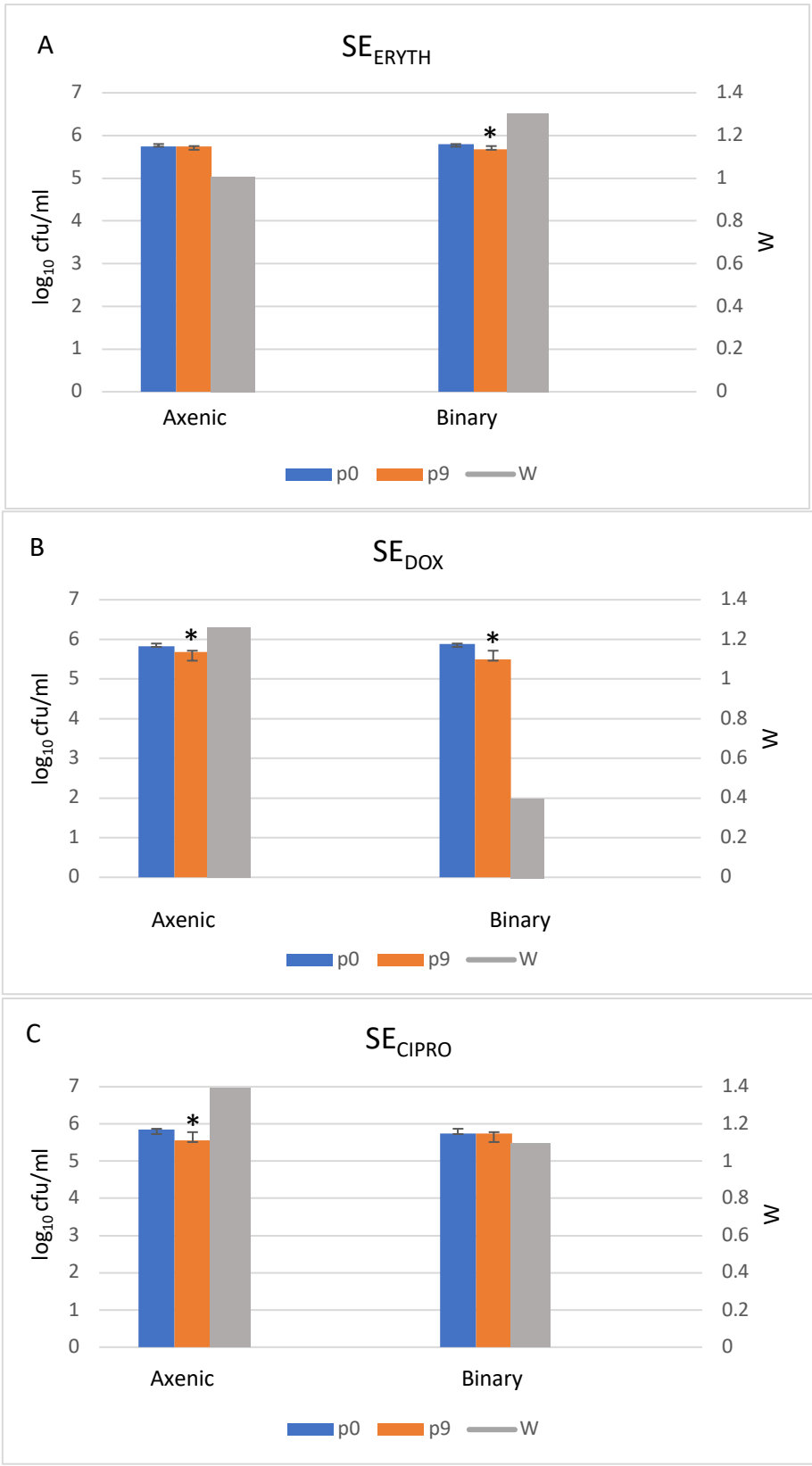

Figure 12. Mean colony forming units with standard deviation error bars (p <0.05). Significant differences in the mean colony forming capacity amongst the various antibiotic-adapted strains when compared to the parent strains at 24 h. SE_{ERYTH} axenic p-value= 0.2 and binary p-value <0.043. **B.** SE_{DOX} axenic p-value < 0.0001 and binary p-value < 0.0001. **C.** SE_{CIPRO} axenic p-value < 0.0001 and binary p-value < 0.0002.

3.6 *Galleria mellonella* (wax worm) virulence assay

Total of 540 larvae tested from group A & B. Data of waxworm survival over two-week period in group A and B shown on the next page. Two PBS injected worms died on Day 4 for both group A & B. 18 non-treated category worms remained healthy throughout the experiments for both groups.

SE_{ERYTH} strain was significantly more virulent than SE and SE_{DOX} strain in Figure 13A**.** 7 SE_{ERYTH} infected waxworms started dying from day 2 with 8 SE_{ERYTH} waxworm surviving on day 4 (Figure 13A). Whereas, on average 14 SE_{DOX} infected waxworms survived on day 4, however, only 2 SE_{DOX} waxworms survived on average. SE infected waxworm started dying on day 3, 12 waxworms survived on day 4.

SE_{ERYTH} strain was significantly more virulent than SE and SE_{DOX} strain in Figure 13B**.** Most SE_{ERYTH} and SE_{DOX} infected waxworms started dying from day 2. Many SE_{ERYTH} infected waxworms did not survive on day 4 with only 4 waxworms surviving on average. Moreover, 7 SE_{DOX} infected waxworms were able to survive on day 4 (Figure 13B). Only few of the SE waxworms died on day 4 with 15 waxworms surviving on day 4.

Table 10. Number of surviving larvae over 4 days following inoculation with SE, SE_{DOX} and SE_{ERYTH}

SE	Day 0	Day 1	Day 2	Day 3	Day 4
SE1	18	18	18	11	12
SE2	18	18	18	12	12
SE3	18	18	18	12	12
Mean	**18**	**18**	**18**	**11.66666667**	**12**
SE_{DOX}					
SE1	18	18	15	5	3
SE2	18	18	16	4	2
SE3	18	18	13	2	1
Mean	**18**	**18**	**14.66666667**	**3.666666667**	**2**
SE_{ERYTH}					
SE1	18	18	10	9	7
SE2	18	18	11	11	8
SE3	18	18	12	11	9
Mean	**18**	**18**	**11**	**10.33333333**	**8**

Surviving larvae in Group A

Table 11. Number of surviving larvae over 4 days following inoculation with SE, SE_{DOX} and SE_{ERYTH}

SE	Day 0	Day 1	Day 2	Day 3	Day 4
SE1	18	18	18	18	15
SE2	18	18	18	18	14
SE3	18	18	18	18	16
Mean	**18**	**18**	**18**	**18**	**15**
SE_{DOX}					
SE1	18	18	16	9	6
SE2	18	18	15	9	7
SE3	18	18	18	7	8
Mean	**18**	**18**	**16.33333333**	**8.333333333**	**7**
SE_{ERYTH}					
SE1	18	18	14	11	4
SE2	18	18	13	10	5
SE3	18	18	15	8	3
Mean	**18**	**18**	**14**	**9.666666667**	**4**

Surviving larvae in Group B

Table 12. One-tailed T-test on surviving groups of larvae

Surviving larvae	SE	SE_{ERYTH}	SE_{DOXY}	PBS	NTC
Mean of A	12	8	2	16	18
Mean of B	15	4	7	16	18
Mean of A & B	13.5	6	4.5	16	18
P-value		0.047733	0.045431		

Average surviving larvae in Group A & B

The assessment of survival rates following *G. mellonella* infection showed that SE_{ERYTH} and SE_{DOXY} strains were significantly virulent compared to *S. epidermidis* parent strains with *p*-value of 0.0477 and 0.0454, respectively**.**

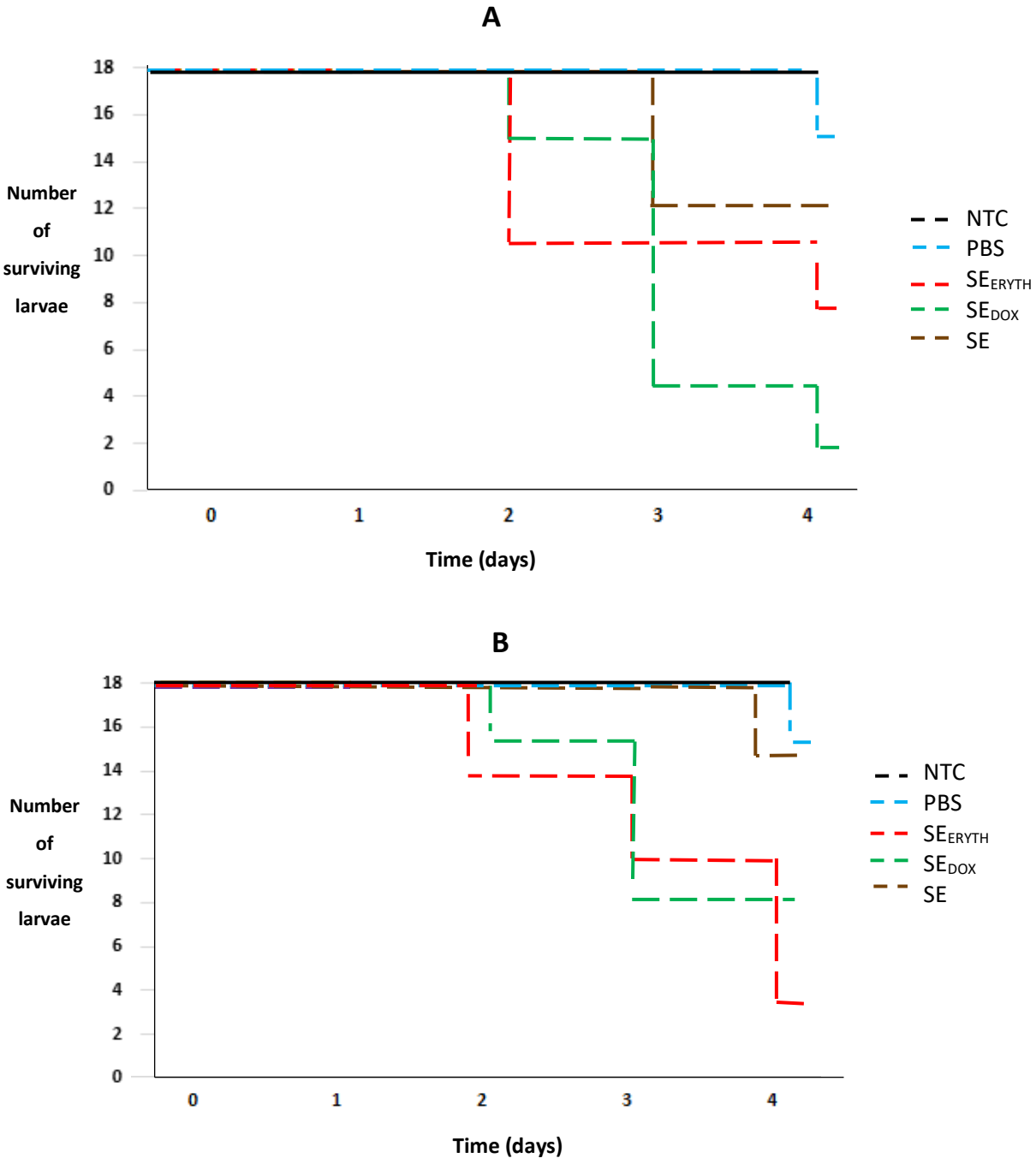

Figure 13. Two independent test results showing a survival curve of the daily number of surviving larvae following *G. mellonella* infection assay **KEY:** Parent strains (**SE**– *S. epidermidis*; Antibiotic-adapted strains ($\mathbf{SE}_{ERYTH,}$ $\mathbf{SE}_{DOX}$); **NTC**– Non-treated category; **PBS**– Phosphate buffered saline.

3.7 MS^E Result

Figure 14. MS^E mass spectrometer profile of antibiotic adapted *S. epidermidis* strains. The y-axis is Base Peak Intensity and the x-axis is Retention Time. A. SE1: SE_{DOX} B. SE_{CIPRO} C. SE SE2: D. SE_{DOX} E. $SE_{CIPRO.}$ Unfortunately, nothing meaningful was detected by the mass spectrometer due to the lack of sample metabolites.

CHAPTER 4

4.0 Discussion

Although there are currently several preventative strategies in place to control infection and closely observed trends in antibiotic resistance, the *S. epidermidis* infection rate is still very high and continues to occur in hospitals and care homes (Otto, 2012; Brown & Clarke, 2017). This research work is based upon the characterisation of *S. epidermidis* taken from human skin, adapted to antibiotics to gain insight on the virulence of antibiotic adapted *S. epidermidis.* This research highlights the potential significance antibiotic adapted *S. epidermidis* may have in prospective diagnosis when treating infected patients. Many studies have determined that opportunistic pathogens such as *S. epidermidis* have virulence factors that commonly assist in advanced bacterial growth and/or spread within or between hosts (Brown, Cornforth & Mideo, 2012).

The technique used to achieve antibiotic susceptibility profiling involved MICs and disk diffusion tests to assess characteristics that link directly to the problem of over resistance and the development of cross-resistance due to repetitive use of antibiotics. The mentioned methods were inexpensive, high-throughput and rapid which provide efficient data that supports the risk of antibiotic resistance evolving (Ferreira *et al*, 2017). However, the zone of inhibition results from disk diffusion tests can be influenced by the thickness or viscidness of the culture medium, the antibiotic concentration the filter disc has absorbed, how fast the antibiotic is diffused and how it interacted with the medium as well as how sensitive the bacteria is to the antibiotic. Also, if an antibiotic is observed to have a strong antibiotic capacity it might not be suitable for medical use as it can possibly cause dangerous side effects in the human biological system. Variables that may influence the reliability of these tests may also limit the interpretation of MIC data. This may occur due to *in vitro* experiment conditions not having the capacity to replicate the host habitat (Srinivasan *et al.,* 2017).

4.1 Antibiotic resistant strains

This research work investigated antimicrobial activity of eleven antibiotics against *S. epidermidis* extracted from the human skin microflora. All bacterial strains developed resistance to erythromycin, while being susceptible to 9 other tested antibiotics except metronidazole. The five isolates (SE1 - SE5; SE_{DOX} SE_{CIPRO} SE_{ERYTH} SE_{CEFAL} SE_{AMOXI}) were found to be multi-resistant, established by the Kirby-Bauer disc diffusion method and MIC results. The antibiogram of the parent and antibiotic-adapted isolates was produced using this standard method as it is constantly and generally utilised for antibiotic sensitivity tests in medical laboratories worldwide (EUCAST, 2019). Parent isolates of *S. epidermidis* that were originally only sensitive to certain antibiotics later formed resistance to them after nine

serial passages (P9); serial passage eventually led to the arise of resistant antibiotic-adapted strains, determined by the absence of zone of inhibition. Resistance is not the matter of if but only of when. There is a constant need for new or improved antibiotics to treat the bacterial populations that come back in resistant form after the first wave of antibiotic treatments. In this project the following antibiotics; (1) doxycycline, (2) erythromycin and (3) ciprofloxacin forced *S. epidermidis* parent strain to adapt.

Penicillin continues to be the most exploited antibiotic while a group of tetracycline antibiotics follow up as the second most commoly used antibiotic universally (van Hoek *et al*., 2011). Doxycycline (1) is a broad-spectrum antibiotic that interrupts the bacteria capability to produce proteins that are important for bacterial growth, replication and to form colonies. Moreover, few bacterial variants have become resistant to this drug, hence, reducing the efficacy of the treatment for certain set of bacterial infections (Otto, 2012). The mode of action for antimicrobial reaction of tetracycline heavily rely on the disruption of translated proteins within the bacteria, hence, destroying the bacteria capacity to duplicate and develop. Nevertheless, the polypeptide translation process can be disarranged within mitochondria belonging to eukaryotic cells, the metabolism appear to be impaired and may cause death, thus, damaging the validity of results from the experiment. Plasmid containing tet genes including *tetL* and *tetK* can encode tetracycline resistance. Similarly, genes *tetO* and *tetM* situated within transposons or chromosome can also encode tetracycline resistance. High percentage of around 92% has been reported for *tetK* gene, however just a single bacterial strain had displayed the *tetM* gene. Various research articles have described the simultaneousness of *tetK* and *tetM* genes within *Staphylococcal* species (Duran *et al*., 2012; Schiwon *et al.,* 2013).

Additionally, some studies showed that *S. epidermidis* resistance can be due to the existence of panton-valentine leucocidin gene (Stamatiou *et al.,* 2013) that is specific to particular strains, these *Staphylococcal* isolates yield and secrete an extracellular toxic two-structured substance. Several *Staphylococci* strains have recently become resistant to tetracycline antibiotics. Doxycycline is a highly lipophilic analogue with an increased *Staphylococcal* activity, previously used in treating urinary tact infections caused by *S. saprophyticus*. The tetracycline resistant strains that contain ribosomal protection (*TetM*) are resistant to both minocycline and tetracycline, whereas those tetracycline resistant strains that have an active efflux (*TetK*) are only susceptible to minocycline (Stamatiou *et al.,* 2013).

Erythromycin (2) is a broad-spectrum macrolide antibiotic which functions against a wide-range of microbes. In regard to the genotype of *Staphylococci*, it forms resistance to erythromycin due to the few genes it carries, several studies (Duran *et al*., 2012; Schiwon *et al.,* 2013) have identified the

prevalence of the ermC gene in Staphylococci including the study carried out by Águila-Arcos *et al*. (2017). The *ermC* gene was identified in all the clinical *Staphylococcus* strains while 72% of the isolates harbored the *ermB* gene. Similarly, Zmantar *et al.* (2011) research displayed a low frequency of *ermB* gene in *S. epidermidis* strains. Vimberga *et al.* (2019) research also found all *S. epidermidis* strains to be resistant to erythromycin, genome sequencing results showed that all of the *S. epidermidis* strains carried *mecA, fosA, blaZ*, *vgaB*, and *vatB* resistance genes. Additionally, *ermA* resistance gene was also found in one of the *S. epidermidis* strains. The presence of macrolide resistance genes positively correlates to the resistance of the isolates to erythromycin.

Ciprofloxacin (3) is a broad-spectrum fluoroquinolone antibiotic which works to kill Gram negative and Gram positive microbes (Marco *et al.,* 2017). The functionality of ciprofloxacin rely on the inhibition of DNA gyrase enzyme which is a type II topoisomerase. It is important for ciprofloxacin to split microbial DNA to stop the cell from dividing/replicating (Marco *et al.,* 2017). Ciprofloxacin antimicrobial activity is not only caused by inhibiting DNA gyrase (topoisomerase II) but also inhibiting type IV topoisomerase as it is needed for microbial DNA replication, recombination, transcription and repair (Marco *et al.,* 2017).

Divakarab *et al.,* (2017) research study showed that *S. epidermidis* from dental infections (caries) had higher ciprofloxacin and erythromycin resistance compared to healthy individuals. These ciprofloxacin and erythromycin resistance isolates had *mecA* gene present and virulence genes e.g. *sea* and *seb* significantly more than healthy individual isolates. As expected, both groups of *S. epidermidis* isolates from dental caries and healthy individuals had different SCCmec types, which may be the case for P0 parent strains and P9 antibiotic adapted strains in this study. Previous research studies have identified various substantial relations between bacterial strains' SCCmec types and antibiotic resistance as well as virulence genes (Divakarab *et al.,* 2017). SCCmec types, III and IV were found to be more resistant than other SCCmec types, mostly resistant to ciprofloxacin and erythromycin, carrying *sea, seg* and *sei* (Divakarab *et al.,* 2017).

4.2 Persister cells in biofilm

Biofilm formation was studied in this research via absorbance of biofilm produced on a 96-well polystyrene microtiter plate. The obtained biofilm data was divided by the planktonic bacteria OD, to find the relative biofilm-forming units, which is an accurate representation of biofilm formed, regardless of planktonic bacteria growth (Stipetic *et al.,* 2016). Due to the significant differences seen in the mean relative biofilm-forming units of the antibiotic-adapted *S. epidermidis* strains and the parent strains, it can be suggested that antibiotic adaptation decreased the ability of the bacteria to produce biofilm in this particular study. The antibiotic-adapted *S. epidermidis* (P9) strains displayed

low significant ($p < 0.05$) fitness level to form biofilms with reduced competence compared to the parent strains. For example, SE_{ERYTH} biofilm significantly increased in density from 24 h to 72 h in all samples apart from SE4, however, when both SE_{ERYTH} and SE parent strains were grown together SE was significantly fitter than SE_{ERYTH} strains. SE3-SE5 SE_{DOXY} and SE_{CIPRO} SE5 samples had gradual biofilm growth and a downregulation of virulence genes, this mechanism is known to cause chronic infections (Johns *et al*., 2015). Some internalisation systems have been reported in *S. epidermidis*, such as SdrG and AtlE. This highlights *S. epidermidis* pathogenic process and the site where bacteria can survive to cause persistent infections (Bresco *et al.* 2017). Biofilm resistance is attributed to "persisters" which are cells capable of surviving the initial effect of an antibiotic, and if left unhindered, regeneration of the biofilm occurs. The possibility of persister cells playing a role in biofilm tolerance was firstly suggested by Kim Lewis (2001), this hypothesis was then reinforced by Levin and Rozen (2006), as they provided mathematical evidence of persister cells hampering bacterial infection treatment. Persistence is one of many examples of phenotypic heterogeneity, persister cells are able to survive stress conditions that genetically homogenous population would not survive. Persister cells are metabolically dormant, hence, concentrations of antibiotics cannot eliminate them (Lewis, 2013). Persister cells breakdown carbon sources and are observed to have a cellular respiration, they develop in a continuous stochastic manner, reaching a rate of 1% in biofilm or in stationary phase (Defraine *et al.,* 2018).

Bacterial cells and persistent strains in the stationary phase of biofilm growth utilise quorum-sensing to control the combined activities of the microbial populace, hence, stimulating greater resistance to antibiotics (Beuchat *et al.,* 2011; Peng *et al.*, 2019). Other influences that can possibly increase microbial resistance and cross-resistance to several physical and chemical stresses include stimulation of stress genes, introduction of antibiotics, synthesis of stress-related proteins such as chaperones (Beuchat *et al.,* 2011; Peng *et al.*, 2019). Furthermore, pathogenic genomic islands that exist on bacterial chromosomes modulate the production of chaperone proteins, quorum sensing signals or antibiotic exposure may augment the production of pathogenic traits, e.g. synthesis of toxins and invasive proteins (Beuchat *et al.,* 2011; Peng *et al.*, 2019).

Some persistence strategies are similar to antibiotic resistance mechanisms utilised by bacteria, for example, efflux pumps play a major role in both anti-infective drug resistance and persistence mechanisms in mycobacteria (Adams *et al.,* 2011) and *E.coli* (Pu *et al*., 2016). The persister cells are not antibiotic-resistant mutants, as they return to being a wild type after additional culturing (Defraine *et al.,* 2018).

The persister cells are usually elevated in immune compromised states due to the inability of the immune system to assist antibiotic eliminating the targeted biofilm cells (Figure 15) (Lewis, 2013). Moreover, persister cells can protect themselves from host defence system via biofilm matrix as well as blood-brain barrier in both biofilm and eukaryotic cells (Buyck *et al.,* 2013). Antibiotics must merge with additional agents to have an increased impact against persisters (Defraine *et al.,* 2018). Recently, persister cells have been eliminated by combining aminoglycosides and specific metabolites in an *in vivo* mouse model, the results showed that persistent cells are 'primed for metabolite uptake and respiration'. Cells that remain within the biofilm may add to persistence, low-level infections, especially in *S. epidermidis* by not expressing Agr (Peng *et al.*, 2019).

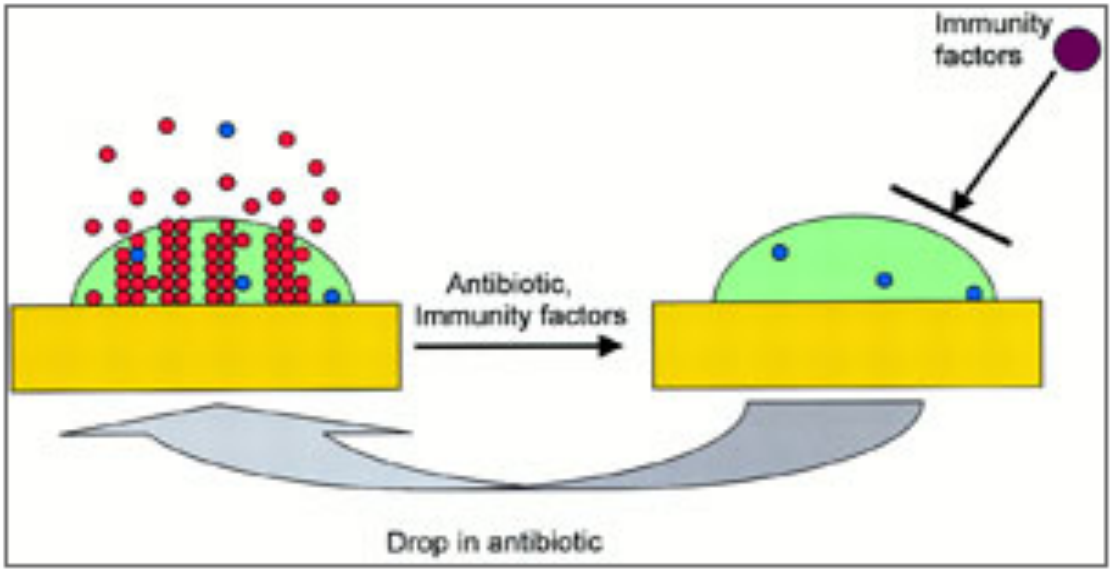

Figure 15. Schematic representation of biofilm resistance centred upon the survival of persisters. A preliminary application of high dosage confirms that the continuous supply of antibiotic eliminates planktonic bacteria alongside the bulk of biofilm bacteria. However, persisters stay active and revive biofilm, thereby instigating relapse of infection (Lewis, 2013).

4.3 Cross resistance

Microbes can adapt to anti-infective drugs by developing a reduced sensitivity, leading to cross-resistance. Microbial cross-resistance is linked to single nucleotide polymorphism, alteration in membrane surface charge and phospholipid profile. Moreover, microbial cross-resistance is associated with modification in expression of enzymes, gene inducers/repressors and lateral transfer of resistant genes (Figure 16) (Candido *et al.,* 2019).

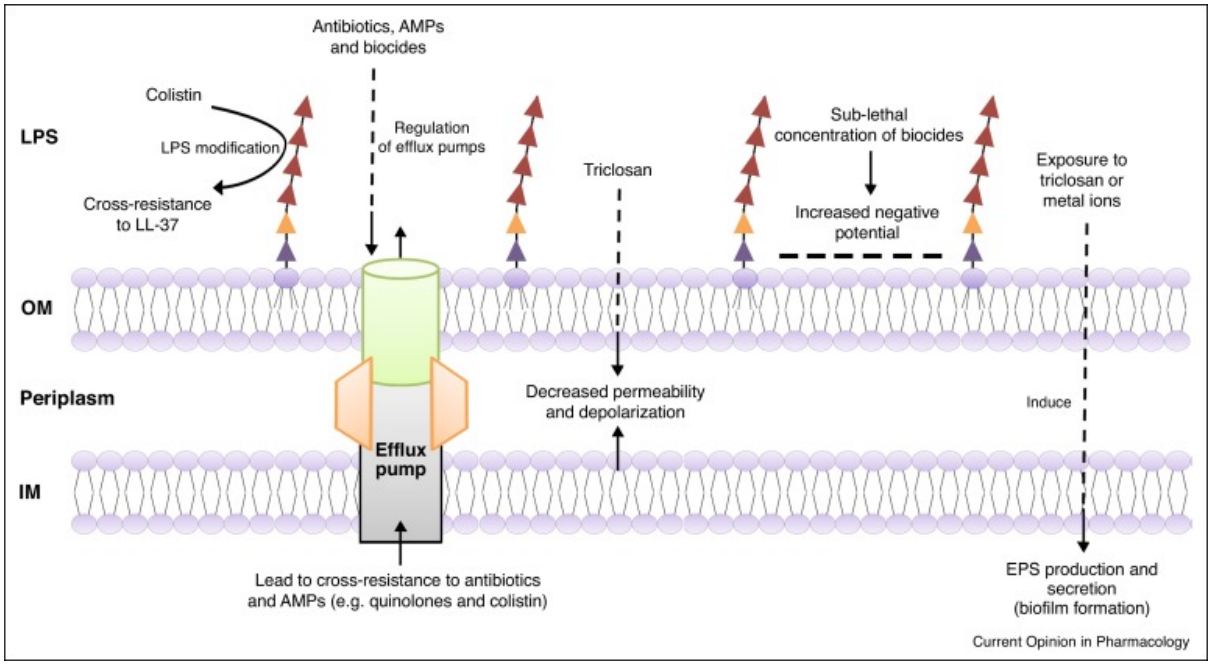

Figure 16. Microbial cross resistance mechanisms to antibiotics and biocide (Candido *et al.,* 2019). When bacteria are exposed to sub-lethal antibiotic concentrations the cell membrane dynamics are modified. The lipopolysaccharide (LPS) is altered by AMPs, such as colistin, possibly leading to resistance to host AMPs e.g. cathelicidin antimicrobial peptide (LL-37). Antibiotics, biocides and AMPs usually regulate efflux pumps by removing anti-infective compounds from the bacteria. Both outer and inner membrane permeability is altered via reduced depolarisation and augmented negative membrane potential. Lastly, contact with biocides, such as triclosan, can stimulate assembly and secretion of extracellular polymeric substances to aid biofilm development (Candido *et al.,* 2019).

4.4 Virulence strains in *G. mellonella*

S. epidermidis strains are generally incapable of causing aggressive infections in healthy hosts due to low virulence factors. The frequency of death in *G. mellonella* is observed in this study and is incidentally caused by microbial pathogenicity. In this research, there is an increase in number of *G. mellonella* larvae dying rapidly after being injected with the most virulent bacteria variant, such as, erythromycin and doxycycline adapted *S. epidermidis* variants. The antibiotic-adapted *S. epidermidis* strains (SE_{ERYTH} and SE_{DOXY}) with higher biofilm formation capacity were able to resist *G. mellonella* immune defense mechanisms and cause infection, this can be related to certain virulence factors. The pathogenicity can be caused by the presence of various virulence substances including adhesin, fimbriae, bacterial capsule and proteins that assist attaining host nutrition, in due course this allows the microbe to survive and persist in the wax worm host (Chiers *et al*., 2010). Furthermore,

some *S. epidermidis* strains are known to produce PSM-mec with cytolytic capacity for neutrophils *in vitro* and PSM-mec has been linked to reduced microbial clearance as well as high mortality rates in *S. epidermidis* infected mice model (Qin *et al.,* 2017). Similarly, this is observed in this study where SE_{ERYTH} and SE_{DOXY} infected insects had much higher mortality rates than parent strain (SE) infected waxworms. An additional possibility is that the SE_{ERYTH} and SE_{DOXY} strains evaded the *G. mellonella* immune system by having a cytotoxic effect on haemocytes, for example, disrupting the actin cytoskeleton, DNA synthesis inhibition and inducing apoptosis (Qin *et al.,* 2017). On the other hand, SE strains with little to no pathogenicity may have quickly been eliminated via insect host defence response, however, other more virulent strains, hence, becoming persistent. The parent strains in general did not cause infections within the insects till day 4 possibly due to haemocytes in *G. mellonella* mediating cellular responses like encapsulation, phagocytosis and clotting (Satyavathi *et al.*, 2014; Brown & Clarke, 2017), thus, successfully clearing sepsis in most wax worms. *G. mellonella* humoral response may have also cleared SE strains via soluble effector substances involving complement-like proteins, melanin, antimicrobial peptides and products produced by proteolytic cascades such as phenoloxidase pathway, which may restrict or kill SE strains (Eleftherianos & Revenis, 2011). The findings from this study is substantiated by multiple relevant research journals that demonstrate how raised level of bacterial virulence is linked to the multiplication of bacteria in *G. mellonella* larvae and in contrast to this, low level of bacteria virulence is associated with microbial clearance (Evans & Rozen, 2012; Loh *et al*., 2013; Norville *et al.,* 2014).

As recommended by Maillard *et al*. (2013) and SCENIHR (2014) it is important to carry out further tests once a consistent alteration in antibiotic susceptibility is observed, this is done to comprehend and report the nature of bacterial alterations. Molecular approaches can be utilised (e.g. microarray analysis or PCR) to study changes within the bacteria caused by constant antibiotic exposure (Yu *et al*., 2012). It is imperative for a researcher to study bacterial transcriptome and proteome as genotypic variations can possibly be resistance markers and provide information on antibiotic resistance mechanisms.

4.5 MS^E analysis

Throughout the extraction method for MS^E analysis intracellular metabolites were exposed to the organic solvent methanol (-48 °C) For an optimal metabolome analysis, this solvent did not physically nor chemically alter or destroy the metabolites (Goodacre *et al*., 2005). Methanol has the capacity to extract as many metabolites possible, both hydrophilic and lipophilic molecules or substances. To quench the metabolic processes of cells, immediate temperature changes are applied at either high (> +80 °C) or low temperature (< -40 °C). Meyer, Liebeke and Lalk (2010) found that cold extraction

procedures were more efficient than a hot extraction (boiling water) for Gram-positive *S. aureus* bacteria. The is due to boiling extraction method degrading some of the metabolites (Meyer *et al.,* 2010). For Gram-positive *S. epidermidis* a mechanical cell disruption is required, this is because the cell wall will not break sufficiently using only an organic solution. For the cell wall disruption glass beads were effectvely used to break the cell wall and release metabolites.

The main experiment for metabolomics failed to gain reliable results for profiling and analysis. However, the first quenching and metabolite extraction method was proven effective during a small trial run at Waters for mass spectrometry analysis. Few extracted metabolite samples (in triplicate) were transported to Waters, the mass spectrometry gave a positive response with high reliable target peaks. This validated the metabolite extraction method which was modified specifically for *S. epidermidis* from Goodacre *et al.* (2005) metabolomics guideline document. For the trial run at Waters, the MHB overnight culture media volumes were 1, 5, 10, 20 & 50 mL to test and optimise how much biomass is needed to get a positive response from MS^E. However, for the main experiment due to the lack of time and Waters MS^E test run deadline the colonies were directly taken from the overnight agar plates into the freezing organic solvent, instead of being grown in culture media at different volumes first, this may have affected the results (Kragh *et al.,* 2018). The inoculation method for the main experiment was inconsistent compared to the trial run, this consequently substantially influenced the experiment result (Kragh *et al.,* 2018).

This research study found no conceivable information from the results received by Waters MS^E analysis (Figure 14). The three typical stages used to process metabolomics data are to first pre-process the raw data for comparison between different data sets. Secondly, to reduce the size of the data to its significant variables and lastly, to store data (Brown *et al.* 2012). Even if an identical analytical MS^E instrument and extraction methods were used, there is no assurance that the entire metabolome has been profiled, the metabolomics community accepts that there is no instrument that can measure everything (Trivedi *et al.,* 2017).

Conventional methods used to measure quantifiable metabolome substances rely on calibration curve or the spike in stable isotope labeled internal standards (Schelli *et al.,* 2017). Nevertheless, methods that rely on calibration curves are time – consuming and susceptible to inaccuracy. Furthermore, methods based on the spikes of stable isotope labeled internal standards are usually expensive and not easily accessible (Schelli *et al.,* 2017). Thus, it is important for improved alternate methods for sensitive, accurate and quantitative metabolite analysis.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS) is capable of separating compounds within samples as it has the power of High Performance Liquid Chromatography (HPLC) and a detector

called Mass Spectrometry (MS) (Schelli *et al.,* 2017). HPLC detectors can detect compounds that have one or more elements including ultra violet absorption, fluorescence or electrical conductivity (Schelli *et al.,* 2017). Substance identification occurs indirectly. The components retention time is paralleled with a standard, if they are the same then it is almost certain that the component has been identified correctly. HPLC detectors have the ability to measure and quantify existing compounds, this is done by relying on the intensity and extent of the pure peaks during known retention times (Schelli *et al.,* 2017). Moreover, HPLC can provide excellent resolution, however the detectors are restricted in their capacity to detect accurately and measure compounds during specific conditions (Schelli *et al.,* 2017). One example of this is when several compounds are inadequately divided on the column. This situation may arise when inspecting for novel antibiotic metabolites as the drug component properties are not identified or if the compound has two isomers.

Characterisation of several extracellular and intracellular metabolites is generally done *in vitro* via numerous analytic devices such as HPLC-MS/MS (Schelli *et al.,* 2017). HPLC-MS/MS machineries have allowed concurrent analysis of multiple metabolites from known biotic processes (Zhong *et al.*, 2018). This approach may offer more precise quantification instead of the conventional universal approach used to characterise untargeted metabolites, enabling the detection of known metabolites for fast and simple study of biotic processes (Dunn *et al*., 2013). Both powerful analytical techniques HPLC and MS/MS can be combined and utilised. Moreover, the HPLC can split a sample into compounds of its component, the compounds are then tested in MS producing a spectra for every chemical entity. The structural information and molecular mass of the sample components can be determined by using the data HPLC MS/MS produces (Dunn *et al*., 2013).

This facility is progressively used to record relative alterations in research fixated on cellular metabolite concentrations stimulated by environmental and genetic perturbations. However, relative concentration fluctuations can be helpful, information on absolute metabolite concentration is an important factor to understand the biochemical responses, enzyme kinetics and other significant biotic systems in living cells (Dunn *et al*., 2013). Consequently, there is a growing need of improved methods to focus on defining absolute metabolite concentrations extracellularly and intracellularly (Dunn *et al*., 2013).

The combination of LC-MS and MS enables more powerful detection with increased sensitivity to detect metabolites accurately (Zhong *et al*., 2018). It is a challenge to differentiate constitutional isomers via HPLC and difficult to detect using MS (You *et al.,* 2014; Schelli *et al.,* 2017). MS detect ions generated by constitutional isomers, however, the isomers are mostly identical thus the variations in mass-to-charge ratio is usually not detected. There are many methods that can be used to overcome

this complication, one particular technique is LC-MS/MS. Ions produced for MS can be further disintegrated, hence, allowing LC-MS/MS to detect similarly arranged isomers (Lu *et al.,* 2006).

4.6 Conclusion

In conclusion, antibiotic adapted *S. epidermidis* strains from human skin showed multi-resistance, including resistance to erythromycin, which is mainly associated with *ermC* gene. The results showed high levels of adaptive resistance toward erythromycin (100%). Erythromycin adapted strain had significantly enhanced fitness advantage (w value- 1.35, *p* value- 0.0427) and were significantly virulent (*p*-value of 0.047733) when compared to the parent strain. It can be concluded that antibiotic resistance continues to pose threat associated with public health in the hospital due to emerging bacterial resistance and multi-drug resistant bacteria. The increased rate of erythromycin, doxycycline and ciprofloxacin resistance among *S. epidermidis* isolates and the cross-resistance amongst the bacterial isolates in this study, emphasise the rapid necessity for adequate and appropriate execution of antibiotic stewardship programmes at all levels. Further investigation is needed to establish resistance determinants. Further *in vivo* studies are also needed to discern the impact that conditions on the skin, such as temperature, dryness, nutrients, horizontal gene transfer, and genetic factors, may have on pathogenicity and virulence.

4.7 Recommendations

- Examination of implant surface *S. epidermidis* biofilms should be monitored and evaluated, the dissemination of bacteria strains resistant to antibiotics should be observed at regular intervals from healthcare settings.
- Strict measurement and precautions should be taken to control the spread of infections; this includes isolating infected patients. Also, the visitors and healthcare employees responsible for patient care should regularly wash their hands after each visit.
- The prescribed antibiotic for patients in healthcare places such as hospitals should be reviewed after every few days, and altered according to the antibiotic sensitivity report, or should fully stop using the given antibiotic if the suspected pathogens are not found in the culture tests.

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APPENDICES

Two W-values for each group of samples are calculated. For p-value, five p0 end samples and p9 end samples were compared for both axenic and binary.

Mean axenic: In (p9 end/p9 start)/In(p0end/p0start)

Mean binary: In (p9 end/p9 start)/In(p0end/p0start)

Table 13. Competitive fitness data of parent and adapted bacterial strains (x 10^{-5})

SE_{ERYTH}	**Axenic p0 start**	**Axenic p0 end**	**Axenic p9 start**	**Axenic p9 end**	**Binary p0 start**	**Binary p0 end**	**Binary p9 start**	**binary p9 end**
SE1	4.49	5.55	4.59	5.68	4.09	7.89	3.40	4.57
SE2	4.48	5.56	4.14	5.41	3.98	6.08	2.56	5.07
SE3	3.97	5.68	4.21	5.44	4.19	6.77	2.98	5.57
SE4	4.37	5.68	3.79	5.36	3.88	5.06	2.14	4.39
SE5	4.19	5.47	4.29	5.55	3.90	5.10	2.20	4.21
mean	4.30	5.59	4.21	5.49	4.01	6.18	2.65	4.76
p-value				0.2				0.043
Standard deviation				0.11				0.93
W-value				1.02				1.35
SE_{DOX}	**Axenic p0 start**	**Axenic p0 end**	**Axenic p9 start**	**Axenic p9 end**	**Binary p0 start**	**Binary p0 end**	**Binary p9 start**	**Binary p9 end**
SE1	5.59	6.99	3.40	4.77	4.82	7.59	3.21	2.54
SE2	5.88	6.78	3.26	4.97	4.94	7.69	2.25	3.45
SE3	4.89	6.27	3.98	4.87	4.96	7.62	2.72	3.26
SE4	5.47	7.06	3.94	4.69	4.85	7.65	2.34	3.65
SE5	5.00	6.10	3.60	4.51	4.99	7.62	2.83	2.88
mean	5.36	6.64	3.63	4.76	4.91	7.63	2.67	3.16
p-value				0.0001				0.0001
Standard deviation				0.33				0.31
W-value				1.27				0.38
SE_{CIPRO}	**Axenic p0 start**	**Axenic p0 end**	**Axenic p9 start**	**Axenic p9 end**	**Binary p0 start**	**Binary p0 end**	**Binary p9 start**	**Binary p9 end**
SE1	6.69	6.73	3.32	3.57	5.89	8.09	3.57	2.99
SE2	6.50	6.69	3.26	3.46	5.98	7.98	3.13	4.57
SE3	6.49	7.89	3.06	3.71	5.59	7.77	3.32	3.46
SE4	6.57	6.57	3.32	3.34	5.33	8.16	2.55	2.95
SE5	6.49	7.34	3.10	3.71	5.43	7.60	2.40	3.46
mean	6.54	7.05	3.21	3.56	5.64	7.92	2.99	3.49
p-value				0.0001				0.0002
Standard deviation				0.16				0.65
W-value				1.39				0.45